# Characterization of Human Telomeric Repeat-Containing RNA Biogenesis and Function

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# Abstract

Telomeres comprise the ends of linear eukaryotic chromosomes and are nucleoprotein complexes that fulfill various crucial functions, as they protect the natural DNA ends from degradation and inappropriate repair. Furthermore, they shorten with every round of DNA replication. This process is thought to contribute to the aging of cells and organisms and makes telomeres a powerful barrier against cancer development. Because of their heterochromatic nature, telomeres have long been considered to be transcriptionally silent genomic areas. Recently, however, it has been demonstrated that telomeres are transcribed in various organisms ranging from yeast to humans to produce long, non-coding RNAs. These transcripts have been named telomeric repeat-containing RNAs (TERRA), are found only within the nucleus, and are associated with telomeres throughout the cell cycle. Mammalian TERRA is transcribed by DNA-dependent RNA polymerase II (RNAPII) using the C-rich telomeric strand as a template, with transcription starting in the subtelomeres and proceeding towards the chromosome end. However, the promoters that drive the transcription of telomeres were not identified, and TERRA could thus be a part of the so-called 'transcriptional noise' that is associated with the pervasive transcription of the human genome. Contradicting this idea, here we report the existence of CpG-island promoters within human subtelomeres. We found that at least 20 human subtelomeres contain a CpG-island and that these CpG-islands are bound by active RNAPII *in vivo*. Furthermore, we identified a TERRA transcription start site directly downstream of the CpG-island promoter and found that TERRA transcription is negatively regulated through cytosine methylation at the CpG-islands. Concerning TERRA functions, the most popular concept is that TERRA antagonizes telomerase activity. Supporting

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# Abstract

this idea, *in vitro* experiments using TERRA-mimicking oligonucleotides have shown that TERRA inhibits telomerase through base-pairing with the telomerase RNA template. This and other observations led to the hypothesis that long telomeres produce more TERRA and inhibit telomerase, whereas short telomeres produce less TERRA and therefore are preferentially elongated. Contradicting this widespread belief, using telomerase overexpression and a telomerase inhibitor, we found that changes in telomere length do not affect steady-state TERRA levels. Furthermore, using a DNA methyltransferase double knockout cell line with high TERRA levels, we show here that endogenous TERRA does not inhibit telomerase in *in vitro* assays. We also report that telomere elongation upon telomerase overexpression happens at the same speed in two isogenic cell lines, one of which has increased TERRA levels, which disproves the idea that TERRA is a telomerase inhibitor *in vivo*. Finally, to identify transcription factors that regulate TERRA expression, we have created stable cell lines that express eGFP under the control of the TERRA CpG-island promoter. These cell lines can now be used for siRNA screening using eGFP fluorescence as a readout.

# Zusammenfassung

Telomere, die Enden von linearen eukaryotischen Chromosomen, sind Nukleinsäure-Protein Komplexe die eine Anzahl von wichtigen Funktionen erfüllen. Sie schützen die natürlichen Enden der DNS vor Degradation und unangebrachten Reparaturmechanismen. Darüberhinaus werden Telomere bei jeder DNS Replikation kürzer, was eine Ursache für das Altern von Zellen und Organismen ist und sie zu einer starken Barriere gegen die Entstehung von Krebs macht. Da Telomere zu den heterochromatischen Bereichen des Genoms gehören wurden sie für lange Zeit als nicht transkribierte Bereiche angesehen. Unlängst wurde jedoch nachgewiesen, dass Telomere in einer ganzen Reihe von Organismen angefangen bei einfachen Hefen bis hin zu menschlichen Zellen transkribiert werden, wobei eine lange nicht-kodierende RNS entsteht. Diese RNS namens TERRA ist ausschliesslich im Zellkern zu finden und bindet während des kompletten Zellzyklus an Telomere. In Säugetierzellen wird TERRA durch die DNS-abhängige RNS-Polymerase II (RNAPII) transkribiert, wobei der Cytosin-reiche DNS Strang als Vorlage dient. Die Transkription startet dabei im Subtelomer und verläuft in Richtung des Endes des Chromosoms. Promotoren die die TERRA Transkription steuern konnten bislang nicht identifiziert werden. Deshalb ist es möglich, dass TERRA zum sogenannten 'Rauschen' gehört, das mit der Transkription des menschlichen Genoms verbunden ist. Dagegen spricht jedoch die Existenz von CpG-Insel Promotoren in den Subtelomeren menschlicher Zellen über die wir hier berichten. Unsere Ergebnisse zeigen, dass mindestens 20 menschliche Subtelomere eine CpG-Insel beinhalten, an die die RNAPII *in vivo* bindet. Darüberhinaus haben wir einen Transkriptions-Startpunkt identifiziert der direkt hinter der CpG-Insel liegt und zeigen, dass die TERRA Transkription durch die Methylierung

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von Cytosinen der CpG-Insel negativ reguliert wird. Was die Funktionen von TERRA betrifft, ist die meistbeachtete Hypothese, dass TERRA die Telomeraseaktivität negativ reguliert. In diesem Zusammenhang wurde *in vitro* gezeigt, dass TERRA die Telomerase durch Basenpaarung mit dem Telomerase RNS-Bestandteil inhibieren kann, wobei künstliche TERRA-imitierende Oligonukleotide verwendet wurden. Dies führte zusammen mit anderen Beobachtungen zu der Hypothese, dass lange Telomere mehr TERRA produzieren wodurch die Telomerase inhibiert wird, während kurze Telomere wenig TERRA produzieren und deshalb bevorzugt durch Telomerase verlängert werden. Gegen diese weit verbreitete Hypothese sprechen jedoch unsere Ergebnisse die wir hier präsentieren. Wir haben Telomerase überexprimiert oder inhibiert und festgestellt, dass die Länge eines Telomeres keinen Einfluss auf seine Transkription hat. Des weiteren haben wir eine DNS-Methyltransferase doppel-knockout Zelllinie mit sehr hohem TERRA Spiegel verwendet und gezeigt, dass zelleigene TERRAmoleküle die Telomerase in *in vitro* Experimenten nicht inhibieren. Wir berichten auch, dass die Verlängerung von Telomeren nach einer Telomerase-Überexpression in zwei genetisch gleichartigen Zelllinien, von denen eine stark erhöhte TERRA Spiegel hat, mit der gleichen Geschwindigkeit stattfindet. Diese Ergebnisse widerlegen die weit verbreitete Theorie, dass TERRA *in vivo* ein Telomeraseinhibitor ist. Zuletzt, haben wir stabile Zelllinien erschaffen, die eGFP unter Kontrolle des TERRA CpG-insel Promotors exprimieren. Diese Zelllinen können genutzt werden um Transkriptionsfaktoren zu identifizieren, die die TERRA Transkritption steuern, indem Kandidaten-Gene mit Hilfe von siRNA herunterreguliert werden und im folgenden die eGFP Fluoreszenz gemessen wird.

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# 1. Declaration of contents

The text of this dissertation has been written by myself, Benjamin Oliver Farnung. The text of the chapter 'Results' in sections 3.1 and 3.2 has been taken and adapted from the following publications:

Nergadze SG[*], **Farnung BO**[*], Wischnewski H, Khoriauli L, Vitelli V, Chawla R, Giulotto E, Azzalin CM (2009) CpG-island promoters drive transcription of human telomeres. RNA 15: 2186-94.

**Farnung BO**[*], Brun CM[*], Arora R, Lorenzi LE, and Azzalin CM (2012) Telomerase efficiently elongates highly transcribing telomeres in human cancer cells. PLoS ONE 7(4):e35714.

The figures presented in this dissertation represent the work of myself, Benjamin Oliver Farnung, as well as of current and/or former members of the groups of Claus Azzalin[1] and Elena Giulotto[2], as listed in the following:

Figures 1, 2, 3, 8, 16, 26, 27, 28, and 29 represent unpublished work of myself.

Figures 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15, and 17 represent published work taken from:

Nergadze SG[*], **Farnung BO**[*], Wischnewski H, Khoriauli L, Vitelli V, Chawla R, Giulotto E, Azzalin CM (2009) CpG-island promoters drive transcription of human telomeres. RNA 15: 2186-94.

Of these figures, figures 6C, 7, 9, 10, 13B and C, and 17 represent my own

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work, while the remaining figures represent work from other current and/or former members of the groups of Claus Azzalin[1] and Elena Giulotto[2].

Figure 18 represents published work from other current members of the group of Claus Azzalin[1] taken from:
**Farnung BO**, Giulotto E, Azzalin CM (2010) Promoting transcription of chromosome ends. Transcription 1: 140-143.

Figures 19, 20, 21, 22, 23, 24, and 25 represent published work taken from:
**Farnung BO**[*], Brun CM[*], Arora R, Lorenzi LE, and Azzalin CM (2012) Telomerase efficiently elongates highly transcribing telomeres in human cancer cells. PLoS ONE 7(4):e35714.
Of these figures, the figures 19, 20, 21, 22, 23, 24 and 25 B and C represent my own work, while figure 25A represents work from another former member of the group of Claus Azzalin[1].

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# 2. Introduction

## 2.1 Telomeres

In archaea and most prokaryotes, the genomic DNA is organized into circular chromosomes (Volff and Altenbuchner, 2000). However, in most eukaryotes, although mitochondria and chloroplasts usually contain circular DNA, consistent with their prokaryotic origin, the genomic DNA is present in linear form (Volff and Altenbuchner, 2000). These linear chromosomes are important for sexual reproduction and are necessary for proper meiosis and recombination between homologous chromosomes (Ishikawa and Naito, 1999). The presence of linear DNA molecules is advantageous but also poses a number of problems, as the natural double-stranded DNA ends have to be protected from exonucleases and degradation. Furthermore, cells need to be able to distinguish the double-stranded DNA ends from DNA double-strand breaks to prevent inappropriate repair, which would lead to fatal chromosome end-to-end fusions (Kurenova and Mason, 1997; Zakian et al., 1990). Finally, because DNA replication is a unidirectional process, the extreme 3' end of a linear DNA molecule cannot be copied, and therefore a piece of DNA is lost during every replication cycle (Olovnikov, 1971; Watson, 1972). As a consequence, eukaryotic linear chromosomes shorten with every round of cell division (Harley et al., 1990). To overcome the aforementioned problems, eukaryotic cells have developed specialized structures at the very ends of their linear chromosomes consisting of repetitive guanosine (G)-rich DNA and proteins (Palm

and De Lange, 2008). Barbara McClintock and Hermann Muller independently described these structures for the first time in the 1930s and found them to be important for chromosome stability (McClintock, 1939; Muller, 1938). Hermann Muller then gave the ends of linear eukaryotic chromosomes their name: telomeres, which is derived from the Greek words 'telos' (end) and 'meros' (part). Even though the pioneering studies of Muller and McClintock on telomeres were followed up by many others, their original conclusions remain valid. In addition, we have learned that telomeres are important not only for genome stability but also in cellular and organismal aging, and they constitute powerful barriers against cancer development (Aubert and Lansdorp, 2008; Hanahan and Weinberg, 2011).

## 2.1.1   Telomeric DNA

To fulfill all of their protective functions, telomeres must be distinguishable from DNA double-strand breaks. Therefore, telomeric DNA in most eukaryotes consists of short tandem repeats that are bound by a number of specialized proteins. In mammalian cells, these tandem repeats comprise the six base pair sequence 5'-TTAGGG/CCCTAA-3'. The length of the telomeric DNA tract varies greatly among eukaryotes and is usually between 10 to 15 kilobases (kb) in human cells. Furthermore, at the actual chromosome end, the G-rich strand forms a single-stranded overhang over its complement, which has a length of 50 to 500 nucleotides (nt) in mammals (Fig. 1A; Palm and De Lange, 2008). The centromere-proximal regions directly adjacent to the telomeres are called subtelomeres and also often contain repetitive DNA elements (Fig. 1A). Using electron microscopy, studies have found that telomeres can form large duplex lariat structures, which have been termed t-loops (Fig. 1B; Griffith et al., 1999). These t-loops are believed to be generated by strand invasion of the telomeric single-stranded overhang into double-stranded telomeric DNA and subsequent base-pairing between the invading strand and the cytosine (C)-rich strand of

**A**



**B**
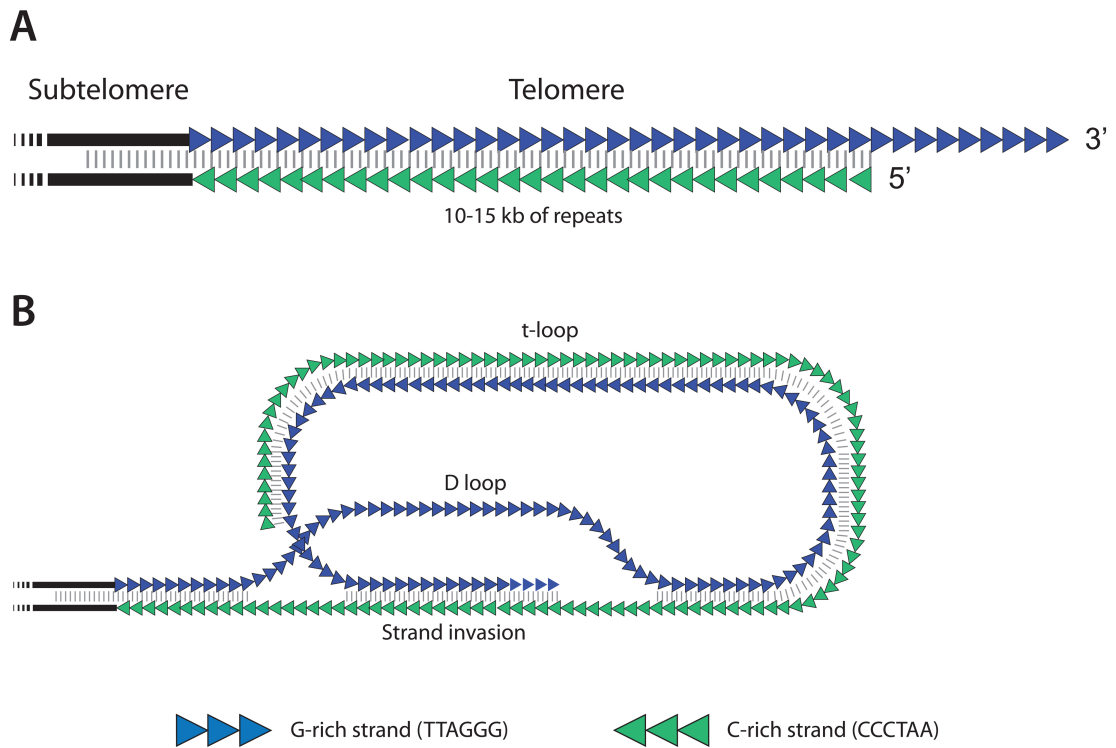


**Figure 1: Telomeric DNA.**
(**A**) Schematic drawing of a telomere without any proteins. (**B**) Schematic drawing of telomeric t-loop and D loop structures. Formation of the t-loop is believed to hide the DNA end from nucleases and inappropriate repair mechanisms.

the telomeric DNA duplex, which in turn leads to the displacement of the G-rich strand and the formation of a single-stranded DNA structure called D loop (Fig. 1B). T-loops have been found in a variety of different organisms, including humans, and are believed to provide a structure that is able to inhibit the DNA damage repair machinery by hiding the actual chromosome end (Palm and De Lange, 2008)

As a global method of gene expression regulation, genomes are separated into heterochromatic and euchromatic regions. Euchromatic regions are characterized by an open DNA conformation that facilitates transcription and is associated with the acetylation of histone tails (Grunstein, 1998; Huisinga et al., 2006; Litt et al., 2001; Noma et al., 2001). In contrast, heterochromatin is a condensed DNA state that is usually marked by low gene density, inhibition of

transcription, histone hypoacetylation and histone methylation (Richards and Elgin, 2002). In the 1970s, telomeres had already been identified as constitutive heterochromatic regions of the genome through chromosome staining experiments (Rodman, 1974). These findings were supported by the discovery of the telomere position effect (TPE), which is a heterochromatin-associated phenomenon that leads to the transcriptional silencing of genes inserted into subtelomeric regions near telomeres and has been described in a variety of organisms, including humans (Gottschling et al., 1990; Matzke et al., 1994; Nimmo et al., 1994; Horn and Cross, 1995; Baur et al., 2004). Today we know that telomeres are indeed enriched in several heterochromatic marks, such as histone H3 trimethylation at lysine 9 (H3K9me3), histone H4 trimethylation at lysine 30 (H4K20me3) and heterochromatin protein 1 (HP1) (Blasco, 2007; García-Cao et al., 2004; Benetti et al., 2007) . Furthermore, mammalian subtelomeres have been shown to contain high levels of DNA methylation at CpG dinucleotides, which is another heterochromatic signature (Blasco, 2007; Benetti et al., 2007).

## 2.1.2   Telomeric proteins: the shelterin complex

Even though telomeric DNA is believed to form t-loops to protect the very end of the chromosome from degradation and inappropriate repair, repetitive telomeric DNA alone is not sufficient for effective end protection. Many of the protective functions exhibited by telomeres are actually performed by specialized proteins that bind to the repetitive telomeric DNA. In mammalian cells, a complex of six proteins is associated with telomeres throughout the cell cycle in the socalled shelterin complex (Palm and De Lange, 2008). The six members of the human shelterin complex are the telomeric repeat binding factors 1 and 2 (TRF1 and TRF2), protection of telomeres 1 (POT1), TRF2- and TRF1-interacting nuclear protein 2 (TIN2), the human ortholog of the yeast repressor/activator protein 1 (RAP1), and TPP1. Of these proteins, TRF1 and

**Figure 2: The shelterin complex.**
Schematic drawing of a telomere together with the shelterin complex. TRF1 and TRF2
bind to double stranded DNA as homodimers. POT1 binds the telomeric single-stranded
overhang. Rap1 is recruited to telomeres by TRF2, while TPP1 is through its interaction
with POT1. TIN2 binds to TRF1, TRF2 and TPP1 and thereby connects the double-strand
binding proteins to the single-strand binding proteins and helps to recruit TPP1 and POT1 to
telomeres.

TRF2 both form homodimers that directly bind to double-stranded telomeric
DNA, whereas POT1 binds to the single-stranded overhang (Fig. 2; Palm and
De Lange, 2008). RAP1 is recruited to telomeres through binding to TRF2,
whereas TPP1 is recruited by binding to POT1 (Fig. 2; Palm and De Lange,
2008). TIN2 takes a central position within the shelterin complex because
it interacts with TRF1, TRF2, and TPP1 and bridges the single-and double-
stranded DNA binding components of shelterin (Fig. 2; Palm and De Lange,
2008). One of the main functions of shelterin is to inhibit the DNA damage
response and repair by the two main DNA damage repair pathways: non-
homologous end-joining (NHEJ) and homologous recombination (HR). Stud-
ies have shown that TRF2 and POT1 are required for the inhibition of the
ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) pro-
tein kinases, respectively, at telomeres; these kinases are responsible for DNA

damage signaling in mammalian cells (Celli and De Lange, 2005; Karlseder et al., 1999; Denchi and De Lange, 2007; Palm and De Lange, 2008). Upon activation, ATM and ATR trigger cell cycle arrest in G1/S- or G2/M- phase and the local accumulation of additional DNA damage response factors (Palm and De Lange, 2008). Therefore, ATM and ATR inhibition at telomeres is crucial for cells to proliferate. In addition to POT1, TPP1 also seems to be involved in ATR inhibition, as its inactivation leads to a DNA damage response at telomeres that is indistinguishable from the response caused by POT1 deletion (Hockemeyer et al., 2007; Denchi and De Lange, 2007; Xin et al., 2007). TIN2 inactivation also causes a DNA damage response at telomeres, and its depletion causes ATM and ATR activation (Kim et al., 2004; Takai et al., 2011). HR at telomeres has been shown to be inhibited by POT1, TRF2, and RAP1 (Palm and De Lange, 2008; Sfeir et al., 2010; Palm et al., 2009). In addition to inhibiting the DNA damage response, shelterin is also crucial for telomere replication and elongation. TRF1, for example, is required to prevent stalling of the replication fork at telomeres and for efficient telomere replication (Sfeir et al., 2009), whereas TIN2, TPP1 and POT1 have been found to function as negative regulators of telomerase-dependent telomere elongation (Houghtaling et al., 2004; Kim et al., 1999; Liu et al., 2004; Smogorzewska et al., 2000; Ye et al., 2004).

### 2.1.3   Telomere length regulation

Because of the need for an RNA primer, DNA polymerases are not able to completely replicate a linear DNA molecule, and telomeres therefore shorten with each cell division cycle (Olovnikov, 1973; Watson, 1972). This phenomenon is called the DNA end-replication problem. Generation of the telomeric single-stranded overhang, which is believed to involve nucleases, is an additional effect that leads to progressive telomere erosion in dividing cells that do not have telomere lengthening mechanisms (Palm and De Lange, 2008). To counteract this loss of telomeric DNA, most eukaryotes express telomerase (Cech,

2004), which is a specialized reverse transcriptase that uses an RNA template to add new telomeric repeats to the 3' end of the telomeric single-stranded overhang (Feng et al., 1995; Greider and Blackburn, 1985, 1987; Lingner et al., 1997; Nakamura et al., 1997). In human cells, however, telomerase is only expressed in the germ line, in stem cells, and during embryogenesis, and most somatic cells do not express telomerase (Gladych et al., 2011). This lack of telomerase expression in somatic cells is believed to be a reason for aging (Aubert and Lansdorp, 2008). Even though the length of individual telomeres can vary within a single cell, the average telomere length is usually kept in equilibrium within a small species-specific range, which is achieved by telomerase regulation through telomere binding proteins (Smogorzewska and De Lange, 2004). This regulation is thought to occur through a negative feedback mechanism where telomeres are bound by negative regulators of telomerase proportional to their length, which ensures preferential elongation of short telomeres (Marcand et al., 1997; van Steensel and de Lange, 1997). One candidate for such a negative regulator of telomerase is the shelterin component TRF1. Studies have shown that TRF1 overexpression leads to telomere shortening, whereas expression of a dominant negative form of TRF1 leads to telomere elongation (van Steensel and de Lange, 1997). In addition to TRF1, TIN2 and TPP1/POT1 have also been implicated in telomerase regulation. Their depletion from telomeres leads to telomerase-dependent telomere elongation, and POT1 competes *in vitro* with telomerase for the single-stranded 3' telomeric overhang (Houghtaling et al., 2004; Kim et al., 1999; Liu et al., 2004; Smogorzewska et al., 2000; Ye et al., 2004; Loayza and De Lange, 2003; Kelleher et al., 2005; Lei et al., 2005). Shelterin components are good candidates not only for telomerase inhibition but also for the recruitment of the low-abundant telomerase to telomeres. Thus far, TPP1 is the only shelterin component that has been shown to directly interact with telomerase, which could indicate its involvement in telomerase recruitment (Abreu et al., 2010;

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Tejera et al., 2010; Xin et al., 2007).

## 2.1.4  The role of telomeres in aging and cancer development

The shortening of telomeres with each cell division leads to consequences that eventually affect not only the cells but also the entire organism. On a cellular level, the limited number of cell divisions of cultured human somatic cells before they stop dividing and enter replicative senescence was first discovered in the 1960s (Hayflick and Moorhead, 1961; Hayflick, 1965). At that time, the association of this phenomenon with telomeres or telomere length was not yet clear (Olovnikov, 1973). A direct connection between telomere length and aging was made for the first time with the discovery that the average telomere length of the sex chromosomes was much longer in sperm cells than in adult somatic cells (Cooke and Smith, 1986). Furthermore, several studies have shown that the replicative potential of primary human fibroblasts can be increased by the overexpression of the telomerase catalytic subunit (hTERT) in these cells (Bodnar et al., 1998; Vaziri and Benchimol, 1998). Today, it is clear that short telomeres in senescent cells trigger a sustained DNA damage signal that contributes to the activation of p53, which is one of the main components of the cellular stress response (d'Adda di Fagagna et al., 2003; Takai et al., 2003; Vousden and Lane, 2007). Several aging disorders have also been linked to telomere dysfunction. Fanconi anemia (FA) and ataxia telangiectasia (AT) are usually autosomal recessive inherited diseases caused by mutations in the Fanconi genes or ATM, respectively. They are characterized by accelerated telomere shortening and problems in telomere replication or repair that cause immunodeficiency and bone marrow failure (Brümmendorf et al., 2001; Leteurtre et al., 1999; Li et al., 2003; Metcalfe et al., 1996). Dyskeratosis congenita (DC) is a premature aging disorder caused by mutations in the genes encoding the telomerase catalytic subunit, the telomerase RNA component

(hTR) or dyskerin, a protein that is associated with active human telomerase (Cohen et al., 2007; Mitchell et al., 1999; Vulliamy et al., 2001, 2005). DC patients suffer from bone marrow failure, abnormal skin pigmentation, nail dystrophy and leukoplakia (Mason, 2003; Mason et al., 2005). Interestingly, short telomeres are a common feature of all DC variants. In mouse models however, telomerase deficiency does not cause symptoms of DC (Blasco et al., 1997; Liu et al., 2000; Yuan et al., 1999), while POT1B (POT1B is one of the two POT1 genes present in rodents) knockout mice, which are also haploinsufficient for telomerase, have shortened telomeres and show clear symptoms of the disease (Hockemeyer et al., 2008). Taken together, these findings indicate a strong relationship between telomere length and cellular and organismal aging.

Enabling replicative immortality is one of the hallmarks of cancer, and because telomeres shorten in normal human somatic cells with every division, telomeres represent a powerful barrier against cancer development (Hanahan and Weinberg, 2011). When telomeres become critically short, they trigger replicative senescence, which is mediated through signaling via the p53 and retinoblastoma (pRB) pathways (Atadja et al., 1995; Beausejour et al., 2003; Farwell et al., 2000; Kim et al., 2002; Marcotte and Wang, 2002). However, acquired mutations may cause cells to not enter senescence and to continue proliferating until they reach a second barrier in the form of a phenomenon called crisis (Wright and Shay, 1992). Crisis occurs when telomeres have lost their protective function, which triggers chromosomal instability and telomere end-to-end fusions and leads to widespread cell death, but also places the cells at risk for malignant transformation (Cheung and Deng, 2008; Ducray et al., 1999). Some cells may be able to survive crises by reactivating telomere lengthening mechanisms. In most cancers, this reactivation is achieved by re-expression of telomerase, whereas relatively few cancers rely on an alternative lengthening of telomeres (ALT) mechanism based on homologous recombina-

tion to maintain functional telomere length (Bryan et al., 1997; Dunham et al., 2000; Muntoni and Reddel, 2005; Reddel et al., 1997). Thus, whereas telomere shortening is a strong barrier against malignant transformation, transient telomere dysfunction promotes genome instability, which facilitates the formation of tumor-promoting mutations (Artandi and DePinho, 2000; Artandi and Depinho, 2010; Cheung and Deng, 2008; Palm and De Lange, 2008).

## 2.2    Telomeric repeat-containing RNA: TERRA

As they are gene-less and heterochromatic in nature, for decades, telomeres were considered to be transcriptionally silent, which was further supported by the discovery of the TPE (Baur et al., 2004; Gottschling et al., 1990). This dogma was overturned with the discovery of long non-coding RNAs transcribed from chromosome ends in human and mouse cells (Azzalin et al., 2007; Schoeftner and Blasco, 2008). These transcripts were named telomeric repeat-containing RNA (TERRA) and were later found to be produced not only in mammals but also in a wide range of organisms, such as yeasts, plants, fishes, and birds (Bah et al., 2012; Luke et al., 2008; Schoeftner and Blasco, 2008; Solovei et al., 1994; Vrbsky et al., 2010). Even though the functions of TERRA are not yet completely clear, its conservation throughout evolution highlights the importance of these transcripts, which to date have been indirectly implicated in telomere length homeostasis, telomere replication, telomeric DNA condensation, and telomerase regulation (Flynn et al., 2011; Poulet et al., 2012; Redon et al., 2010; Schoeftner and Blasco, 2008; Chawla et al., 2011; Luke et al., 2008).

### 2.2.1    TERRA biogenesis

TERRA is produced using the C-rich telomeric strand as a template for transcription that starts within the subtelomere and proceeds towards the chromo-
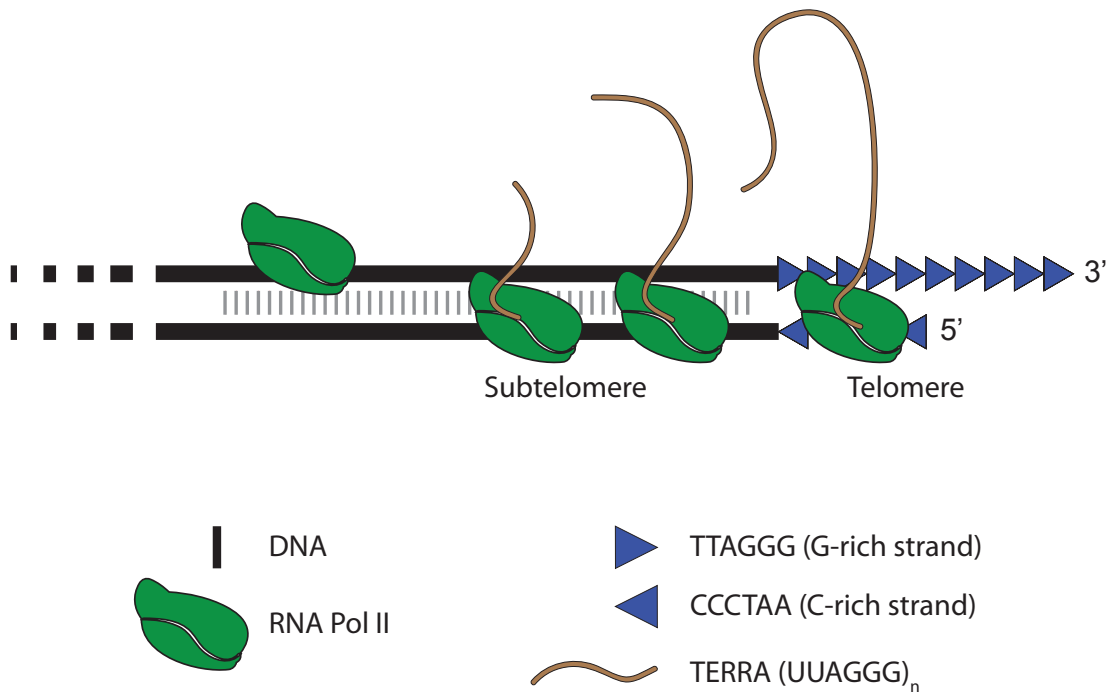
**Figure 3: TERRA transcription.**
Simplified drawing showing TERRA transcription from telomeres. Transcription starts within the subtelomere using the cytosine-rich telomeric strand as a template. TERRA therefore consists of a subtelomeric part and (5'-UUAGGG-3')$_n$ repeats.

some end (Fig. 3); TERRA is therefore composed of subtelomeric sequences and (5'-UUAGGG-3')$_n$ repeats (Azzalin et al., 2007; Schoeftner and Blasco, 2008). In mammalian cells, the G-rich telomeric strand is not transcribed, whereas in Schizosaccharomyces pombe and Saccharomyces cerevisiae, several different telomeric transcripts have been identified, some of which originate from transcription of the telomeric G-rich strand and are called ARIA and ARRET (Bah et al., 2012; Greenwood and Cooper, 2012; Luke et al., 2008). TERRA is produced by DNA-dependent RNAPII-dependent transcription of telomeres (Fig. 3), is only found in the nucleus, and is very heterogeneous in length with lengths of 100 bases to up to 9 kb (Azzalin et al., 2007; Schoeftner and Blasco, 2008). Furthermore, the clear differences in TERRA abundance in different mammalian cell lines point towards cell type- and species-specific TERRA regulation (Azzalin et al., 2007; Azzalin and Lingner, 2008; Schoeft-

ner and Blasco, 2008). Additionally, there is evidence that TERRA abundance correlates with telomere length, as, for example, HeLa cell lines bearing very long telomeres (HeLa I.2.11) have higher TERRA steady-state levels than normal HeLa cells (Azzalin et al., 2007; Schoeftner and Blasco, 2008). Studies have also shown that TERRA molecules have a canonical 7-methylguanosine (m-7G) cap structure, as do mRNAs generated by RNAPII transcription (Porro et al., 2010). A small fraction of TERRA ($\sim$7%) is also polyadenylated, and this modification strongly increases the TERRA half-life (Azzalin et al., 2007; Porro et al., 2010).

### 2.2.2  TERRA localization

Mammalian TERRA has been shown to exclusively occur in nuclear RNA fractions, and RNA fluorescence *in situ* hybridization (FISH) experiments demonstrated that TERRA forms discrete foci during interphase (Azzalin et al., 2007). Furthermore, the combination of RNA FISH and indirect immunofluorescence using antibodies directed against shelterin components revealed that these TERRA foci partially co-localize with telomeres (Azzalin et al., 2007; Schoeftner and Blasco, 2008). This association of TERRA with telomeres has even been demonstrated in transcriptionally silent metaphase cells, which suggests the existence of post-transcriptional mechanisms to retain TERRA at telomeres (Azzalin et al., 2007). The localization of non-telomeric TERRA foci is not entirely clear, but in mouse embryonic fibroblasts (MEFs), some TERRA foci have been shown to overlap with the X inactive-specific transcript (XIST) RNA that coats the inactive X-chromosome in female cells (Schoeftner and Blasco, 2008). In addition, TERRA also associates with both sex chromosomes in male and female mouse embryonic stem cells (ESCs) (Zhang et al., 2009), but it is not yet clear whether these phenomena are specific to mouse cells or whether TERRA binds to chromosome ends *in cis*, *in trans*, or both. Nevertheless, the binding of TERRA to telomeres seems to be tightly controlled,

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as indicated by the discovery of proteins regulating TERRA localization. Several proteins normally involved in nonsense-mediated mRNA decay (NMD), which is a pathway that degrades faulty RNA molecules, have been found to interfere with TERRA localization to telomeres (Azzalin et al., 2007). UPF1, hEST1A/SMG6 and SMG1 are primarily found in the cytoplasm but have also been directly implicated in nuclear processes such as S-phase progression, DNA damage detection and repair, telomere capping, and telomerase regulation(Azzalin and Lingner, 2006; Brumbaugh et al., 2004; Redon et al., 2007; Reichenbach et al., 2003; Snow et al., 2003). The down-regulation of UPF1, hEST1A/SMG6, or SMG1 by shRNA has been shown to increase the number of telomere-associated TERRA foci, whereas the TERRA steady-state levels and half-life were not affected (Azzalin et al., 2007). Furthermore, the down-regulation of any of these three factors also leads to the sudden loss of entire telomeric tracts (Azzalin et al., 2007; Chawla et al., 2011). The possibility that these effects are directly caused by UPF1 or hEST1A/SMG6 depletion rather than by DNA damage at telomeres is supported by the finding that all three proteins do localize to telomeres at low levels (Azzalin et al., 2007; Chawla et al., 2011).

### 2.2.3   TERRA regulation

There is evidence that the heterochromatic environment at telomeres affects TERRA transcription. For example, the treatment of human cells with Trichostatin A (TSA), which is an inhibitor of class I and class II histone deacetylases, leads to an increase in cellular TERRA levels (Azzalin and Lingner, 2008). Furthermore, cancer cells that use the ALT mechanism to maintain their telomeres were shown to have a decreased density of CpG methylation within subtelomeric regions and lower TERRA levels than telomerase-positive cancer cells (Ng et al., 2009). Similarly, cells from patients suffering from immunodeficiency, centromere instability, and facial anomalies (ICF) syndrome, which is a disor-

der caused by mutations in the DNA methyltransferase (DNMT) 3b gene, were found to have higher TERRA levels and hypomethylated subtelomeric DNA (Yehezkel et al., 2008). In agreement with these findings, mouse cells deficient in Suv3-9h and Suv4-20h histone methyltransferases also have higher TERRA levels when compared with wild-type cells (Schoeftner and Blasco, 2008). However, several results directly contradict these findings. For example, mouse cells deficient in the telomerase RNA component have shorter telomeres and lower TERRA levels along with decreased (sub-)telomeric H3K9 and H4K20 methylation and increased levels of histone acetylation (Benetti et al., 2007). In addition, mouse DNMT1 or DNMT3a/b knockout cells have decreased TERRA levels when compared with wild type cells (Schoeftner and Blasco, 2008). Thus, TERRA levels may possibly be regulated by different mechanisms in mouse and human cells. Other proteins that have been implicated in mammalian TERRA regulation are the retinoblastoma family proteins pRB1, p107, and p130; the nuclear lamina component lamin A (LMNA); the shelterin component TRF2; and the histone variant H3.3 (Benetti et al., 2008; Elsaesser et al., 2010; Goldberg et al., 2010; Gonzalez-Suarez et al., 2009). In addition to these proteins, telomere length has also been implicated in TERRA regulation. It has been suggested that long telomeres produce and/or accumulate more TERRA than short telomeres, even though the cell lines used in these experiments were not isogenic (Porro et al., 2010; Redon et al., 2010; Schoeftner and Blasco, 2008). TERRA has also been reported to be down-regulated during S-phase, but whether this is achieved by increasing TERRA degradation or by decreasing transcription is unknown (Flynn et al., 2011; Porro et al., 2010).

### 2.2.4  Putative TERRA functions

Since TERRA was discovered, one of the main questions in the field has been which functions are fulfilled by telomere transcription and the respective

transcripts. Even today, this question remains largely unanswered because of the nuclear localization of TERRA and its transcription from many loci in the genome, which make standard approaches such as the down-regulation of transcripts using short interfering RNAs (siRNA) very difficult. Nonetheless, there is a single study where TERRA was down-regulated using siRNA molecules. In this case the depletion of TERRA has been shown to cause a loss in cell viability, telomeric aberrations, the formation of telomere dysfunction-induced foci (TIFs), and diminished recruitment of the origin recognition complex (ORC) to telomeres (Deng et al., 2009). These results would suggest that TERRA may play basic roles in maintaining telomere integrity; however, it is not clear how TERRA down-regulation by siRNA works, because TERRA is found exclusively in the nucleus, while the components of the RNAi machinery reside in the cytoplasm. Additionally, there is the possibility that these findings are secondary effects that result from the induction of a DNA damage response at telomeres caused by the transfection of TERRA-like siRNA molecules, because the transfection of $(5'\text{-TTAGGG-}3')_n$ oligonucleotides into human cells is known to cause a strong DNA damage response (Milyavsky et al., 2001). A different study suggested that TERRA is important for proper telomere replication. In this study, TERRA was proposed to help mediate the switch from binding of the telomeric overhang by replication protein A (RPA), which binds single-stranded DNA during replication, back to binding by POT1 after successful telomere replication (Flynn et al., 2011). Finally, TERRA has also been implicated in the inhibition of telomerase and the regulation of telomere length. TERRA is complementary in sequence to the RNA template used by telomerase to extend telomeres and therefore could inhibit telomerase simply by base-pairing with the template (Redon et al., 2010; Schoeftner and Blasco, 2008). This hypothesis is apparently supported by several indications: TERRA levels are lower in high-grade tumor cells than in low-grade tumor cells (Schoeftner and Blasco, 2008). Furthermore, TERRA levels are

low during mouse embryogenesis when telomerase is active (Schoeftner and Blasco, 2008), and artificial TERRA-like oligonucleotides have been shown to inhibit telomerase in *in vitro* experiments (Redon et al., 2010; Schoeftner and Blasco, 2008). These findings have led to the hypothesis that telomeres could auto-regulate their length.  Higher TERRA levels produced by long telomeres supposedly cause telomerase inhibition, whereas lower TERRA levels at short telomeres could favor telomerase-mediated telomere elongation (Redon et al., 2010; Schoeftner and Blasco, 2008).

# 3. Results

## 3.1 CpG-island promoters drive transcription of human telomeres

### 3.1.1 Human subtelomeres contain active CpG-island promoters

The subtelomeric DNA sequences associated with the long arm telomeres of human chromosomes X/Y (Xq/Yq) and 10 (10q) were isolated nearly 20 years ago and named TelBam3.4 and TelSau2.0, respectively (Brown et al., 1990).The two sequences share a conserved repetitive region that extends for ~1.6 kb (nucleotides 2110-3117) and ~1.3 kb (nucleotides 408-1789) to ~280 nt upstream of the terminal array in TelBam3.4 and TelSau2.0, respectively (Fig. 4A and B). This conserved region contains three different repetitive DNA tracts: the most centromere-proximal tract comprises tandemly repeated 61-base-pair (bp) units (five repeats in TelBam3.4 versus six repeats in Tel-Sau2.0); a second, more distal tract comprises 29-bp tandem repeats (nine repeats versus 18 repeats); and a third tract comprises five tandemly repeated 37-bp DNA units in both sequences (Fig. 4A and B). We refer to the tandem repeat-containing region as '61-29-37' repeats and to the ~280 nt region between the last 37-bp repeat and the telomeric hexamers as 'pre-tel' (Fig. 4A and B). Five additional subtelomeres containing TelBam3.4-like sequences

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(Fig. 5: 3q, 6p, 9q, 12p, 20p) were previously identified using *in situ* hybridization (Brown et al., 1990). One intrachromosomal locus at 2q13 also contains 61-29-37 repeats (Fig. 5) and corresponds to an ancestral telomere-telomere fusion point (Ijdo et al., 1991). The 61-29-37 repeats possess a remarkably high (67% to 86% ) overall content of CpG dinucleotides, with a peak corresponding to the 29- and 37-bp repeat tracts (Fig. 4C and D). High CpG contents are typical of CpG islands, which are found in broad-type promoters of most eukaryotic RNAPII-transcribed genes (Sandelin et al., 2007). Indeed, a bioinformatic analysis of TelBam3.4 and TelSau2.0 sequences predicted the existence of CpG-islands spanning the 29- and 37-bp repeats (Fig. 4C and D).

To experimentally test the promoter activity of this region, we generated promoter reporter plasmids where progressive 5' deletions of a ∼3.5-kb sub-
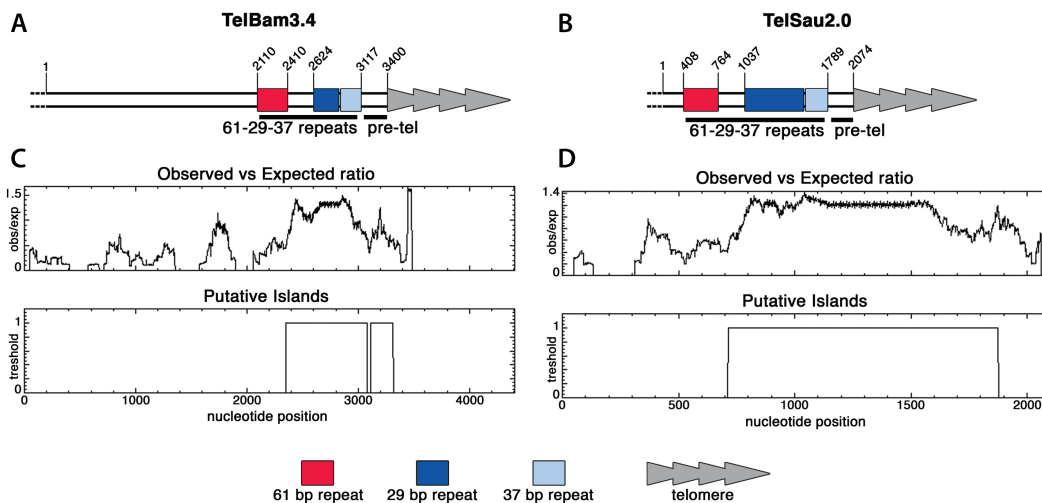


**Figure 4: CpG-island promoters at TelBam3.4 and TelSau2.0 subtelomeres.**
(**A** and **B**) The schematic organization of TelBam3.4 and TelSau2.0 subtelomeric sequences is sketched. Nucleotide positions are indicated by numbers and refer to the sequences deposited in the database (TelBam3.4: M57752.1; TelSau2.0: M57753.1). The 61-bp tandem repeats are indicated by red boxes, the 29-bp repeats by dark blue boxes and the 37-bp repeats by light blue boxes. (**C** and **D**) Graphs show the ratio between observed and expected CpG dinucleotides in the TelBam3.4 and TelSau2.0 sequences and the prediction of putative CpG-island promoters; we utilized the CpGPlot/CpGReport tool at EMBOSS (Rice et al., 2000). Nucleotide positions are indicated by numbers. Two different scales were used for TelBam3.4 (**C**) and TelSau2.0 graphs (**D**).

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3.1. CpG-island promoters drive
transcription of human telomeres

Results



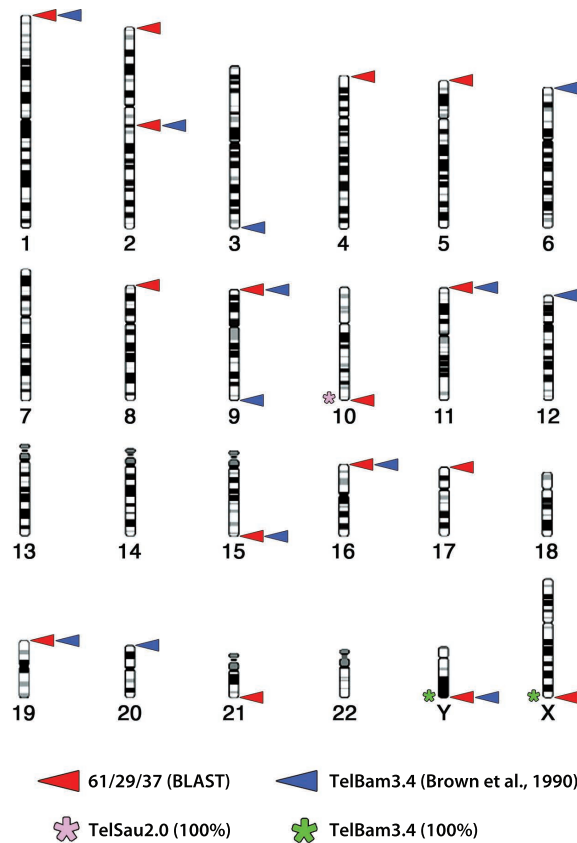**Figure 5: Chromosomal distribution of 61-29-37 repeats, TelBam3.4 and TelSau2.0 sequences.**
Chromosome numbers are indicated below each ideogram. Red arrows indicate chromosomal loci containing 61-29-37 repeats as derived from BLAST analsis. Blue arrows indicate chromosomal loci detected in *in situ* hybridization experiments performed using the TelBam3.4 DNA as a probe (Brown et al., 1990). Asterisks indicate 100% sequence identity to TelBam3.4 and TelSau2.0 complete sequences.

telomeric DNA tract composed of 61-29-37 repeats were inserted upstream of an enhanced green fluorescence protein (eGFP) reporter gene (Fig. 6A). Fluorescence microscopy of human HeLa cells transfected with reporter plasmids revealed that a ~1-kb sequence comprising the 29- and 37-bp repeat tracts was sufficient to induce cellular eGFP expression (Fig. 6B). Quantification of eGFP mRNA by quantitative reverse transcription polymerase chain reaction (qRT-PCR) showed that the identified promoter is approximately five times less efficient than the strong cytomegalovirus (CMV) promoter (Fig. 6C). Thus, 29-

37 subtelomeric repeats contain sequences with promoter activity.

TERRA has been shown to originate from transcription by RNAPII (Azzalin and Lingner, 2008; Schoeftner and Blasco, 2008). Therefore we performed chromatin immunoprecipitation experiments on formaldehyde cross-
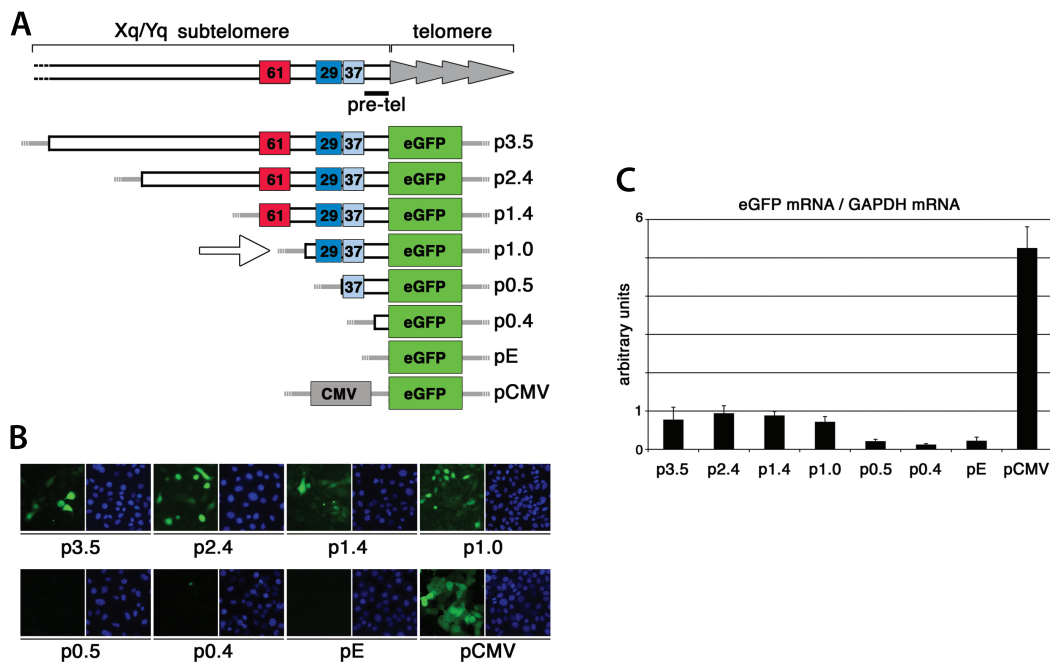


**Figure 6: Promoter reporter assays.**
(**A**) A ~3.5-kb DNA fragment from Xq/Yq subtelomeres was cloned upstream of an eGFP cDNA (green box). The fragment contains five 61-bp tandem repeats (red box), ten 29-bp repeats (dark blue box), and five 37-bp repeats (light blue box). The original reporter plasmid was digested with restriction enzymes in order to generate plasmids containing serial 5' deletions. Plasmid names are given on the right. pE: promoter-less eGFP negative control plasmid; pCMV: cytomegalovirus promoter eGFP positive control plasmid. Gray lines indicate plasmid backbone sequences. The arrow points to the reporter plasmid containing the minimal DNA tract sufficient to induce eGFP expression. (**B**) Reporter plasmids containing the puromycin resistance gene were transfected into HeLa cells. Puromycin was added 24 h after transfection, and 4 d later cells were fixed and imaged by fluorescence microscopy to detect eGFP expression (green, left panels) and DAPI stained DNA (blue, right panels). (**C**) HeLa cells were transfected with the indicated reporter plasmids and five days after transfection total RNA was extracted. RNA was reverse transcribed using random hexamer oligonucleotides and cDNA was PCR amplified using primer pairs specific for eGFP and GAPDH sequences. All values obtained for the reverse transcribed samples were first background-corrected using corresponding minus RT controls, and eGFP values were then normalized using the corresponding GAPDH values. Bars and error bars indicate averages and standard deviations derived from triplicate measurements of cDNA from two independent experiments.

linked chromatin from U2OS cells using independent antibodies against the phosphorylation-activated RNAPII large subunit (Fig. 7A; Hirose and Ohkuma, 2007), to test whether RNAPII binds to TERRA CpG-island promoters. Using probes to detect 61-29-37 repeats, telomeric (5'-TTAGGG-3')$_n$ and Alu repeat sequences, we found active RNAPII to be enriched at 61-29-37 and telomeric repeats over repetitive Alu sequences *in vivo* (Fig. 7B). Overall, these results reveal the existence of CpG-island promoters within human subtelomeres that are located in close proximity to the telomeric tracts on roughly half of all human subtelomeres and are bound by active RNAPII *in vivo*.
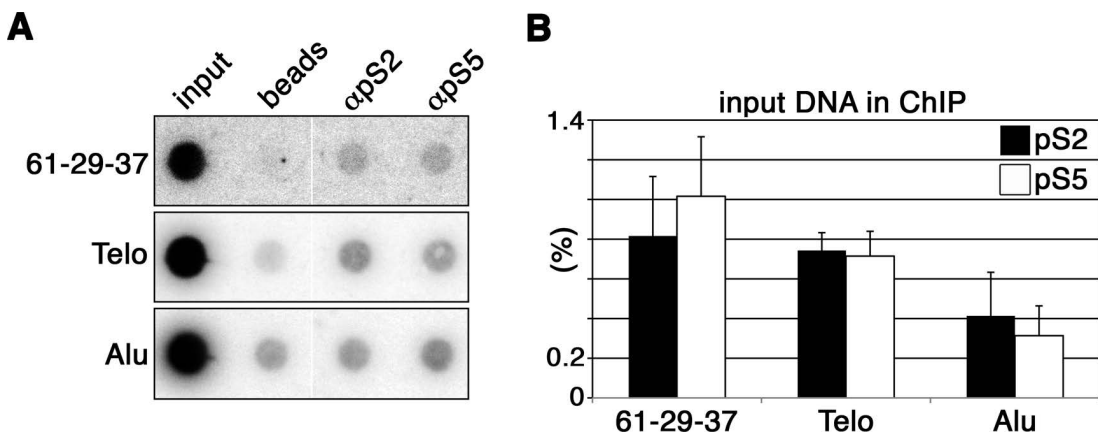


**Figure 7: RNAPII binds to 61-29-37 repeats *in vivo***
(**A**) Formaldehyde cross-linked chromatin from U2OS cells was immunoprecipitated using antibodies raised against phosphorylated serine 2 (pS2) and serine 5 (pS5) from the C-terminal repeat of human RNAPII. Immunoprecipitated DNA was dot-blotted and hybridized with radioactive DNA probes detecting 61-29-37 repeats.  The same blot was stripped and re-hybridized sequentially to detect repetitive telomeric (positive control) and Alu sequences. (**B**) The bar graph shows the fraction of input DNA immunoprecipitated in the different samples, after subtraction of the background signal measured for control reactions performed using only beads. Bars and error bars represent averages and standard deviations from three independent immunoprecipitations.

## 3.1.2 Subtelomeric CpG-island promoters drive transcription of TERRA molecules

To determine whether TelBam3.4 and TelSau2.0 subtelomeres are transcribed *in vivo*, we performed northern blot analysis of nuclear RNA extracted from human telomerase-negative primary lung fibroblasts (HLF) and U2OS tumor cells as well as telomerase-positive HeLa and HEK293T tumor cells using strand-specific DNA probes, which correspond to the TelBam3.4 and TelSau2.0 pre-tel sequences (Fig. 9A). The TelBam3.4 probe is 100% identical to Xq/Yq pre-tels; 92%-98% identical to 8p, 9p, 15q, and 19p pre-tels; and 90%-91% identical to 10q and 21q pre-tels. The TelSau2.0 probe is 100% identical to 10q pre-tel sequences and 89%-95% identical to 8p, 9p, 16p, 15q, 19p, and 21q pre-tels. The two probes are therefore expected to detect partially overlapping families of transcripts originating from various chromosome ends,



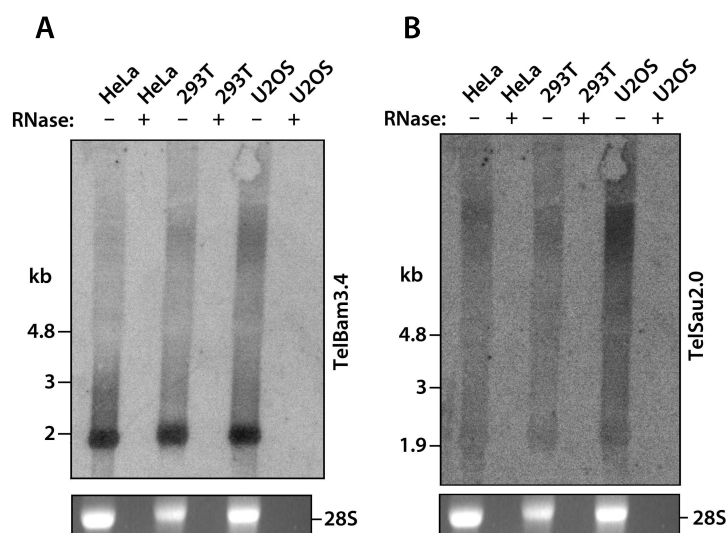**Figure 8: TelBam3.4 and TelSau2.0 northern blot signals stem from RNA.**
(**A** and **B**) .Northern blot analysis of RNA from the indicated cell lines that has been RNase treated (+) or left untreated (−). The membrane was first hybridized with a TelBam3.4 probe and subsequently stripped and hybridized with a TelSau2.0 probe. Ethidium Bromide stained 28S rRNA transcripts are shown to confirm equal loading.

**Figure 9: TERRA transcription from TelBam3.4 and TelSau2.0 subtelomeres.**
(**A**) Schematic representation of a 61-29-37 repeat-containing chromosome end. Note that the sketch is not to scale. (**B**) Nuclear RNA extracted from the indicated cell lines was electrophoresed and hybridized with radioactive strand-specific probes corresponding to TelBam3.4 or TelSau2.0 pre-tel sequences (black bar in A). (**C**) The blots hybridized with TelBam3.4 probes were stripped and probed for total TERRA. Ethidium bromide-stained 28S and 18S rRNA transcripts are shown to confirm equal RNA loading. Standard molecular weights are on the left in kilobases (kb).

even when the hybridizations are performed at higher stringency. After high-stringency washes, both probes detected RNA species varying in length from ~500 b up to more than 5 kb in the different cell lines tested (Fig. 8 and 9B). The radioactive signals were completely abolished upon ribonuclease A (RNase A) treatment (Fig. 8A and B), which confirmed that they originated from RNA. We also obtained smeared patterns of hybridization when we hybridized the same filters with telomeric probes, although the telomeric signal

extended to molecular weights lower than 100 b (Fig. 9C). These results suggest that transcripts originating from TelBam3.4 and TelSau2.0 subtelomeres may constitute a fraction of TERRA molecules. The total cellular TERRA probably also comprises transcripts derived from subtelomeres devoid of TelBam3.4 and TelSau2.0 sequences, as well as from 5' processing of TelBam3.4 and TelSau2.0 RNA molecules. Interestingly, whereas the signal detected with the TelBam3.4 and TelSau2.0 probes remains fairly constant in all tested cell lines, the signal detected using telomeric probes indicates a two- to eight-fold increase in total TERRA levels in U2OS cells, which suggests that in these cells, a substantial fraction of the TERRA transcripts does not contain TelBam3.4 and TelSau2.0 sequences. Although the molecular mechanisms leading to increased TERRA levels in U2OS cells remain unclear, a connection may exist between TERRA cellular expression and the decreased density of CpG methylation within subtelomeric regions in ALT positive cancer cells like U2OS (Ng et al., 2009; Royle et al., 2008). To determine whether TelBam3.4 and TelSau2.0 subtelomeres are indeed transcribed into TERRA molecules,
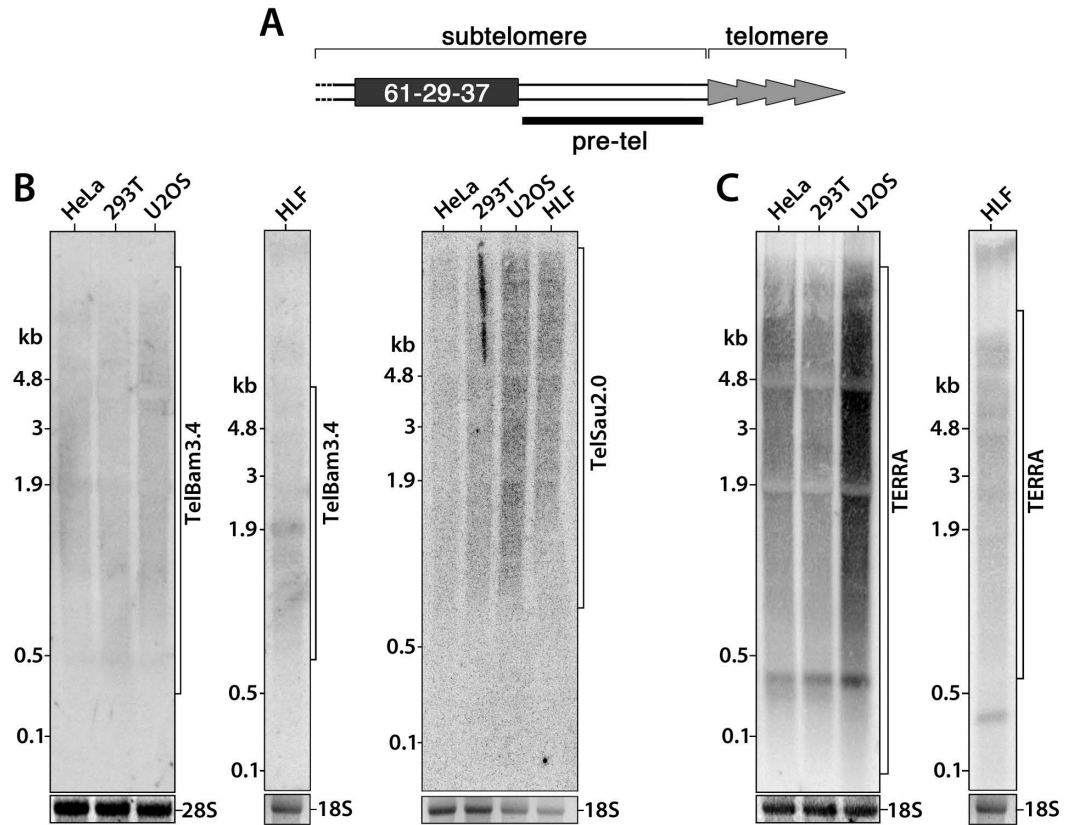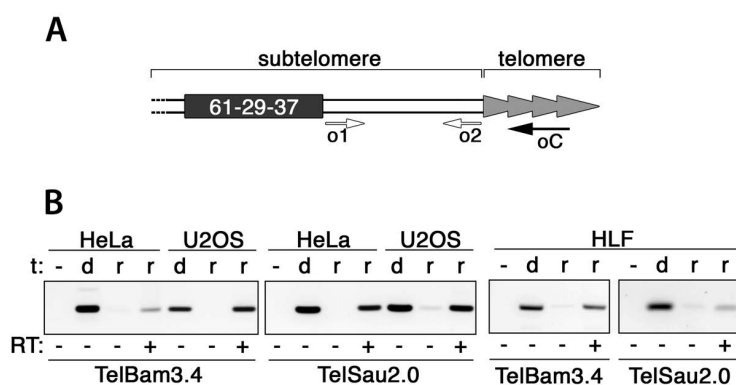


**Figure 10: Transcription of TelBam3.4 and TelSau2.0.**
(**A**) Schematic representation of a 61-29-37 repeat-containing chromosome end. Note that the sketch is not to scale. (**B**) Nuclear RNA (r) from the indicated cell lines was reverse transcribed (RT) using oC oligonucleotides, and cDNA was PCR amplified using o1 and o2 oligonucleotides specific for TelBam3.4 or TelSau2.0 pre-tel sequences (oligonucleotide positions are indicated by arrows in A). PCR products were run in agarose gels and stained. Control PCR reactions were performed using genomic DNA (d) as template (t).
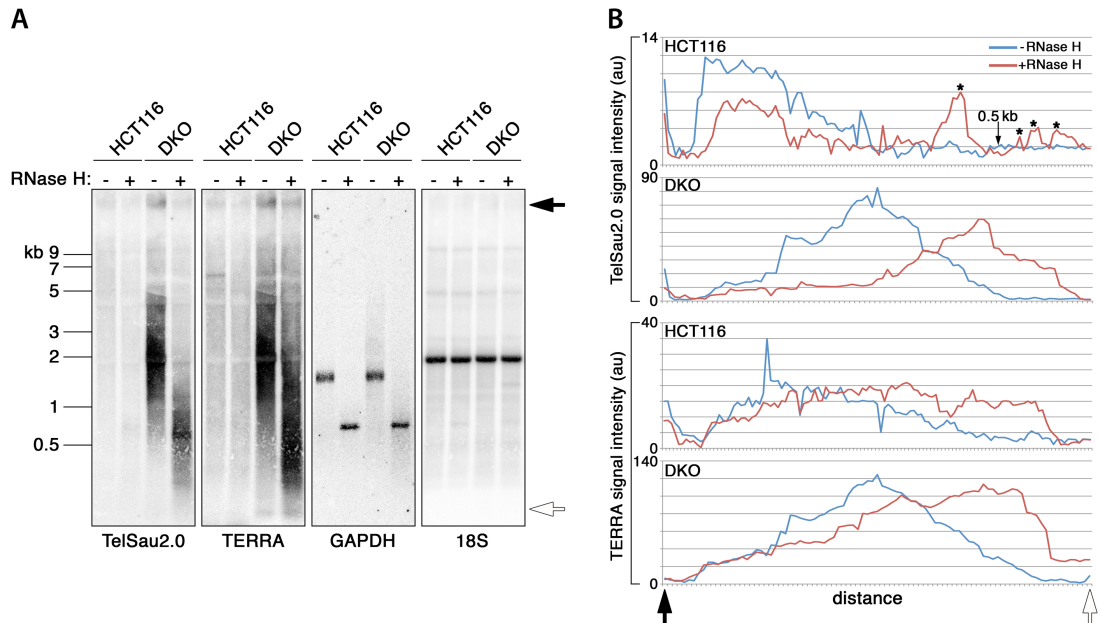
**Figure 11: Transcripts from TelSau2.0 and TelBam3.4 subtelomeres constitute a substantial fraction of total TERRA molecules.**
(**A**) Nuclear RNA from the indicated cell lines was annealed to a DNA oligonucleotide 100% and 70% complementary to TelSau2.0 and TelBam3.4 pre-tel transcripts, respectively. The same RNA was also annealed to a DNA oligonucleotide complementary to GAPDH mRNA. RNA was incubated with RNase H or left untreated, electrophoresed, and hybridized with radioactive strand-specific probes corresponding to TelSau2.0 pre-tel sequences. The same blot was stripped and probed sequentially to detect total TERRA, GAPDH (positive control for RNAse H reaction), and 18S (control for RNase H specificity and loading control) RNA sequences. Standard molecular weights are shown on the left in kilobases (kb). (**B**) Charts showing the distribution of signal intensity for the Northern blots shown in A. The asterisks indicate RNase H-induced peaks in the TelSau2.0 hybridization profile of HCT116 RNA. The peaks are likely to correspond to TelSau2.0-like pre-tel sequences from which the telomeric RNA tracts have been removed by the RNase H treatment. The black and white arrows indicate the lane area used for signal quantifications. Similar shifts in the telomeric profiles were obtained when RNA was incubated only with TelSau2.0 oligonucleotides, omitting the GAPDH oligonucleotides from the reactions (data not shown).

we employed an RT-PCR protocol previously developed to detect TERRA transcripts (Azzalin et al., 2007). We reverse transcribed nuclear RNA using a telomeric (5'-CCCTAA-3')$_5$ oligonucleotide (Fig. 10A, oC; Table 1), complementary to the (5'-UUAGGG-3')$_n$ sequence present in TERRA molecules. We then PCR-amplified the obtained cDNA with unique primer pairs corresponding to the TelBam3.4 and TelSau2.0 pre-tel sequences immediately adjacent

to the telomeric tracts in the genome (Fig. 6A: o1 and o2; Table 1). We obtained amplicons that matched the size (Fig. 10B) and sequence (data not shown) of amplicons obtained in PCR reactions performed on genomic DNA, thereby proving that the tested subtelomeres are indeed transcribed and that the transcription continues into the telomeric tract. To reinforce these findings, we annealed nuclear RNA prepared from telomerase-positive HCT116 human cancer cells with a TelSau2.0 DNA oligonucleotide adjacent to the telomeric tracts in the genome (Fig. 10: o2; Table 1). The oligonucleotide is 100% and 70% complementary to sequences from TelSau2.0 and TelBam3.4 pre-tel transcripts, respectively. We then treated the RNA with RNase H, which specifically digests RNA molecules engaged in DNA/RNA hybrids, and subjected the digested RNA to northern blot analysis with radioactive telomeric probes. A quantitative analysis of the obtained hybridization profiles reproducibly revealed that the RNase H treatment resulted in a shift in the telomeric hybridization profile toward lower molecular weights (Fig. 11). Taken together, these results indicate that TERRA molecules originating from the TelBam3.4 and TelSau2.0 subtelomeres exist in different human cell lines and constitute a substantial fraction of total TERRA.
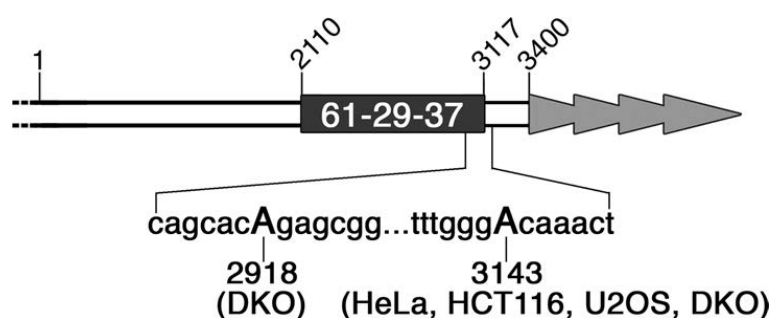


**Figure 12: TERRA transcription start sites.**
RACE experiments performed on nuclear RNA from HeLa, U2OS, and HCT116 cells and from HCT116-derived cells knockedout for DNA methyltransferases 1 and 3b (double KO, DKO) identified the capitalized adenines as transcription start sites at TelBam3.4 subtelomeres. Numbers indicate nucleotide positions as they appear in the database entry M57752.1.

We then used rapid amplification of cDNA ends (RACE) to isolate intact 5'
ends of TelBam3.4 transcripts from cDNA obtained by reverse transcription of
HeLa, HLF, HCT116, and U2OS nuclear RNA with the telomeric oC oligonu-
cleotide (Fig. 10; Table 1). Our RACE protocol permits the amplification of
RNA molecules with a methylated 5' cap only, thereby preventing the detection
of uncapped and degraded RNA species. In all cell lines, we identified one
unique transcription start site located 27 nt downstream from the last 37-bp
repeat (Fig. 13) that is conserved on chromosomes 8p, 9p, 15q, 16p, and 19p,
as well as on Xq/Yq subtelomeres. Although our extensive analysis of RACE
products identified only one transcription start site, we cannot exclude the pos-
sibility that alternative, perhaps more rare, start sites may exist in the same
cells. Nevertheless, the transcription of a fraction of TERRA molecules begins
immediately downstream from the 61-29-37 repeats and continues towards the
chromosome ends. Because the physical distance between the identified tran-
scription start site and the telomeric tract is ∼250 nt (Fig. 12D), whereas the
length of TelBam3.4 transcripts visualized by northern blotting varies between
∼500 and >5000 b (Fig. 9B), we infer that transcription can proceed through
the telomeric tract for several kilobases.

### 3.1.3  TERRA promoters are methylated at CpG dinucleotides and cytosine methylation regulates TERRA cellular levels

DNMT-mediated methylation of cytosines at promoter CpG dinucleotides neg-
atively regulates transcriptional activity (Esteller, 2007; Suzuki and Bird, 2008).
Furthermore, DNMT3b-deficient human cell lines established from patients
affected by ICF syndrome are characterized by hypomethylated subtelom-
eric DNA and increased TERRA expression compared with normal cells from
healthy individuals (Yehezkel et al., 2008). Promoter CpG methylation may
therefore regulate TERRA expression.

We digested HLF, HeLa, and U2OS genomic DNA with the methylation-sensitive HpaII restriction enzyme or with its methylation-insensitive isoschizomer MspI and performed Southern blot hybridizations with radiolabeled DNA probes that corresponded to the 61-29-37 repeats. Whereas MspI digestion generated prominent hybridization bands ranging in size from 100 to 500 bp, additional bands of higher molecular weight, i.e. up to greater than 23 kb, appeared in HpaII-digested samples (Fig. 13A), which indicates
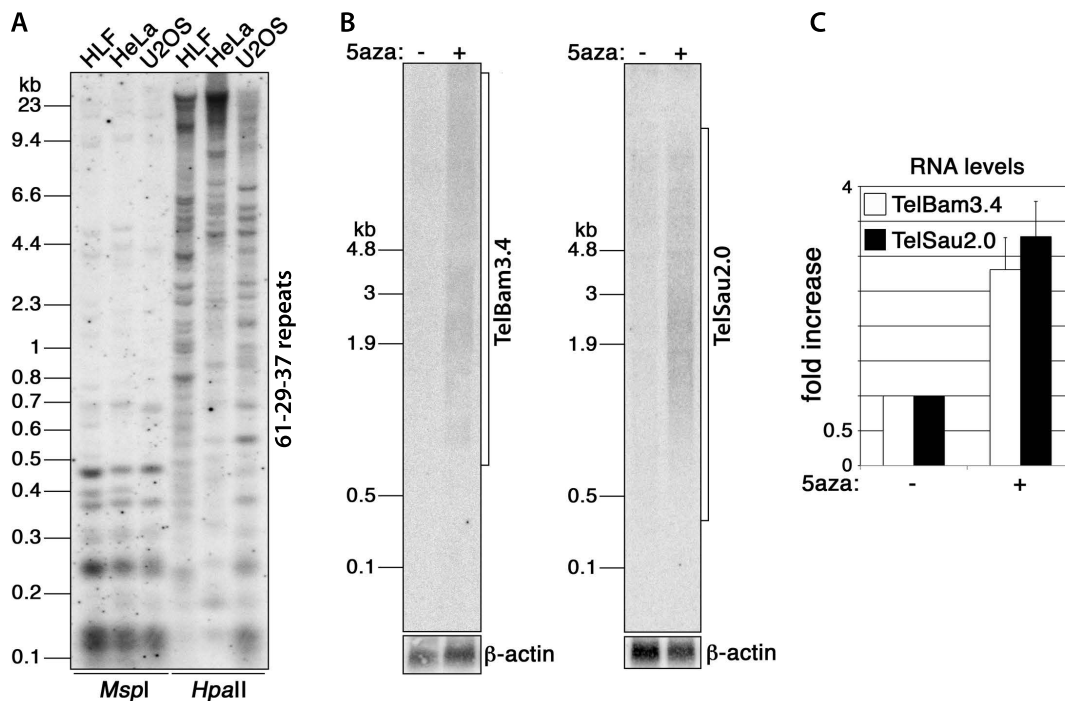


**Figure 13: Cytosine methylation at 61-29-37 repeats.**
(**A**) Genomic DNA extracted from the indicated cell lines was digested with the methylation-sensitive HpaII restriction enzyme or with its methylation-insensitive isoschizomer MspI. Digested DNA was electrophoresed, blotted, and hybridized with radioactive DNA probes detecting 61-29-37 repeats. Standard molecular weights are shown on the left in kilobases (kb). (**B**) HeLa cells were treated with 5-azacytidine (5aza) for 48 hours or left untreated. Nuclear RNA was extracted, electrophoresed and hybridized using strand-specific TelBam3.4 or TelSau2.0 radioactive probes. The same blots were stripped and re-probed for β-actin. Standard molecular weights are shown on the left in kilobases (kb). (**C**) TelBam3.4 and TelSau2.0 transcripts from northern blots were quantified and values were expressed as fold increase over untreated samples after normalization using the β-actin signal. Bars and error bars indicate averages and standard deviations from three independent experiments.
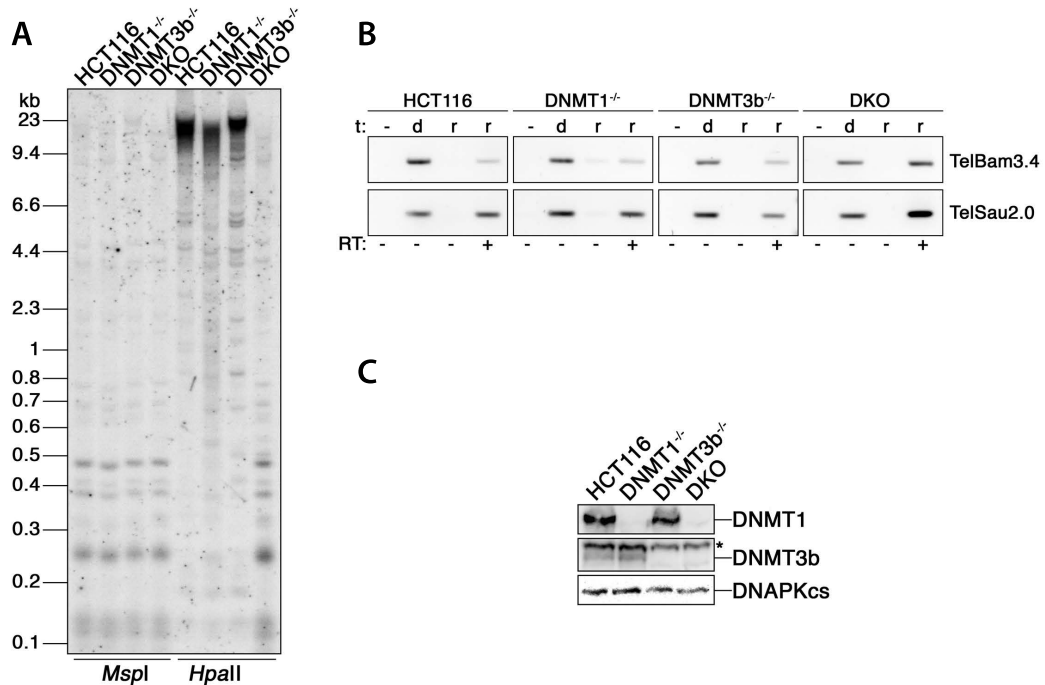
**Figure 14: 61-29-37 repeats methylation and TelBam3.4 and TelSau2.0 transcription in DNMT knock-out cell lines.**

(**A**) Genomic DNA extracted from the indicated cell lines was digested with the methylation-sensitive HpaII restriction enzyme or with its methylation-insensitive isoschizomer MspI. Digested DNA was electrophoresed, blotted, and hybridized with radioactive DNA probes detecting 61-29-37 repeats. DNMT1$^{-/-}$ and DNMT3b$^{-/-}$ are HCT116-derived clonal cell lines knocked-out for the DNA methyltransferases 1 and 3b, respectively. Double KO (DKO) are HCT116-derived clonal cell lines knocked-out for both methyltransferases. Standard molecular weights are shown on the left of each blot in kilobases (kb). (**B**) Nuclear RNA (r) from the indicated cell lines was reverse transcribed (RT) using telomeric oC oligonucleotides and cDNA was PCR amplified using o1 and o2 oligonucleotides (Fig. 10A;Table 1) specific for TelBam3.4 or TelSau2.0 pre-tel sequences. PCR products were run in agarose gels and stained. Control PCR reactions were performed using genomic DNA (d) as template (t). (**C**) To confirm ablation of DNMT1 and DNMT3b genes, western blot analysis was performed using nuclear protein extracts from the indicated cell lines. Western blots were probed using rabbit polyclonal antibodies against human DNMT1 (Abcam, ab16632), DNMT3b (Abcam, ab16049) and DNAPKcs (Bethyl Laboratories, A300-516A; loading control). The asterisk indicates an unspecific band detected by the anti-DNMT1 antibody.

that 61-29-37 repeats are methylated *in vivo*.

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The 61-29-37 repeat methylation appears to be nearly complete in HeLa cells, whereas a lower degree of methylation is observed in HLF and U2OS cells as demonstrated by increased size heterogeneity of the hybridization bands in HpaII-digested samples (Fig. 13A). This is consistent with recent observations showing that telomerase-positive human cancer cells carry sub-telomeric DNA that is hypermethylated when compared to the subtelomeric DNA of telomerase-negative cancer or primary human cells (Ng et al., 2009; Tilman et al., 2009; Vera et al., 2008). Next, to test whether the methylation of 61-29-37 repeats affects TERRA transcription, we treated cells with the DNMT inhibitor 5-azacytidine, followed by northern blot analysis (Fig. 13B). Quan-



| | TelBam3.4 | TelSau2.0 | TERRA |
|---|---|---|---|
| HCT116 | 1 | 1 | 1 |
| DNMT1$^{-/-}$ | 1.2 | 1.2 | 1.1 |
| DNMT3b$^{-/-}$ | 1.3 | 1.3 | 1.2 |
| DKO | 6.1 | 9.8 | 4.8 |
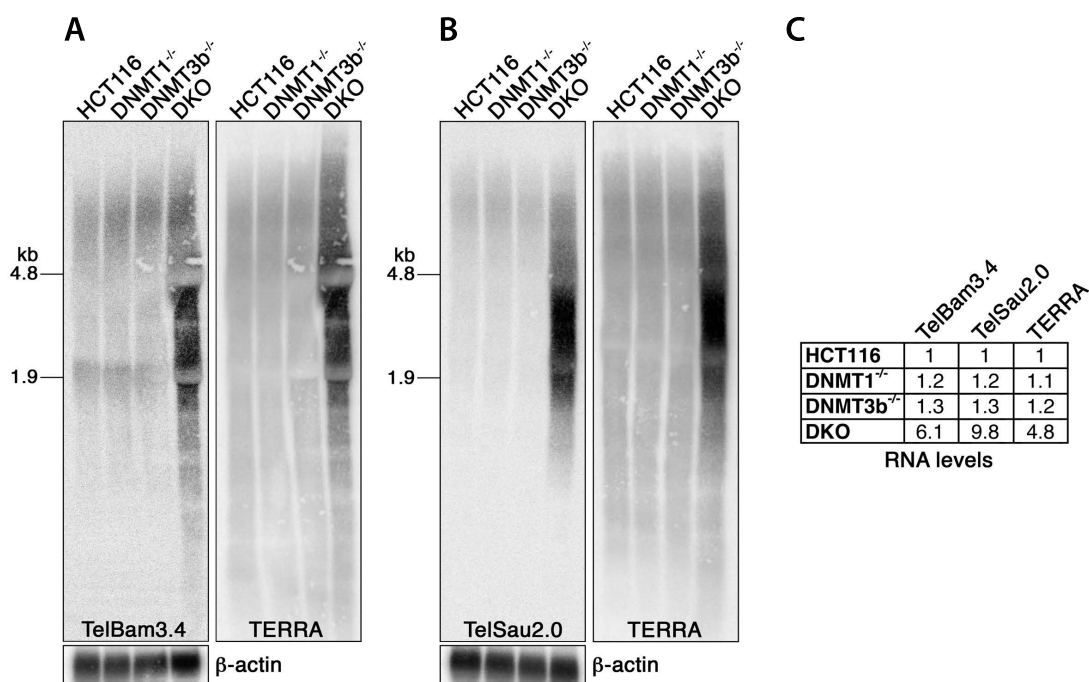
RNA levels

**Figure 15: TERRA levels in DNMT knock-out cell lines.**
(**A**, **B**) Nuclear RNA was extracted from the indicated cell lines, electrophoresed, blotted, and hybridized using strand-specific TelBam3.4 or TelSau2.0 radioactive probes. The same blots were stripped and re-probed sequentially for total TERRA and β-actin. Molecular weights of 28S (4.8-kb) and 18S (1.9-kb) rRNA are shown on the left. (**C**) Quantifications of TelBam3.4, TelSau2.0 and total TERRA transcripts shown in A and B. Northern blot signals were quantified, normalized through the relative β-actin signals and expressed as fold increase over parental cells.
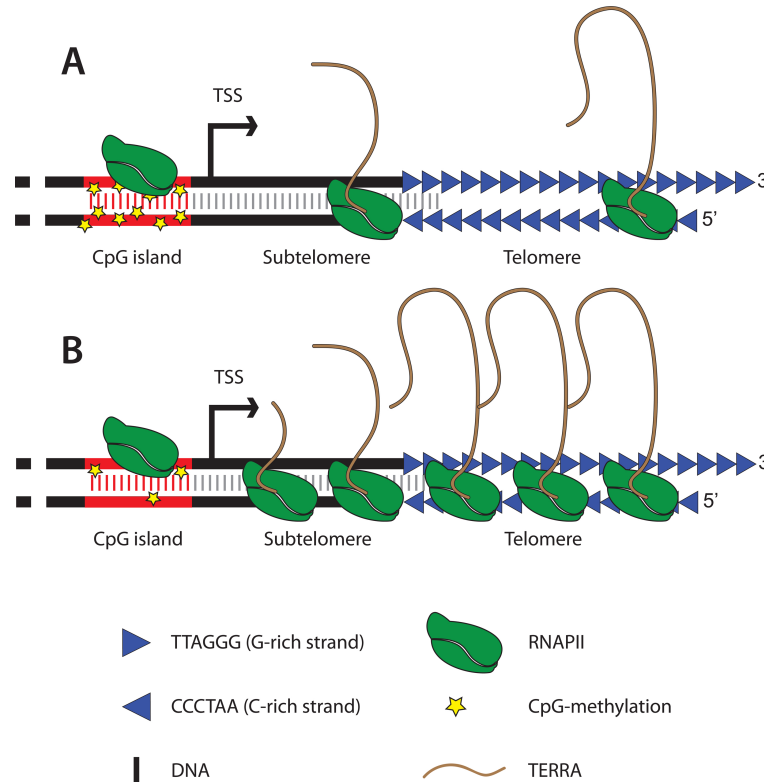
**Figure 16: CpG methylation regulates TERRA transcription.**
Simplified drawing of TERRA transcriptional regulation.  (**A**) Increased CpG methylation
at TERRA CpG-island promoters leads to TERRA transcriptional repression.  (**B**) Loss of
subtelomeric CpG-methylation leads to an increase in TERRA transcription.

tification of TelBam3.4 and TelSau2.0 transcripts revealed a roughly three-fold
increase in steady-state levels upon 5-azacytidine treatment (Fig.  13C). To
identify the DNMTs responsible for the methylation of the 61-29-37 repeats, we
used human HCT116 cells lacking DNMT1 (DNMT1$^{-/-}$), DNMT3b (DNMT3b$^{-/-}$)
or both (DKO) methyltransferases (Fig. 14C; Rhee et al., 2000, 2002). We an-
alyzed the methylation state of the 61-29-37 repeats in these cell lines and
found only modest changes in the single knockout cells.  In DKO cells, how-
ever, the methylation of the 61-29-37 repeats was completely abolished, which
implies that the DNMT1 and DNMT3b enzymes cooperatively maintain DNA
methylation at the 61-29-37 repeats in HCT116 cells (Fig.  14A). Next, we
confirmed by RT-PCR that TelBam3.4 and TelSau2.0 subtelomeres are tran-

scribed in HCT116 cells and the derived DNMT knock-out cell lines (Fig. 14B). Furthermore, northern blot analysis revealed dramatically increased steady-state levels of TelBam3.4, TelSau2.0, and total TERRA transcripts in DKO cells compared with the parental, DNMT1$^{-/-}$, and DNMT3b$^{-/-}$ cells (Figs. 11A, 15). The extra TelBam3.4 and TelSau2.0 transcripts detected in the DKO cells (Figs. 11, 15A and B) appear to be true TERRA molecules for several reasons. First, RT-PCR experiments using telomeric oC oligonucleotides for reverse transcription produced TelBam3.4 and TelSau2.0 amplicons whose sequences matched the corresponding genomic sequences both in parental and DKO cells (Fig. 14B). Second, RACE experiments performed on nuclear RNA prepared from DKO cells identified the same TelBam3.4 transcription start site used in parental HCT116 cells (Fig. 12, nucleotide 3143) in 31 of the 41 analyzed positive RACE plasmid clones and a second transcription start site within the first 37-bp repeat (Fig. 12, nucleotide 2918) in the remaining 10 clones. Third, RNase H digestion of DKO nuclear RNA, that was previously an-
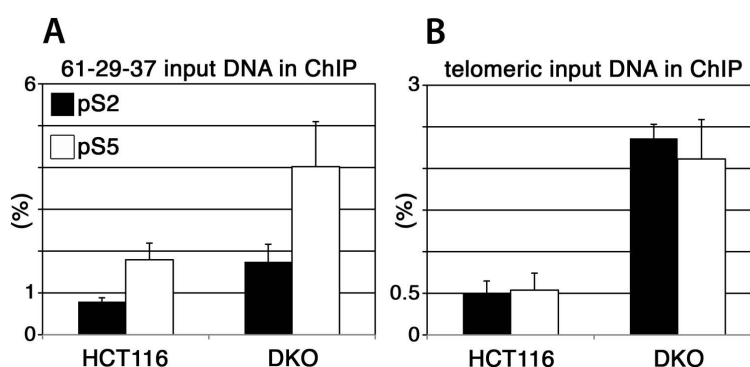


**Figure 17: Increased binding of RNAPII to 61-29-37 repeats in DKO cells.**
(**A**, **B**) Formaldehyde cross-linked chromatin from the indicated cell lines was immunoprecipitated using antibodies raised against phosphorylated serine 2 (pS2) and serine 5 (pS5) from the C-terminal repeat of human RNAPII. Immunoprecipitated DNA was dot-blotted and hybridized sequentially with radioactive DNA probes detecting 61-29-37 and telomeric repeats. Graphs show the fraction of input DNA immunoprecipitated in the different samples, after subtraction of the background signal measured for control reactions performed using only beads. Bars and error bars represent averages and standard deviations of three independent immunoprecipitations.

nealed with TelSau2.0 DNA oligonucleotides induced a substantial shift of the TERRA hybridization profile toward lower molecular weights (Fig. 11A and B). In conclusion, CpG methylation appears to modulate the cellular abundance of TERRA (including TERRA transcribed from TelBam3.4 and TelSau2.0 subtelomeres) as well as the usage of independent transcription start sites. We hypothesize that CpG methylation represses TERRA promoter transcriptional activity (Fig. 16). Consistent with this hypothesis, ChIP experiments revealed increased levels of active RNAPII binding to 61-29-37 and telomeric repeat DNA in DKO cells compared with parental HCT116 cells (Fig. 17). However, we cannot exclude the possibility that impaired degradation of TERRA transcripts could occur in DKO cells, thereby contributing to the increased steady-state levels.

## 3.2 Telomerase efficiently elongates highly transcribing telomeres in human cancer cells

### 3.2.1 TERRA steady-state levels are maintained independently of telomere length in human cancer cells

The proposed role for TERRA in inhibiting telomerase activity has suggested a model in which long telomeres accumulate or produce more TERRA, thereby preventing telomerase from further extending them (Porro et al., 2010; Redon et al., 2010; Schoeftner and Blasco, 2008). Correlative evidence using nonisogenic mammalian cells lines of different origins seems to support this hypothesis, as does the finding that DKO cells with highly increased TERRA levels harbor shorter telomeres compared with HCT116 cells (Fig. 18; Schoeftner and Blasco, 2008). In contrast, recent studies in budding yeast have shown that experimentally induced over-elongation or shortening of telomeres does not alter TERRA levels or RNAPII occupancy at the chromosome ends (Iglesias et al., 2011). We therefore tested whether a correlation exists between TERRA levels and telomere length in isogenic human cells with telomeres of different lengths. We stably infected cervical cancer HeLa cells with retro-
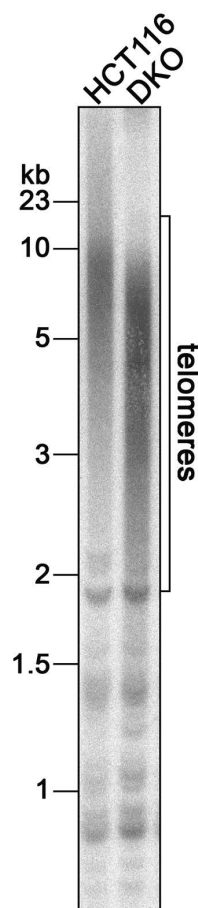


**Figure 18: DKO cells have shorter telomeres than HCT116 cells.**
TRF analysis of genomic DNA from HCT116 and DKO cells. Molecular weights are indicated on the left. Note that telomeres in DKO cells are shorter than in parental HCT116 cells.

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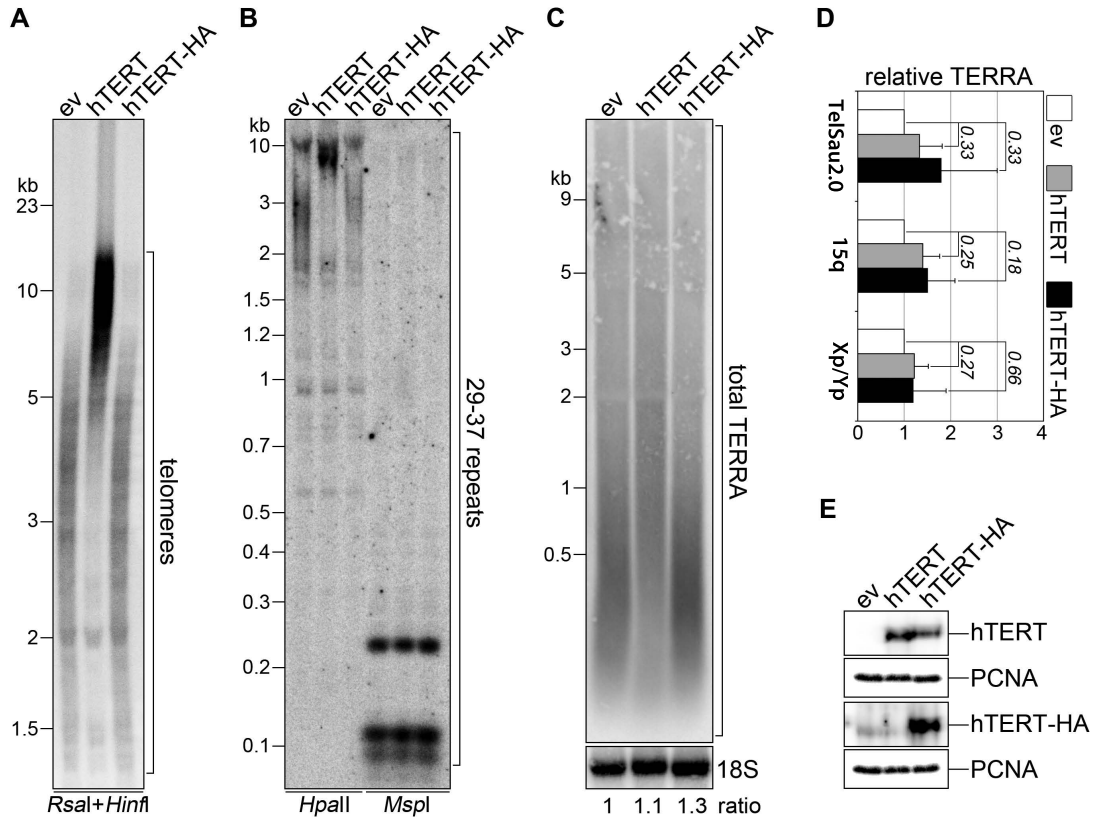
**Figure 19: TERRA steady-state levels are not affected by telomere elongation in HeLa cells.**
(**A**) TRF analysis of HeLa cells infected with empty vector (ev), hTERT or hTERT-HA retroviruses. DNA was digested with RsaI and HinfI restriction enzymes and hybridized with telomeric probes. (**B**) The same DNA as in A was digested with HpaII (methylation sensitive) or MspI (methylation insensitive) restriction nucleases and hybridized with a probe detecting the 29-37 bp repeats of TERRA promoters. (**C**) Nuclear RNA was hybridized using telomeric probes to detect total TERRA and successively with 18S rRNA probes to control for loading. Numbers at the bottom are the ratios between TERRA and 18S signal expressed as fold increase over ev-infected samples. Molecular weights are on the left in kilobases. (**D**) qRT-PCR analysis of the steady-state levels of TERRA transcripts originating from TelSau2.0, 15q and Xp/Yp chromosome ends. Bars are averages from three independent experiments expressed as fold increase over ev-infected samples. Error bars and numbers are standard deviations and P-values (two-tailed t-test), respectively. (**E**) Western blot analysis of infected cells using anti-hTERT (to detect all hTERT molecules), anti-HA (to detect hTERT-HA) and anti-PCNA (loading control) antibodies.

viruses expressing the catalytic subunit of human telomerase (hTERT). As a control, we infected the same cells with empty vector (ev) retroviruses or with retroviruses expressing hTERT C-terminally fused to an influenza hemagglu-

tinin epitope tag (hTERT-HA). hTERT-HA displays normal catalytic activity *in vitro* but fails to elongate telomeres *in vivo*, likely because of impaired recruitment to telomeres (Counter et al., 1998). Stably infected populations were se-
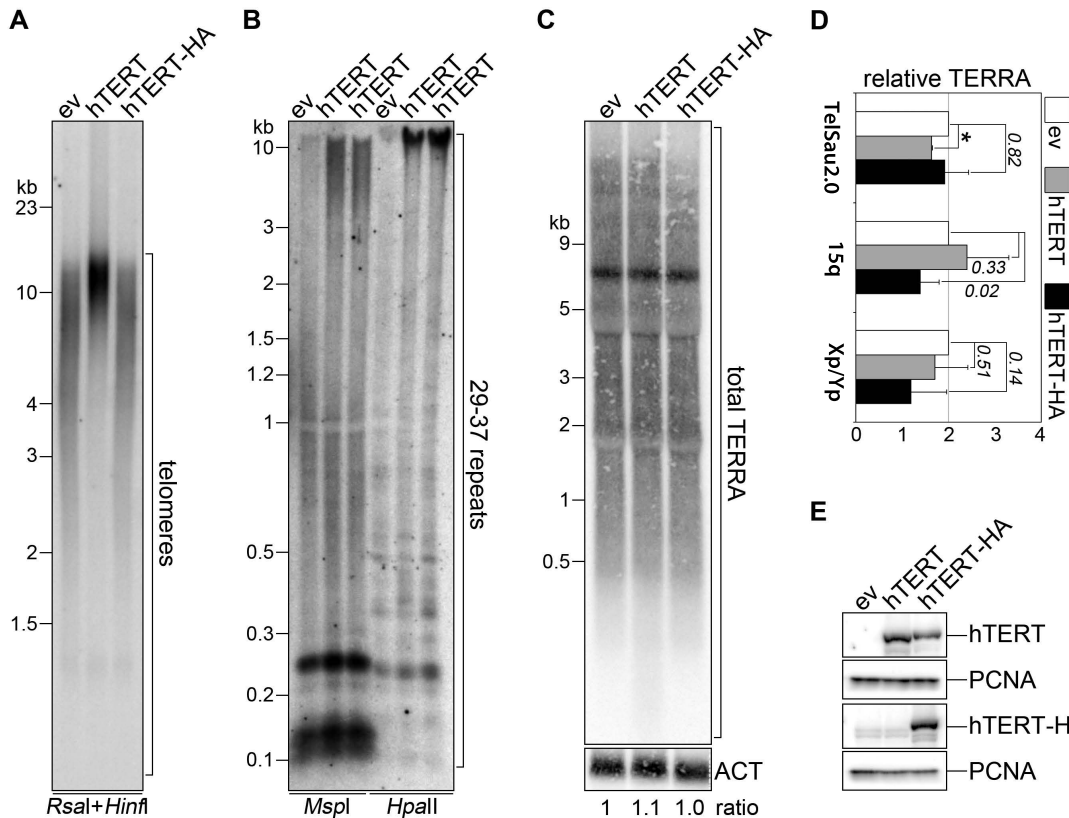


**Figure 20: TERRA steady-state levels are not affected by telomere elongation in human primary fibroblasts.**

(**A**) TRF analysis of human lung primary fibroblasts (HLF) infected with empty vector (ev), hTERT or hTERT-HA retroviruses. DNA was digested with RsaI and HinfI restriction enzymes and hybridized with telomeric probes. (**B**) The same DNA as in A was digested with HpaII (methylation sensitive) or MspI (methylation insensitive) restriction nucleases and hybridized with a probe detecting the 29-37 bp repeats of TERRA promoters. (**C**) Total RNA was hybridized using telomeric probes to detect total TERRA and successively with β-actin (ACT) probes to control for loading. Numbers at the bottom are the ratios between TERRA and actin signals expressed as fold increase over ev-infected samples. Molecular weights are on the left in kilobases. (**D**) qRT-PCR analysis of the steady-state levels of TERRA transcripts originating from TelSau2.0, 15q and Xp/Yp chromosome ends. Bars are averages from three independent experiments expressed as fold increase over ev-infected samples. Error bars and numbers are standard deviations and P-values, respectively. *: $P < 0.01$ (two-tailed t-test). (**E**) Western blot analysis of infected cells using anti-hTERT (to detect all hTERT molecules), anti-HA (to detect hTERT-HA) and anti-PCNA (loading control) antibodies.

lected and maintained in culture for several population doublings (PDs). Telom-
ere restriction fragment (TRF) analysis of genomic DNA indicated that, at the
time the experiments were performed, hTERT infection led to a substantial
elongation of bulk telomeres: TRFs with lengths of 2 to 5 kb were found in ev-
infected cells and between 5 and more than 15 kb in hTERT-infected cells (Fig.
19). As anticipated, hTERT-HA expression did not alter the telomere length, al-
though the expression occurred at levels comparable to those of hTERT (Fig.
19A and E). We then digested genomic DNA with MspI or with its CpG methy-
lation sensitive isoschizomer HpaII and hybridized the DNA to radiolabelled
probes corresponding to the 29-37 repeats found within TERRA promoters.
Consistent with what we have previously shown (Fig. 13A), we found that the
29-37 repeats are largely methylated in HeLa cells.  The restriction pattern
detected with 29-37 repeat probes was not altered in hTERT-expressing cells
compared with ev- and hTERT-HA control cells, which indicates that telom-
ere elongation does not affect the methylation state of TERRA promoters (Fig.
19B). Northern blot analysis of total RNA using telomeric probes did not reveal
any substantial changes in total TERRA cellular levels in hTERT-infected cells
when compared with the ev- or hTERT-HA-infected controls (Fig. 19C). A size
shift of TERRA molecules towards higher molecular weights was evident in
cells with elongated telomeres, which suggests that, at least in these cell lines,
telomere length may influence the length of TERRA molecules (Fig. 19C). Con-
sistent with the northern blot analysis, quantitative real-time PCR (qRT-PCR)
measurements of TERRA molecules transcribed from chromosome arms Tel-
Sau2.0, 15q and XpYp revealed no statistically significant changes in TERRA
steady-state levels upon telomere elongation (Fig. 19D).

Next, we performed experiments analogous to the experiments described
above in primary human lung fibroblasts. Therefore we infected HLF cells with
ev-, hTERT- and hTERT-HA-expressing constructs, selected stably infected
populations, and ensured that the telomeres were significantly elongated in

hTERT-expressing cells when the experiments were performed (Fig. 20A and E). Confirming what we found in HeLa cells, also in HLF cells telomere elongation did not affect the methylation of the 29-37 repeats (Fig.20 B). Northern blot and qRT-PCR analyses revealed that TERRA levels were unchanged in hTERT-expressing HLF cells compared to ev- or hTERT-HA-expressing cells
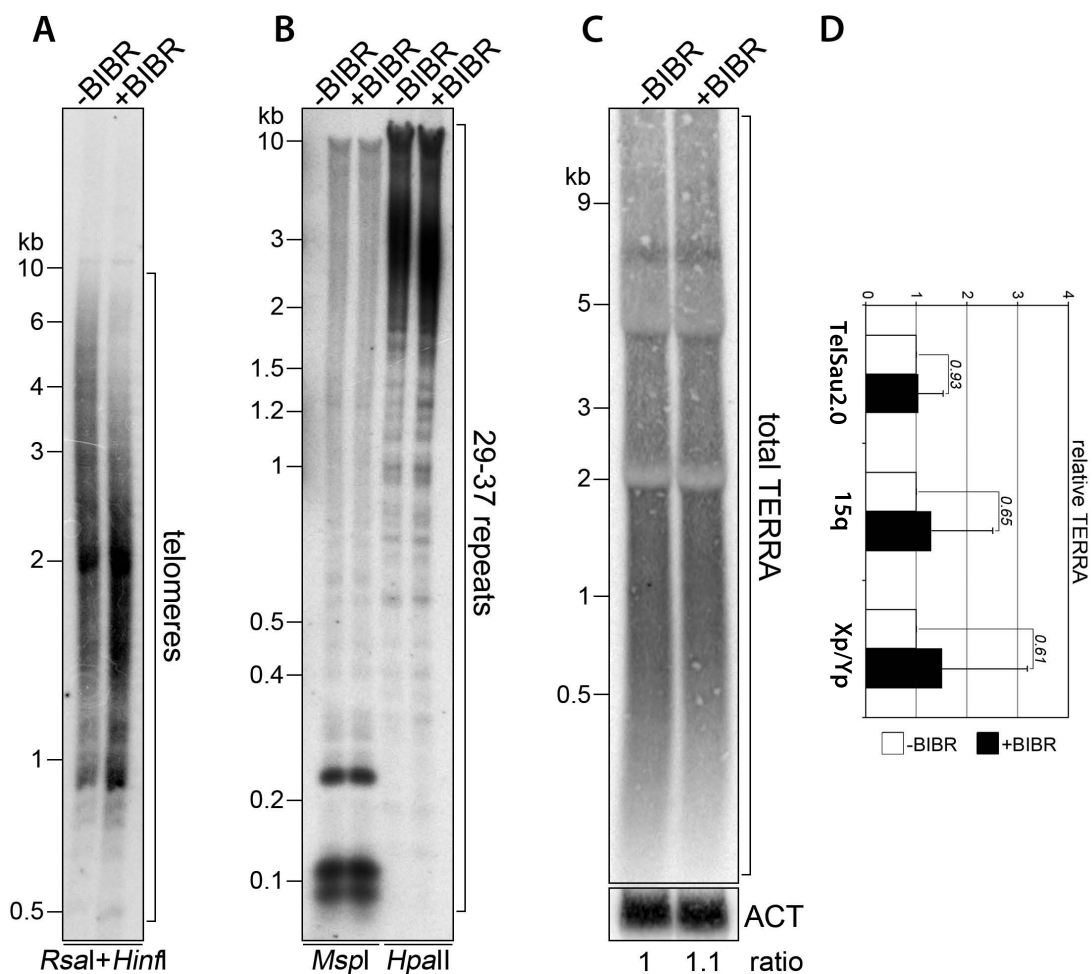


**Figure 21: TERRA steady-state levels are not affected by telomere shortening in HeLa cells.**

(**A**-**D**) HeLa cells were treated with the telomerase inhibitor BIBR1532 (+BIBR) for 19 weeks or left untreated (-BIBR). Telomere length, TERRA promoter methylation and total or chromosome specific TERRA steady-state levels were analyzed as in Fig. 18. Total RNA was used for all experiments. (**C**) For northern blot analysis of total TERRA β-actin (ACT) was used as a normalization control. Molecular weights are on the left in kilobases (kb).

(Fig. 20C and D). These results match our findings in HeLa cells, and therefore we conclude that telomere elongation does not affect TERRA levels, at least in the tested cell lines.

In a third and complementary approach, we cultured HeLa cells in the presence of the telomerase inhibitor BIBR1532 (BIBR) (Damm et al., 2001) for approximately 19 weeks. BIBR treatment led to a substantial shortening of bulk telomeres: the telomere length was mostly found to be between 2 and 5 kb in untreated control cells and between 1 and 3 kb in BIBR-treated cells (Fig. 21A). The treated cells divided in manner similar to that of the untreated cells, which indicates that the telomeres were long enough to sustain normal cell growth (data not shown). Telomere shortening did not induce any substantial change in the methylation state of the 29-37 repeats or in the total or chromosome-specific TERRA steady-state levels (Fig. 21B-D). Altogether, these experiments indicate that, as recently shown for budding yeast (Iglesias et al., 2011), telomere length alterations do not affect TERRA promoter methylation or TERRA levels in human cells. We conclude that TERRA transcription is not regulated by telomere length, at least in the cell types that we analyzed.

## 3.2.2 DNA methyltransferase-deficient cells have normal telomerase activity

The elevated TERRA levels in DKO cells make them a suitable cellular system to test the effects exerted by TERRA on telomerase activity. We stably infected DKO and HCT116 parental cells with hTERT or ev retroviruses and selected stable populations. Possibly because of the different transcriptional states of the two cell lines, we repeatedly found that over-expressed hTERT protein levels were more than two-fold higher in parental than in DKO cells (Fig. 22A). As shown by qRT-PCR and northern blot, the steady-state levels of TERRA molecules transcribed from 61-29-37 repeat-containing TelSau2.0 and 15q chromosome ends were dramatically increased in DKO cells (Figs. 22B,
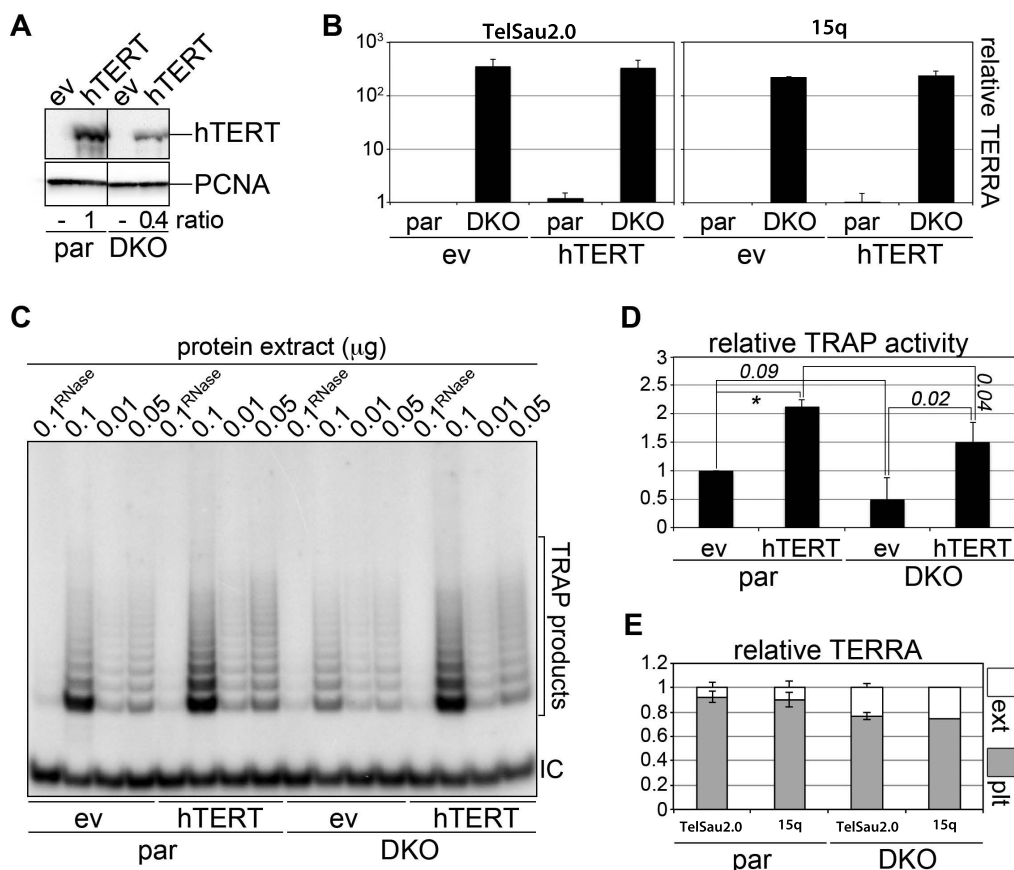
**Figure 22: Cells deficient for DNMT1 and 3b display normal telomerase activity.**

(**A**) Western blot analysis of hTERT-expression in HCT116 parental (par) or DNMT1 and 3b double KO (DKO) infected cells. Numbers at the bottom are the ratios between hTERT and PCNA (loading control) signals, expressed as fold increase over hTERT-infected parental cells. (**B**) qRT-PCR analysis of the steady-state levels of TERRA transcripts originating from TelSau2.0 and 15q chromosome ends expressed as fold increase over ev-infected par cells. Bars and error bars are averages and standard deviations from three independent experiments. (**C**) Analysis of TRAP amplification products from the indicated cells lines. Three different amounts of total proteins were used for each cell line and to control for specificity one sample was pre-treated with RNase A. Control primers amplifying an internal control (IC) were included in all reactions. (**D**) Quantification of telomerase activity in the indicated samples using qRT-PCR-based TRAP assays. Bars and error bars are averages and standard deviations from three independent experiments, after normalization through ev-infected parental cell samples. Numbers indicate P-values (*: P $<$0.01; two-tailed t-test). (**E**) qRT-PCR-based quantification of TelSau2.0 and 15q TERRA transcripts in TRAP extracts (ext) or in cell pellets left after extraction (plt). Relative TERRA amounts are expressed as fractions of total TERRA molecules from pellet plus extract. Bars and error bars are averages and standard deviations from three independent experiments.

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11A, and 15B and C) and, consistent with the results described above for HeLa and HLF cells, stable expression of hTERT did not alter TERRA amounts in either cell line (Fig. 22B). We prepared protein extracts and analyzed telomerase activity using quantitative TRAP assays. No significant difference in telomerase activity was observed in ev-infected par and DKO cells, whereas hTERT infection led to a substantial increase in telomerase activity in both cell lines (Fig. 22C and D). The TRAP activity was slightly higher in hTERT-infected par cells than in hTERT-infected DKO cells, and we ascribe this difference to the lower amounts of total hTERT protein expressed in the DNMT-deficient cell line (Fig. 22A, C and D). RNA isolated from TRAP extracts contained TelSau2.0 and 15q TERRA molecules, although a large fraction of them (approximately 90% and 80% for par and DKO cells, respectively) remained in the cell pellets left after extraction (Fig. 22E), likely because most TERRA is chromatin-associated (Azzalin et al., 2007; Bah et al., 2012; Schoeftner and Blasco, 2008). Still, considering that the TelSau2.0 and 15q TERRA molecules are ∼300-fold more abundant in DKO cells than in par cells (Fig. 22B), we estimate that the DKO TRAP extracts contained ∼600-fold more TERRA molecules than the parental extracts. In conclusion, we report that TRAP activity is similar in cells with drastically different TERRA levels, which is in stark contrast with results obtained *in vitro* using short TERRA-like oligonucleotides (Redon et al., 2010; Schoeftner and Blasco, 2008). We believe that the short RNA oligonucleotides employed *in vitro* do not behave similarly to the natural, long TERRA molecules, possibly because of their use in high, non-physiological concentrations.

### 3.2.3 Efficient telomerase-mediated telomere elongation in DKO cells

To determine how telomerase elongates telomeres in par and DKO cells, we prepared genomic DNA from infected cells 2, 5 and 9 days after hTERT infection. We then performed TRF analysis using probes that specifically detect

TelSau2.0 subtelomeric sequences or telomeric repeat probes that detect bulk telomeres. Although TelSau2.0 and bulk TRFs were substantially shorter in DKO cells than in the parental cells, they both underwent progressive lengthening throughout the chosen time course in cells infected with hTERT but not in ev control cells (Fig. 23A). Thus, telomerase is able to elongate telomeres both in par and DKO cells, including TelSau2.0 telomeres, which are extensively transcribed in DKO cells (Figs. 22B, 11A, and 15B and C). To obtain more quantitative data, we used single telomere length analysis (STELA), which is a PCR-based approach that permits the precise measurement of the length of individual telomeres (Baird et al., 2003). STELA of 15q telomeres confirmed that the average telomere length in DKO cells is lower than that in parental cells (5.9 kb and 3.8 kb in par and DKO cells, respectively; Fig. 23B and C). Nevertheless, as already suggested by the TRF analysis, 15q telomeres were efficiently elongated in both cell lines infected with hTERT retroviruses (Fig. 23D). Via quantitative analysis of STELA products and normalization through the population doubling (PD) time calculated for the two cell lines (17.7 h and 29.6 h for par hTERT and DKO hTERT cells, respectively), we estimated that the telomerase elongation rates were $\sim$204 bp/PD in parental cells and $\sim$186 bp/PD in DKO cells (Fig. 23C and D). This slight difference in telomere elongation rate does not correlate with the greatly increased level of 15q TERRA in DKO cells (Fig. 22B) and can likely be ascribed to the different hTERT protein levels detected in infected par and DKO cells (Fig. 22A).

Trypan blue staining and fluorescence-activated cell sorter (FACS) analysis of Annexin V and propidium iodide-stained cells did not reveal any major differences in the fraction of dead or apoptotic cells in the different cell lines and did not show substantial changes in the distribution of cells among the different phases of the cell cycle (Fig. 24A). These findings indicate that the higher PD time measured for DKO cells is likely attributable to a longer cycling time of these cells, which is further corroborated by the evident delay in
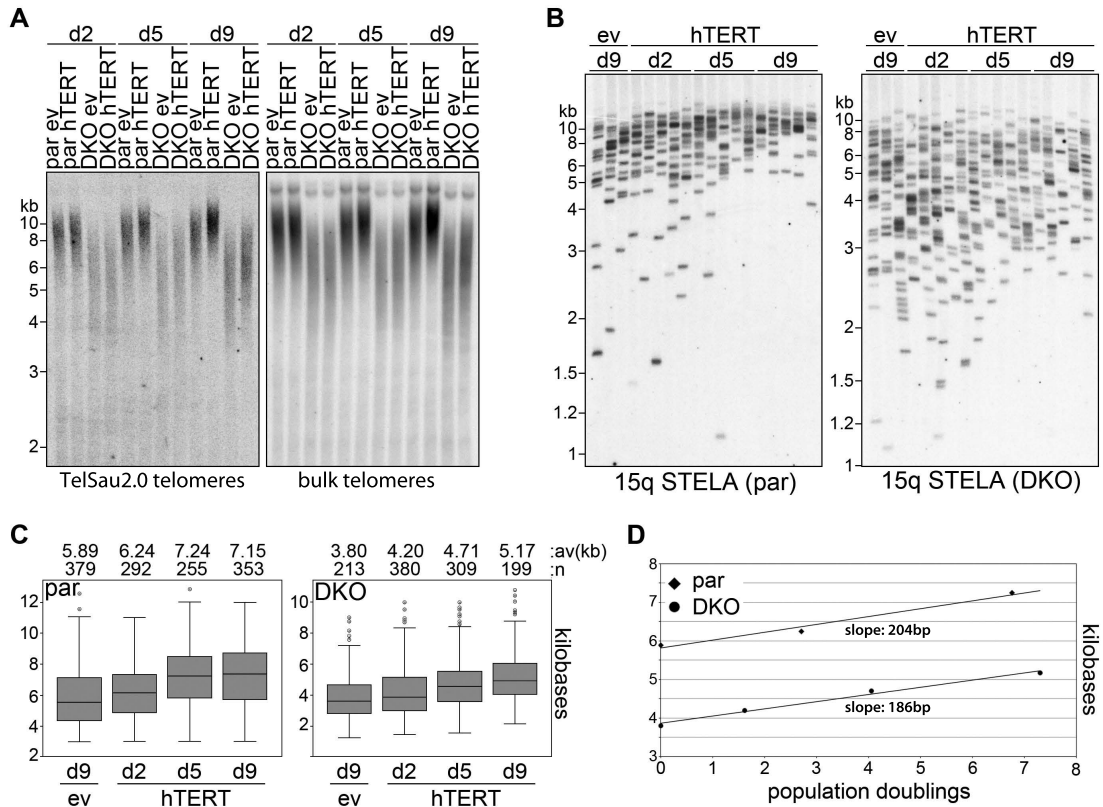
**Figure 23: Efficient telomerase-mediated elongation of telomeres in DKO cells.**
(**A**) TRF analysis of HCT116 parental (par) and DKO cells infected with empty vector (ev) or hTERT retroviruses. Genomic DNA was collected 2, 5 and 9 days (d) after infection, digested with RsaI and transferred to a nylon membrane.  The same membrane was first hybridized with a probe detecting TelSau2.0 TRFs and successively with a probe detecting total telomeres.  (**B**) STELA of 15q telomere length using the same DNA as in A. Marker molecular weights are on the left in kilobases. (**C**) Box plot representation of 15q telomere lengths.  Average telomere lengths in kilobases and the number of total telomeres analyzed (n) are indicated for each sample. (**D**) 15q telomere elongation rates expressed as average telomere length at different population doublings.  Note that for par cells, only three time points were used because no statistically significant difference in average telomere length was measured between day 5 and day 9.

cell cycle progression observed for synchronized DKO cells released from a nocodazole block (Fig.  25A). Normal telomere elongation in hTERT-infected DKO cells does not support the notion that TERRA functions as a telomerase inhibitor *in vivo*.  Still, we considered the possibility that TERRA transcripts in DKO cells are unable to inhibit telomerase because they do not properly

localize to telomeres or because they are drastically down-regulated during S-phase, which is when telomerase acts (Porro et al., 2010; Zhao et al., 2009).

**A**



| cell state | par ev | par hTERT | DKO ev | DKO hTERT |
|---|---|---|---|---|
| alive (trypan blue) | 99±0.1% | 99±0.2% | 98±1% | 99±0.3% |
| apoptotic (AV) | 3±2.1% | 5±4.3 % | 2±0.2% | 2±0.8% |
| <G1 (PI) | 3±0.7% | 4±0.7% | 3±0.6% | 2±1.7% |
| G1 (PI) | 36±1.5% | 34±4.5% | 37±8.9% | 37±9.6% |
| S (PI) | 34±3.4% | 35±7.8% | 31±2.1% | 33±1.6% |
| G2/M (PI) | 19±0.9% | 16±3.3% | 15±2.1% | 15±2.3% |
| >G2/M (PI) | 8±4.7% | 11±9.9% | 14±5.1% | 13±5.3 |

**B**



**C**
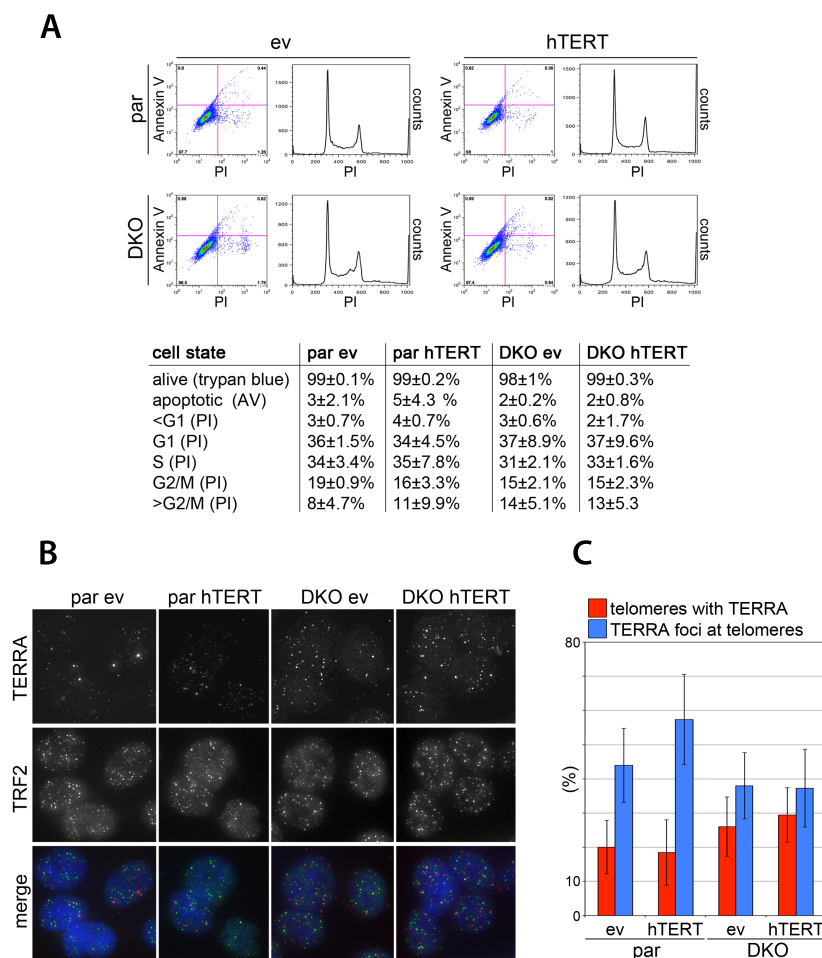


**Figure 24: Cellular state and TERRA localization in par and DKO cells upon telomere elongation.**
(**A**) Top: examples of flow cytometric analysis of Annexin V and propidium iodide stained HCT116 parental (par) and DKO cells. Bottom: quantifications of alive cells (trypan blue negative cells), apoptotic cells (Annexin V - AV - positive cells) and cells in the different phases of the cell cycle as judged by propidium iodide (PI) staining. Values are averages and standard deviations form three independent experiments. (**B**) Examples of anti-TRF2 indirect immunofluorescence combined with TERRA RNA FISH in par and DKO cells infected with hTERT or empty vector (ev) retroviruses. In the merge panels TRF2 is in green, TERRA in red and DAPI-stained DNA in blue. (**C**) Quantification of co-localization of TRF2 and TERRA foci. Bars and error bars are averages and standard deviations of co-localization events per nucleus. For each condition 50 nuclei were analyzed.
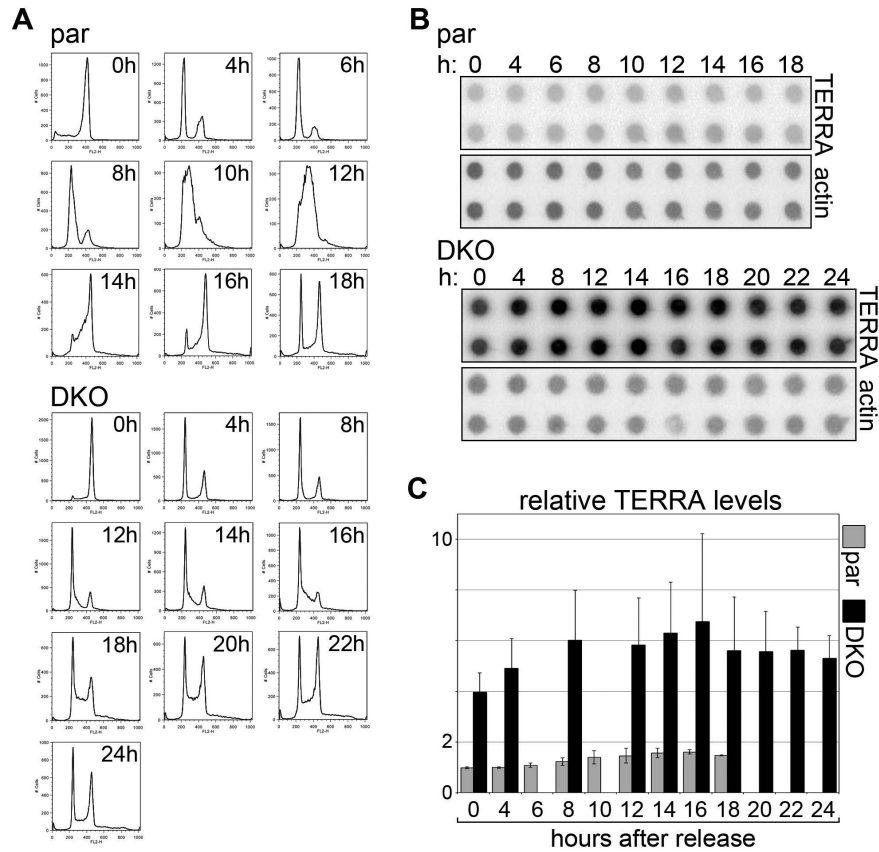
**Figure 25: TERRA steady-state levels in synchronized par and DKO cells.**
(**A**) FACS profiles of propidium iodine stained HCT116 parental (par) and DKO cells blocked in G2/M using nocodazole and released into the cell cycle for the indicated hours. (**B**) Dot-blot analysis of total RNA isolated at indicated hours after release. The same membranes were first hybridized with telomeric probes (to detect TERRA), stripped and re-hybridized with β-actin probes to control for loading. (**C**) Quantification of dot blots as in B. TERRA values were normalized through the corresponding actin values and expressed as fold increase over par cells at time 0h. Bars and error bars represent averages and standard deviations from two independent experiments.

We combined indirect immunofluorescence with RNA fluorescence *in situ* hybridization (IF/RNA-FISH) to simultaneously detect the telomeric factor TRF2 and TERRA molecules. DKO cells contained more and brighter TERRA foci than the parental cells, and hTERT infection did not alter the overall pattern of TERRA hybridization. Approximately 20% and 30% of TRF2 foci co-localized with TERRA foci in par and DKO cells, respectively (Fig. 24B and C), which

most likely reflects the fact that the increased levels of TERRA in DKO cells facilitated the detection of TERRA foci. In contrast, the fraction of TERRA foci that co-localized with telomeres was similar in both cellular backgrounds, which indicates that the intrinsic ability of TERRA to remain associated to telomeric heterochromatin does not require intact DNMT activity (Fig. 24B and C). Importantly, hTERT expression did not alter the co-localization rates in either cell line. Hence, the fraction of TERRA associated with telomeres is similar in par and DKO cells, and telomere elongation does not noticeably affect TERRA localization. We then blocked par and DKO cells in G2/M phase and released them synchronously into the cell cycle for a time sufficient to allow progression through S-phase (Fig. 25A). We prepared total RNA at different time points and dot-blot hybridized it to TERRA and actin probes. The TERRA levels remained stable throughout the entire time course in both cell lines (Fig. 25B and C). Altogether, these observations suggest that telomerase-mediated telomere elongation is not substantially impaired in DKO cells, whereas TERRA transcription rates and steady-state levels are dramatically increased (Figs. 11A, 15, and 22B). This lack of robust inhibition does not stem from the mislocalization of TERRA or from its down-regulation during S-phase. This finding is in line with our observations that telomerase activity is unaffected in the same cell lines and strongly contradicts the widely accepted notion that TERRA is a telomerase inhibitor *in vivo*.

## 3.3   Stable cell lines to screen for TERRA promoter regulators and binding factors

### 3.3.1   Creating stable 61-29-37-eGFP cell lines

TERRA has been shown to be down-regulated during S-phase (Porro et al., 2010); however, it is not known how this down-regulation is achieved. Down-regulation could be mediated through increased TERRA degradation or through decreased TERRA transcription, which is possibly transcription factor-dependent. After successfully identifying promoters that drive TERRA transcription we wondered whether these promoters are bound by transcription
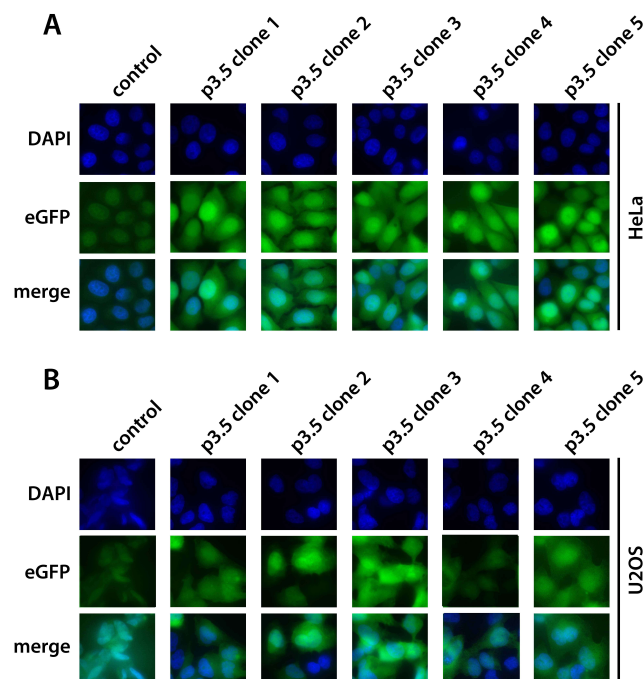


**Figure 26: 61-29-37-eGFP HeLa and U2OS cell lines.**
(**A**, **B**) Microscopy images of HeLa and U2OS cells, which have been transfected with a construct to express eGFP under control of the 61-29-37 promoter (Fig. 6A, p3.5) in order to create stable cell lines. For each cell line five eGFP expressing clones were isolated. Cells were fixed and stained with DAPI before imaging.

factors that modulate TERRA expression during the cell cycle. Therefore, we wanted to establish a cellular system with a simple readout, that would allow us to screen for TERRA transcription factors using siRNA. To create stable cell lines expressing eGFP under the control of the 61-29-37 promoter (61-29-37-eGFP), we used construct p3.5 from Fig. 6A for the transfection of telomerase-positive (HeLa) and telomerase negative (U2OS) human cancer cells. After transfection, five eGFP expressing clones were selected by microscopy from the HeLa and U2OS cell lines (Fig. 26A and B).

Next, we isolated total RNA from all ten p3.5 clones and performed northern blot and qRT-PCR analysis to confirm eGFP expression. As expected, we found eGFP to be expressed in all clones; however the expression levels var-
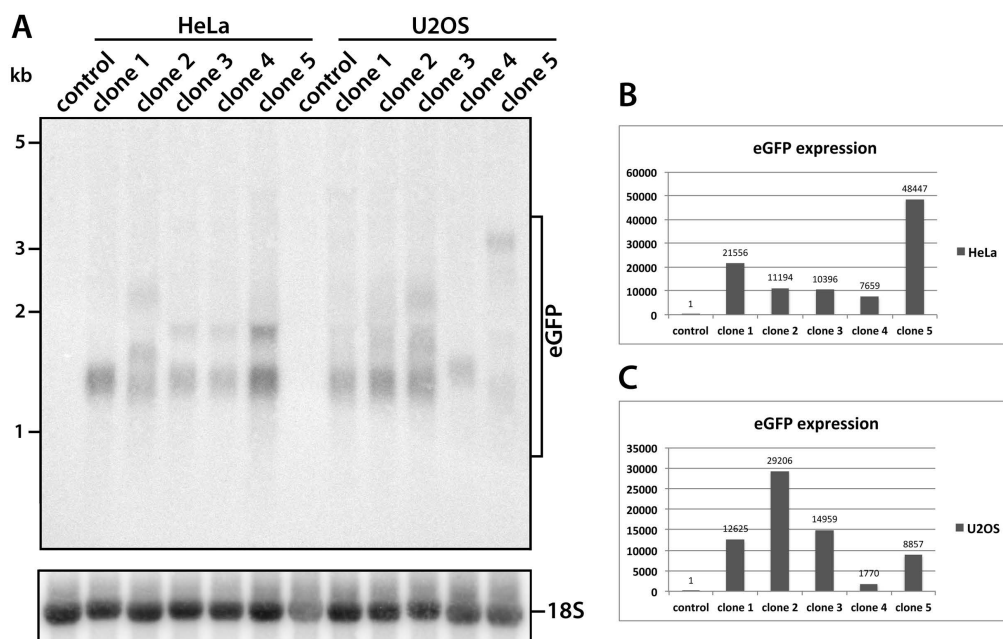


**Figure 27: eGFP expression in 61-29-37-eGFP HeLa and U2OS clones.**
(**A**) Northern blot analysis of total RNA from all ten eGFP expressing clones. The membrane was first hybridized with a probe to detect eGFP transcripts then stripped and rehybridized with an 18S loading control probe. Kilobases (kb) are shown on the left. (**B, C**) qRT-PCR analysis of RNA from all ten 61-29-37-eGFP expressing clones. Expression levels relative to GAPDH expression shown are relative to HeLa or U2OS control cells not expressing eGFP, which have been set to one.
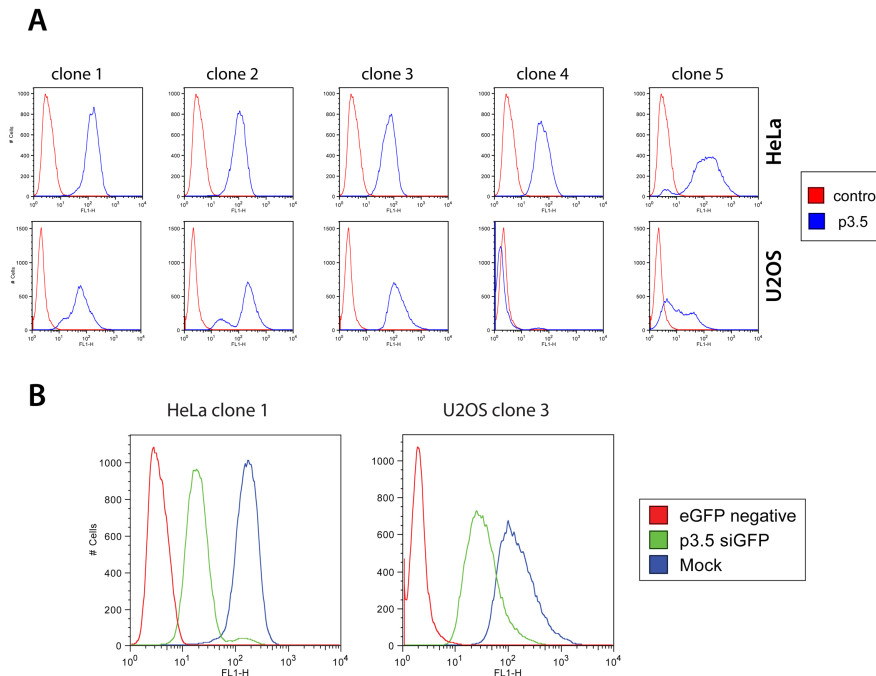
**Figure 28: Flow cytometric analysis of eGFP signal intensity in 61-29-37-eGFP HeLa and U2OS cells.**
(**A**) eGFP expression analysis by flow cytometry of indicated cell lines and clones. Cell count (y-axis) is plotted against eGFP fluorescence intensity (x-axis). (**B**) eGFP expression analysis by flow cytometry of indicated HeLa p3.5 clones and normal HeLa cells. HeLa p3.5 clones have either been mock or eGFP siRNA treated. Cell count (y-axis) is plotted against eGFP fluorescence intensity (x-axis). Note that eGFP siRNA treatment leads to a clear decrease in eGFP signal intensity in both clones.

ied up to several fold among the different clones (Figure 27A-C). This variation in eGFP expression most likely stems from multiple insertions of the construct in some clones and/or from differences in the chromatin environment at the different sites of insertion. Furthermore, northern blot analysis revealed that several p3.5 clones produce not only one but also two or three eGFP transcripts of different sizes (Fig. 27A). This is unlikely to be caused by the usage of different transcription start sites, because the size differences between the transcripts are too big; a more likely explanation is again multiple insertions of the construct into the genome.

## 3.3.2  Methods for screening

To test whether flow cytometry is suitable to screen for TERRA transcription factors, we analyzed the eGFP expression of all ten p3.5 clones using a flow cytometer. We found that for all clones except U2OS p3.5 clone 4, the obtained eGFP signal was clearly distinguishable from HeLa and U2OS control cell signals (Fig. 28A). Next, we selected HeLa p3.5 clone 1 and U2OS p3.5 clone 3 and transfected them with either GFP siRNA or mock-treated them. GFP siRNA transfection led to a clear decrease in the eGFP signal intensity in both clones compared with non-target siRNA transfection (Fig. 28B). Therefore, flow cytometry would be a suitable method for an siRNA screen to identify TERRA transcription factors using the 61-29-37-eGFP clones. A drawback of flow cytometry is that a large number of cells is required, which does not permit siRNA transfection in small-scale formats, as required in large-scale screens. This limitation brought us to test whether automated microscopy was suitable
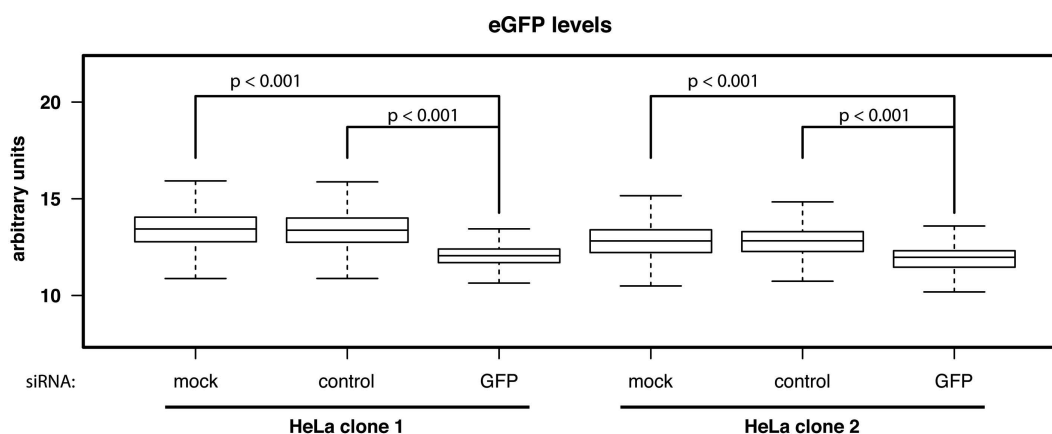


**Figure 29: Analysis of eGFP fluorescence in 61-29-37-eGFP HeLa clones by automated microscopy.**
Cells from cell lines and clones indicated were mock, control siRNA or eGFP siRNA treated for 72h. Cells were then fixed and stained with DAPI and imaged with an automated microscope. Average eGFP fluorescence for each nucleus was calculated and blotted in box plots. Outliers are not shown in the graph. Note that eGFP siRNA treatment leads to a highly significant down-regulation of eGFP fluorescence signal intensity. p-values from a Mann-Whitney-U-test are indicated.

for screening, as this technique permits siRNA transfection in a 96- or 384-well plate format. We used HeLa p3.5 clones 1 and 2 and transfected them with non-target or GFP siRNA before the cells were fixed and stained with DAPI. This procedure was followed up by automated microscopy and bioinformatics analysis of the average eGFP signal for each sample. We found that GFP siRNA transfection led to a highly significant decrease in the eGFP signal in both clones as compared with non-target siRNA-transfected or mock-transfected samples (Fig. 29), which makes this method suitable to screen for TERRA transcription factors. To perform the actual screen, a few more tests will be necessary to identify the best siRNA transfection conditions and the best time point for microscopy after transfection. Identifying a transcription factor that regulates TERRA expression would be an important step, as this discovery could be used to study the effects of TERRA overexpression or down-regulation *in vivo*.

# 4. Discussion

## 4.1 CpG-island promoters drive transcription of human telomeres

The recent development of new experimental techniques has permitted the analysis of transcription on a genome-wide level (Berretta and Morillon, 2009), which led to the surprising finding that the vast majority of the genome, i.e. more than 93% in human cells, is transcribed, even though $<2$ % of the mammalian genome are protein-coding (Bertone et al., 2004; Carninci et al., 2005; Cheng et al., 2005; He et al., 2008; Kapranov et al., 2007; Kim et al., 2005; Wilusz et al., 2009). These findings make the discovery that even heterochromatic telomeres are transcribed seem less surprising. Therefore, one of the main questions after the discovery of TERRA was whether telomeric transcription and TERRA fulfill a function or if they are instead part of the so called transcriptional 'noise' associated with the pervasive transcription of the human genome (Berretta and Morillon, 2009; Struhl, 2007). However, the conservation of telomeric transcription throughout evolution from yeast to humans (Azzalin et al., 2007; Bah et al., 2012; Greenwood and Cooper, 2012; Luke et al., 2008; Schoeftner and Blasco, 2008; Solovei et al., 1994; Vrbsky et al., 2010) is a strong indication for its importance. Furthermore, the discovery of CpG-island promoters within the subtelomeres of human cells that we report here (see sections 3.1.1 and 3.1.2) is an important step that underscores the impor-

tance of telomeric transcripts. The existence of promoters dedicated to driving the transcription of human subtelomeres and telomeres supports the notion that transcription and/or TERRA must be important for proper telomere function. This idea is further supported by the finding that even telomeres that are apparently devoid of CpG-island promoters, such as 11q and Xp/Yp, produce TERRA molecules, as has been demonstrated by RT-PCR (Figs. 5, 19D, 20D, and 21D; Azzalin et al., 2007). Thus, all telomeres in human cells are assumed to be transcribed, although this has not been formally demonstrated, and the promoters driving the transcription of telomeres devoid of 61-29-37 repeats are unknown. One possibility is that there are other CpG-islands located within the subtelomeres of telomeres without 61-29-37 repeats. Alternatively, ill-defined subtelomeric sequences in the databases (Riethman et al., 2004; Riethman, 2008) may have caused us to underestimate the actual number of subtelomeres containing 61-29-37 repeats. In mice, zebrafish or plants, no information concerning the nature of TERRA promoters is available to date. In budding yeast, however, research has shown that in mutants lacking the 5' to 3' RNA exonuclease Rat1p, telomeres are likely to be transcribed by run-on transcription from genes located in close proximity to the telomeric tracts (Luke et al., 2008). Because human subtelomeres contain various genes with a gene density of one gene per 30 kb, on average (Linardopoulou et al., 2005), we cannot exclude the possibility that run-on transcription from upstream genes also contributes to the total TERRA levels in human cells.

The methylation of CpG dinucleotides within CpG-island promoters by DN-MTs is a common epigenetic method to negatively regulate transcription and assure the proper regulation of gene expression (Esteller, 2007; Kulis and Esteller, 2010; Suzuki and Bird, 2008). We found that TERRA promoters are also negatively regulated by CpG methylation (see section 3.1.3), which is supported by several findings from other groups. For example, as already mentioned above, cells from patients with ICF syndrome have diminished sub-

telomeric methylation patterns and higher TERRA levels compared with control cells (Deng et al., 2010; Yehezkel et al., 2008). In contrast to this finding, DNMT-deficient mouse ESCs show subtelomeric CpG-island hypomethylation along with lower TERRA levels (Gonzalo et al., 2006; Schoeftner and Blasco, 2008). This apparent contradiction could be the result of different TERRA regulatory mechanisms in mouse and human cells, especially as, to date, TERRA promoters have not yet been characterized in mouse cells. Interestingly, CpG hypermethylation at promoter regions of certain tumor suppressor genes has been shown to be a common feature of different types of cancer (Kulis and Esteller, 2010). This finding raises the intriguing possibility that TERRA could also act as a tumor suppressor. Supporting this hypothesis is the finding that TERRA steady-state levels are higher in primary cells (HLF) and ALT cells (U2OS) than in telomerase-positive HeLa and HEK293T cancer cells (Fig. 9B). Similarly, TERRA promoter methylation is higher in HeLa cells than in HLF and U2OS cells (Fig. 13A).

## 4.2   Telomerase efficiently elongates highly transcribing telomeres in human cancer cells

After the discovery of TERRA, several findings supported the notion that TERRA could be an inhibitor of telomerase activity. Because of its sequence complementarity to the telomerase RNA component, TERRA is able to inhibit telomerase activity *in vitro* (Redon et al., 2010; Schoeftner and Blasco, 2008). In addition, telomeric transcription driven by a galactose-inducible promoter has been shown to lead to telomere shortening in Saccharomyces cerevisiae (Sandell et al., 1994), and cells from patients with ICF syndrome have been shown to have higher TERRA levels and shorter telomeres (Yehezkel et al., 2008). Finally, DKO cells with strongly increased TERRA steady-state levels have shorter telomeres than HCT116 parental cells (Figs. 11A, 15, and

18). These findings, together with evidence that long telomeres apparently produce or accumulate more TERRA than short telomeres (Schoeftner and Blasco, 2008), led to a model in which telomeres auto-regulate their length through TERRA expression (Porro et al., 2010; Redon et al., 2010; Schoeftner and Blasco, 2008). However, when we tested whether there is a general effect of telomere length on TERRA steady-state levels or TERRA promoter methylation levels, we found no causal relationship between these features (see section 3.2.1). These results are in agreement with recent findings in budding yeast, where the use of a telomerase fusion protein led to telomere elongation, which did not affect TERRA levels (Iglesias et al., 2011). Furthermore, in the same study, telomerase deletion led to telomere shortening and a slight increase in TERRA levels in budding yeast (Iglesias et al., 2011). Our results possibly deviate from what has been reported before because other authors compared non-isogenic cell lines from different species, whereas we compared human isogenic cell lines (see section 3.2.1; Schoeftner and Blasco, 2008).

The lack of a positive correlation between telomere length and TERRA levels (Figs. 19, 20, and 21) led us to test whether TERRA inhibits telomerase *in vivo*, because most evidence supporting this hypothesis is either indirect or derived from *in vitro* experiments (Luke et al., 2008; Redon et al., 2010; Sandell et al., 1994; Schoeftner and Blasco, 2008; Yehezkel et al., 2008). Using DKO cells, we found that endogenous TERRA is not able to inhibit telomerase activity in TRAP assays *in vitro* (Fig. 22). These results stand in stark contrast to those from TRAP and direct telomerase assays, where artificial, short TERRA-like RNA molecules were shown to strongly inhibit telomerase activity (Luke et al., 2008; Schoeftner and Blasco, 2008). A possible explanation for this contradiction is the use of high non-physiological concentrations of TERRA-like RNA molecules in these *in vitro* assays, or that artificial TERRA may simply not behave like endogenous TERRA because of its length and/or

because it does not contain subtelomeric sequences. When we measured the telomere elongation rate after infection with telomerase in HCT116 and DKO cells, we found only minor differences that we attribute to different telomerase expression levels in the different cell lines (Figs. 23 and 22A). These finidings strongly contradict the popular notion that TERRA is a telomerase inhibitor *in vivo* and are supported by additional data from our laboratory. We have shown that the transcriptional induction of a newly seeded telomere over a long time period does not affect its length (Farnung et al., 2012). Furthermore, telomere re-elongation after induced telomere shortening in the same cells using BIBR was found to occur at the same speed in transcriptionally induced and non-induced telomeres (Farnung et al., 2012). In addition, it was recently demonstrated that the telomere shortening of budding yeast telomeres that are forced to transcribe is derived from a replication-dependent loss of telomeric DNA sequences and is not caused by telomerase inhibition by TERRA (Maicher et al., 2012). Taken together, these recent data make it seem quite unlikely that TERRA is a general inhibitor of telomerase activity *in vivo*, even though we cannot rule out the possibility that TERRA may inhibit telomerase in certain cell lines.

Disproving the popular notion that TERRA is a telomerase inhibitor again raises the question of TERRA's function(s). One proposed model suggests that TERRA contributes to proper telomere replication by inhibiting hnRNPA1, which displaces RPA from telomeric single-stranded DNA (Flynn et al., 2011). This model is supported by the finding that TERRA is down-regulated in late S-phase (Porro et al., 2010), which would allow hnRNPA1 to remove RPA from single-stranded telomeric DNA post-replication to allow POT1 to bind again and to ensure telomere protection (Flynn et al., 2011). Even though this hypothesis is logical, it is mostly based on *in vitro* experiments and awaits confirmation *in vivo*. In another study, siRNA-mediated down-regulation of TERRA caused an increase in telomere dysfunction-induced foci, aberrations at telom-

eres, and the loss of telomeric heterochromatin, which suggests that TERRA plays an important role in maintaining functional telomeres (Deng et al., 2009). However, whether the DNA damage at telomeres in this case really results from TERRA down-regulation or is caused by the transfection of short telomeric RNA molecules is unclear because the transfection of short telomeric DNA oligonucleotides induces a strong DNA damage response at telomeres (Milyavsky et al., 2001). Alternatively, TERRA was proposed to interact with the N-terminal basic domain of TRF2 (Deng et al., 2009) and therefore to inhibit DNA condensation by TRF2 (Poulet et al., 2012). The caveat of this study is again that short artificial TERRA-like RNA molecules have been used, which seem to not effectively mimic endogenous TERRA molecules, at least in TRAP and direct telomerase assays (Fig. 22C and D; Redon et al., 2010; Schoeftner and Blasco, 2008).

## 4.3 Stable cell lines to screen for TERRA promoter regulators and binding factors

After identifying CpG-island promoters that drive TERRA transcription and showing that TERRA does not inhibit telomerase activity *in vivo*, we were searching for a suitable tool to study TERRA function *in vivo*. TERRA is a nuclear and minimally abundant RNA that is transcribed from many loci within the cell and is difficult to overexpress or down-regulate. Although DKO cells are a useful system to study the effects of TERRA overexpression, they have to be used with caution because they have a genome-wide loss of DNA methylation and largely altered gene expression patterns. Therefore, almost any effects seen in DKO cells could be indirect or secondary and caused by global gene expression changes. Therefore, a system that permits the upregulation of all cellular TERRA transcription would be useful to complement a system developed in our laboratory with a single transcriptionally inducible telomere (Farnung et al.,

2012). The identification of a transcription factor (TF) that binds the TERRA promoters and modulates their activity would be an important step in this direction. Knockdown or overexpression of the TF would then permit the study of the effects of TERRA up- and down-regulation on telomeres and cells. The HeLa and U2OS cells we created that stably express eGFP under control of the 61-27-39 promoter (see section 3.3.1) are a suitable system to screen for TERRA regulators and TFs. For the screening itself, different approaches could be taken that we already tested and that rely on siRNA-mediated knockdown of candidate genes. Screening using a flow cytometer for the readout would be one approach (see Fig. 28); however, with this method larger-scale screens become difficult and expensive because a high number of cells and therefore a large transfection volume is required. As an alternative readout system, an automated microscope can be used to image 96- and 384-well plates (see Fig. 29). Average eGFP intensities can then be calculated using specialized software. This approach has the advantage that large-scale screens would be much easier to perform. However, some problems must still be overcome before a screen can be started. Even though the tests using siRNA to knock down eGFP directly were successful (see Figs. 28 and 29), the screening for a TERRA TF would be a challenge, because in this case, the loss or increase in eGFP fluorescence would be secondary to the down-regulation of the TF. Therefore, the effects are expected not to be as strong as those obtained by direct eGFP knockdown and may appear delayed, which may necessitate two siRNA transfections of cells. An additional problem may be that, in the eGFP-expressing HeLa cell line, and especially in the U2OS cell line, expression levels vary substantially from cell to cell (Fig. 28B, note width of the curves on the logarithmic scale), which will make the identification of weak regulators of TERRA transcription difficult. Nevertheless, screening for TERRA regulators seems to be promising and could provide a great tool to study the functions of TERRA.

# 5. Materials and Methods

## 5.1 DNA sequence analysis

We aligned the TelBam3.4 and TelSau2.0 sequences with the entire human genome using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). To analyze CpG dinucleotide contents and predict CpG-islands, we utilized the CpGPlot/CpGReport at the European molecular biology open software suite program (EMBOSS; http://www.ebi.ac.uk/Tools/emboss/cpgplot/) (Rice et al., 2000).

## 5.2 Plasmid constructions

Since TelBam3.4 and TelSau2.0 plasmids (Brown et al. 1990) were no longer available, we subcloned a ∼3-kb BamHI-EcoRI human genomic DNA fragment from the bacterial artificial chromosome clone RP11-34P13. This fragment is identical to a portion of the TelBam3.4 sequence and contains 61-, 29-, and 37-bp repeats. The pre-tel fragment comprised between the last 37-bp repeat and the telomeric repeats was obtained by PCR on human genomic DNA using primers constructed on the TelBam3.4 sequence. We constructed a plasmid containing the eGFP cDNA under control of the CMV promoter and the puromycin resistance gene using the pEGFN1 and pPUR plasmids (Clontech). We then substituted the CMV promoter with the ∼3.5-kb TelBam3.4 fragment

and obtained deletions using appropriate restriction enzymes or substituting the CMV promoter with PCR amplified fragments. We checked all constructs by sequencing. Retroviral plasmids pBABE-puro-hTERT and pBABE-puro-hTERT-HA were from Bob Weinberg's laboratory and were purchased from Addgene (plasmids 1771 and 1772). pMD-VSVG and pMDGAG/POL plasmids to produce retroviruses were obtained from Joachim Lingner's laboratory in Lausanne.

## 5.3  Cell lines and tissue culture procedures

HLF (human lung fibroblasts), HeLa (human cervical carcinoma), HEK293T (human embryonic kidney), U2OS (human osteosarcoma), and HCT116 (human colon carcinoma), HCT116 DKO and T-Rex-HeLa (Invitrogen) cells were cultured in high-glucose D-MEM (Gibco Life Technologies) supplemented with 10% of fetal calf serum, nonessential amino acids (Invitrogen), penicillin, and streptomycin (Invitrogen). Where indicated, 10 mM 5-azacytidine (Sigma Aldrich), or 1 μM of BIBR1532 (Tocris Bioscience) were added to the culture medium. Plasmid transfections were performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. For promoter reporter assays, we selected positively transfected cells 24 hours after transfection in medium containing 1 μg/ml puromycin for 4 days.

In order to produce retroviruses HEK293T cells were transfected with pMD-VSVG, pMDGAG/POL, and either pBABE-puro-EV or pBABE-puro-hTERT or pBABE-puro-hTERT-HA plasmids using Lipofectamine 2000 reagent (Invitrogen). 24 and 48 hours post transfection HEK293T cell culture medium was filtered using a 0.45 μm sterile filter (Sarstedt) and added to HeLa or HLF cells for infection, selection was started 24 hours after the second infection. Selection of EV, hTERT or hTERT-HA infected cells was done using 1.5 μg/ml Puromycin (Sigma-Aldrich). Positively infected stable cell lines were maintained with 0.75 μg/ml Puromycin.

For cell cycle synchronization HCT116 parental and HCT116 DKO cells were blocked in G2/M phase with 60 ng/ml Nocodazole (Sigma-Aldrich) at 40% confluency for 14h and at 60% confluency for 18h, respectively. G2/M arrested cells were collected by mitotic shake-off before releasing them by washing three times with culture medium and adding fresh culture medium.

For cell cycle analysis cells were harvested at indicated times post release, stained with propidium iodide (PI, Sigma) and subjected to flow-cytometer analysis. For PI staining, cells were washed in 1x PBS and fixed in ice-cold 70% ethanol. Cell pellets were treated with 25 µg/ml RNase A in 1x PBS for 15 min at 37° C, and stained with 50 µg/ml PI for 15 min at 4° C. Cells were analyzed using a BD FACS Calibur flow cytometer and FlowJo software.

To analyze eGFP expression levels by flow cytometry cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature; analysis was done using a BD FACS Calibur flow cytometer and FlowJo software.

To analyze cell viability, cells were stained with 0.2% trypan blue (Sigma) and counted using the Cellometer Auto T4 instrument (Nexcelom Bioscience). Apoptotic cells were scored using the Annexin V-FITC Apoptosis Detection Kit (eBioscience Diagnostics) according to manufacturers instructions.

## 5.4   RNA preparation and analysis

Nuclear RNA was prepared by lysing $1\text{-}5\text{x}10^6$ cells in 300 ul ice-cold cytoplasm lysis buffer (50 mM Tris-HCL pH 8.0; 140 mM NaCl; 1.5 mM $MgCl_2$; 0.5% (v/v) IGEPAL CA-630 (FLUKA); 1mM DTT; 10mM ribonucleoside vanadyl complex) for five minutes on ice. Cells were spun down for 3 min at 2000 rpm at 4° C and washed with 200 µl cytoplasm lysis buffer. From here on nuclear RNA preparation was identical to total RNA preparation.

For total RNA isolation cells were harvested by trypsinization or scraping and total or nuclear RNA was prepared using the NucleoSpin RNAII Kit (Macherey-Nagel) according to manufacturer's instructions. One or two ad-

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ditional DNaseI (Qiagen) treatments were performed according to the manufacturer's protocol for northern blot, and RT-PCR and qRT-PCR experiments, respectively, in order to eliminate any DNA contaminations.

For RNase H experiments, we mixed 10 mg of nuclear RNA with 600 pmol of o2 (TelSau2.0) oligonucleotide and 600 pmol of GAPDH oligonucleotide (see Table 1). We incubated the RNA/DNA mix at 65° C for 4 min and at room temperature for 20 min. We then added 1 Unit of RNase H (New England Biolabs) to the mix and allowed digestion at 37° C for 1 h, before subjecting the RNA to northern blot analysis.

For Northern blots, we electrophoresed 10-20 mg of total or nuclear RNA in 1.2% formaldehyde agarose gels and blotted it to nylon membranes. For RNA doblots 1 μg of total RNA was blotted to the membranes. Membranes were UV cross-linked and hybridized for ∼18 h in Church buffer successively with TERRA, TelBAm3.4, TelSau2.0, 18S, GAPDH, eGFP, or β-actin radiolabeled probes. Hybridization with TelBam3.4, TelSau2.0, and eGFP probes was done at 64° C, and with TERRA, 18S, GAPDH, and β-actin probes at 50° C. Post hybridization washes were performed at the same temperatures as the hybridization in 2x SSC, 0.2% SDS. The final washes were done with 0.2x SSC, 0.2% SDS for TelBam3.4, TelSau2.0, and eGFP probes, with 1x SSC, 0.2% SDS for 18S, GAPDH, and β-actin probes and with 0.5x SSC, 0.2% SDS for TERRA probes. Radioactive signals were detected using phosphor screens and a Typhoon FLA 9000 instrument (GE Healthcare) and quantified with Quantity One (Bio-Rad) and with ImageQuant (GE Healthcare) software.

The TERRA probe was a mixture of 1-5 kb long telomeric DNA fragments synthesized by ligating double stranded telomeric oligonucleotides followed by PCR amplification. To obtain a telomeric strand specific probe, the TERRA probe was random primer labeled with $P^{32}$-dCTP and cold dTTP and dATP for detection of the $(5'\text{-UUAGGG-}3')_n$ containing transcripts. The TelBam3.4, TelSau2.0, 15q and eGFP probes were genomic DNA PCR products obtained

using P1 and P2 oligonucleotides, and labeled by primer extension using P2 onligonucleotides (see Table 1). The β-actin, GAPDH, and 18S rRNA probes were 5' end-labeled DNA oligonucleotides (see Table 1).

For qRT-PCR analysis 2-5 μg of total RNA were reverse-transcribed using SUPERSCRIPT II or SUPERSCRIPT III reverse transcriptases (Invitrogen), according to the manufacturer's protocol. Reverse transcriptions were performed using random hexamers (New England Biolabs) or oC primers (see Table 1), and P3 and P4 or P5 and P6 oligonucleotides pairs (see Table 1) were used for PCR reactions. The PCR reactions were performed for 45 cycles (10 sec at 98° C, 30 sec at 60° C) using the LightCycler 480 SYBR Green I Master mix (Roche) and a Rotor-Gene Q instrument (Qiagen). Averages, standard deviations, and P-values (two-tailed Student's t-test) were calculated using Microsoft Excel software. For absolute quantification we used standard curves from plasmids of known concentrations.

For RT-PCR experiments, 1 μg of nuclear RNA was reverse-transcribed with oC oligonucleotides (see Table 1) using the SUPERSCRIPT III Reverse Transcriptase (Invitrogen) at 55° C, according to the manufacturer's instructions. The obtained cDNA was PCR amplified for 15 sec at 98° C, 20 sec at 55-60° C and 30 sec to 2 min at 72° C for 30-45 cycles using o1 and o2 oligonucleotides (Table 1). Sequencing of the amplification products shown in Fig. 10B demonstrated their specificity.

We performed 5' RACE experiments with the FirstChoice RLM-RACE Kit (Ambion) using the oC oligonucleotide for reverse transcription and the Tel-Bam3.4 gene-specific oligonucleotides oR1 and oR2 for PCR (see Table 1). We cloned RACE products into the pDRIVE vector (Qiagen) and sequenced 18 to 41 independent colony plasmids for each cell line.

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## 5.5   Telomere length analysis

For genomic DNA isolation cells were harvested by trypsinization, and DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega) according to manufacturer's instructions. For telomere restriction fragment (TRF) analysis of bulk telomeres, 5 μg genomic DNA were digested with RsaI and HinfI or RsaI alone as indicated. Restriction fragments were resolved by electrophoresis in 0.7% agarose gels. Gels were either dried or transferred to nylon membranes (GE Osmonics). DNA was denatured in 0.4 N NaOH, 0.6 M NaCl, and hybridized overnight with radiolabeled probes containing a mixture of 1-5-kb-long telomeric DNA fragments (TERRA probes), or with radiolabeled probes corresponding to TelSau2.0 subtelomeres (TelSau2.0 probes). Hybridizations were performed for ∼16 hours at $50°$ C for TERRA probes and at $64°$ C for TelSau2.0 probes. Post hybridization washes were performed at $50°$ C in 0.5x SSC, 0.2% SDS for TERRA probes and at $64°$ C in 0.2x SSC, 0.2% SDS for TelSau2.0 probes. Radioactive signals were detected using phosphor screens and a Typhoon FLA 9000 instrument (GE Healthcare).

For STELA experiments 2 μg of genomic DNA were digested with EcoRI. Digested DNA was then purified with the GeneClean Turbo Kit (MP Biomedicals). For the ligation step, 1 μl of 10 μM telorette 4 oligonucleotide was added to 20 ng of digested DNA. The reaction mix was incubated at $60°$ C for 10 minutes and cooled down at RT before adding 0.1 μl of 100mM ATP, 0.1 μl of 400 Units/μl T4 DNA ligase (NEB) and 1μl of 10x T4 DNA ligase buffer. The reaction mix was brought to 20 μl with water and incubated at $35°$ C for 12 hours and at $70°$ C for 15 min. For the following step we prepared 15 μl-PCR reaction containing 400 pg ligated DNA, 0.5 μM TelTail reverse primer, 0.5 μM forward primer (see Table 1: R3 (15q)), 0.3 mM dNTPs, 5 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 0.5 mM $MgCl_2$, 1.5 Units of Taq Thermoprime Polymerase (ABgene) and 0.15 Units of Pwo polymerase (Roche).

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STELA PCR reactions were performed as follows: 15 sec at 94° C, 30 sec at 65° C, and 10 min at 68° C for 25 cycles. PCR products were separated in 0.8% agarose gels, which were subsequently dried, denatured and hybridized with radiolabeled probes corresponding 15q subtelomeres (15q probes). Hybridizations were as described above for TRF analysis excepted for 15q probe hybridization, which was done at 50° C and post hybridization washes at 50° C in 1x SSC, 0.2% SDS. Radioactive signals were detected using phosphor screens and a Typhoon FLA 9000 instrument (GE Healthcare).

STELA analysis was performed using QuantityOne and R software. For each lane, which represents one PCR reaction, the lowest intensity band was considered to be one telomere. All other bands in the same lane were calculated to be one or more telomeres of the same size according to their fold increase in intensity over the lowest intensity band. Finally, all telomeres from all lanes and for each sample were used to calculate average telomere length and Box-Whisker-plots. The data in Box-Whisker-plots is represented as follows from bottom to top: 1) the lowest value excluding outliers (data points, which are less then 1.5 times the lower quartile), 2) the lower quartile, 3) the median 4) the upper quartile and 5) the highest value excluding outliers (data points, which are more then 1.5 times the upper quartile). Outliers not contained in the whiskers are represented as dots.

## 5.6   DNA methylation analysis

Genomic DNA was collected as described above for telomere length analysis. For TERRA promoter methylation analysis, genomic DNA for each sample was digested overnight separately with MspI and HpaII, and afterwards separated on a 1.2% agarose gel. DNA was then transferred onto a Nylon membrane (GE Osmonics) and denatured in 0.4 N NaOH, 0.6 M NaCl. Hybridization was performed for ~18 h at 60° C with $P^{32}$-labeled 655 bp-long DNA fragments containing 9x29bp repeats and 4x37bp repeats from the TelBam3.4 human

subtelomere (Brown 1990), or for $\sim$18 h at 64$°$ C with P$^{32}$-labeled probes generated by random primer labeling of a $\sim$1-kb DNA fragment comprising 61-29-37 repeats. We performed post-hybridization stringency washes in 0.2x SSC, 0.2% SDS at the same temperature as the hybridization, and detected and quantified radioactive signals as for northern blots.

## 5.7  Chromatin immunoprecipitation (ChIP)

We cross-linked cells in 1% formaldehyde for 30 min at room temperature. We resuspended cell pellets in 1% SDS, 10 mM EDTA, and 50 mM Tris-HCl (pH 8); sonicated them using a Bioruptor (Diagenode); and diluted extracts in 150 mM NaCl, 20 mM Tris-HCl (pH 8), 1% Triton X-100, and 2 mM EDTA. We performed immunoprecipitations using rabbit polyclonal antibodies raised against phosphorylated serine S2 or serine S5 (A300-654A and A300-655A, Bethyl Laboratories) from the human RNAPII C-terminal repeat. After isolating immunocomplexes using protein A and G beads and purifying immunoprecipitated DNA with the Wizard SV gel and PCR cleanup system (Promega), we dot-blotted DNA onto nylon membranes and hybridized it with P$^{32}$-labeled 61-29-37 repeat probes as for DNA methylation analysis. After signal detection, we stripped the filters and hybridized them sequentially with P$^{32}$-labeled probes detecting telomeric and Alu repeat (see Table 1) sequences.

## 5.8  Western blotting

For each sample 1x10$^6$ cells were collected by trypsinization, and lysed by boiling for 10 minutes in 2x Lämmli buffer. Proteins were separated using an 8% SDS-polyacrylamide gel and transferred onto a nitrocellulose (GE Osmonics) using a semi-dry transfer system (BIO-RAD). Membranes were blocked for 1h at room temperature in PBS-T containing 5% low fat milk powder (PBS-T-M). Primary antibody hybridization was done over night at 4$°$ C in PBS-T-M and

secondary antibody hybridization for 50 minutes at room temperature in PBS-T-M. Signals were detected using ChemiGlow reagent and the FluorChem imaging system (both Cell Biosciences). Primary antibodys used were anti-PCNA (PCNA antibody sc-56, mouse monoclonal, SantaCruz Biotechnology), anti-HA-tag (C29F4, rabbit, Cell Signaling), anti-hTERT (anti-human telomerase, rat monoclonal, Cat. # 95, Diesse Diagnostica ITA), anti DNAPKcs (A300-516A Bethyl Laboratories), anti DNMT1 (rabbit polyclonal, Abcam), and anti DNMT3b (rabbit polyclonal, Abcam). Secondary HRP-coupled anti-mouse, anti-rabbit, and anti-rat antibodies were obtained from Santa Cruz Biotechnology.

## 5.9 Telomere repeat amplification protocol (TRAP) assays

qRT-PCR based TRAP assays were performed using the Quantitative Telomerase Detection Kit (Catalog No. MT3011, Allied Biotech) according to manufacturer's instructions. For gel based TRAP assays $1 \times 10^6$ cells were collected by trypsinization and lysed for 20 minutes on ice in TRAP extraction buffer (0.25 mM sodium deoxocholate, 1% Nonidet P-40, 150 mM NaCl, 10 mM Tris HCl pH 7.5, 1mM $MgCl_2$, 1mM EGTA, 10% glycerol, 1mM dTT, 1x protease inhibitor complex). Subsequently the lysate was centrifugated for 5 min at 4° C at 4500 rcf, the supernatant was recovered, and the protein concentration was determined using standard Bradford assay protocols. For each sample 0.1 µg, 0.05 µg and 0.01 µg protein were mixed in a 50 µl reaction with a final concentration of 1x TRAP buffer (40 mM Tris-HCl pH 8.3, 13 mM $MgCl_2$, 126 mM KCl, 0.01% (v/v) Tween 20, 2mM EGTA, 0.2 mg/ml BSA), 25 µM dNTPs, 2 ng TS primer, and 2 µCi alpha-P$^{32}$-dGTP. RNAse treatment of TRAP samples was done by adding 10 µg RNase A to the sample and incubate for 20 min at 37° C. All TRAP mixtures were incubated for 30 minutes at 30° C, and then

heated up to 94° C, before 2 Units Taq polymerase, 2 ng ACX primer, 0.024 fg TSNT primer, and 0.1 µg NT primer all in 1x TRAP buffer were added to the reaction mix. This was followed by 27 PCR cycles of 94° C for 30 seconds and 60° C for 30 seconds. The reactions were then mixed with 6x gel loading buffer (60% glycerol, 0.1% Bromophenol blue, and 0.1% Xylene Cyanol), and separeted on a 15% non-denaturing polyacrylamide gel. The gel was dried for 2h at 75° C and exposed to a phosphor screen. Radioactive signals were detected using a Typhoon FLA 9000 instrument (GE Healthcare).

## 5.10   RNA fluorescence in situ hybridization (FISH)

Cells were plated on Coverslips at 50% confluency a day before the experiment was performed. The next day, after a brief wash in 1x PBS, a pre-extraction step was performed in CSK buffer (Azzalin et al., 2007) for 7 minutes on ice. Following this treatment the cells were fixed in 4% paraformaldehyde at RT for 10 minutes. The composition of the blocking solution was 1x PBS with 5% BSA and 0.1% Tween-20, with the addition of 10 mM Vanadyl Ribonucleoside Complex (NEB). After 30 minutes in the blocking solution at room temperature, the cells were incubated with Mouse Anti TRF2 clone 4A794 antibody (Millipore 05-521) diluted 1:200 for 1 hour at room temperature in a humid chamber. Donkey anti mouse secondary antibody conjugated with Alexa 488 (Invitrogen) was used at a dilution of 1:2000. All washes were performed in 1x PBS containing 0.1% Tween-20. After the Immunofluorescence protocol cells were fixed once more in 4% paraformaldehyde. Followed by permeabilization in the CSK buffer at room temperature for five minutes. The samples were next washed in 2x SSC. The cells were subjected to dehydration in an ethanol series, followed by an overnight hybridization at 37° C with the TERRA probe labeled with cy3-dCTP (Perkin Elmer). For strand specific detection of telomeric RNA, the TERRA probe was random primer labeled with cy3-dCTP, dTTP and dATP (for detection of the 5'-UUAGGG-3' strand). The post hybridization

washes were performed with 50% formamide in 2x SSC at 39° C followed by washes in 2x SSC. After staining with DAPI the coverslips were mounted in Vecta Shield (Vector labs). Images were taken as Z stacks 0.2 µms apart using the Deltavision Multiplexed system (Applied Precision). For analysis TRF2 foci and TERRA foci were assumed to be 0.7 µm in size, and counted using the spot detection application of Imaris software (Bitplane). TRF2 and TERRA foci were considered to be colocalizing if they were 0.6 µm or lass apart, using the colocalization application of Imaris software (Bitplane).

## 5.11   Automated microscopy and data analysis

Cells were plated in 96-well plates (Greiner: 655090) and transfected with siRNA using Lipofectamine RNAiMax and Qiagen siRNAs according to manufacturer's instructions. 72h post transfection cells were permeabilized using 0.5% Triton X-100 in 1x PBS, fixed with 4% paraformaldehyde and stained with DAPI. Microscopy was done on a MD ImageXpress Micro microscope using 10x and 20x objectives. Image analysis was done using Cell Cognition software (www.cellcognition.org) by only taking DAPI positive regions (nuclei) into account. The average eGFP fluorescence per nucleus was then calculated using an R script.

## 5.12   eGFP Microscopy

Cells were plated on coverslips at 50% confluency one day before the experiment was performed. Cells were fixed in 3.7% formaldehyde, permeabilized in 0.5% Triton X-100 in 1x PBS, and stained with DAPI. The coverslips were then mounted on an object carrier using DABCO and sealed with nail polish. Images were taken using a Zeiss LiveCell Station microscope using 20x and 40x objectives.

# Materials and Methods

| Oligo name | Oligo sequence (5'-3') | Application |
|---|---|---|
| P1 (TelBam3.4) | TAAAAATGTTTCCCGGTTGC | PCR |
| P2 (TelBam3.4) | CACCCTCACCCTAAGCACAT | PCR |
| P1 (TelSau2.0) | CCGTTTGCTGCCCTGAATAA | PCR |
| P2 (TelSau2.0) | CCCGAACCTGAACCCTAACC | PCR |
| P1 (15q) | AGCTGCGTTGTCCTCTGCAC | PCR |
| P2 (15q) | AACCCTAACCACATGAGCAACG | PCR |
| P1 (eGFP) | ATGGATCCATGGTGAGCAAGGGCGAG | PCR |
| P2 (eGFP) | CGAGATCTGAGTCCGGACTTGTACA | PCR |
| beta-actin | GTGAGGATCTTCATGAGGTAGTCAGTCAGGT | RNA hybridization |
| 18S | CCATCCAATCGGTAGTAGCG | RNA hybridization |
| GAPDH probe | GGGTGGAATCATATTGGAACATGTAAACCATGTAG | RNA hybridization |
| oC | CCCTAACCCTAACCCTAACCCTAACCCTAA | RT |
| P3 (TelBam3.4) | CACCCTCACCCTAAGCACAT | qRT-PCR |
| P4 (TelBam3.4) | AAGCAAAAGCCCCTCTGAAT | qRT-PCR |
| P3 (TelSau2.0) | CCGCATCGAGGGTGAATAAA | qRT-PCR |
| P4 (TelSau2.0) | TCTGACGCTGCACTTGAACC | qRT-PCR |
| P5 (TelSau2.0) | GAATCCTGCGCACCGAGAT | qRT-PCR |
| P6 (TelSau2.0) | CTGCACTTGAACCCTGCAATAC | qRT-PCR |
| P3 (15q) | CAGCGAGATTCTCCCAAGCTAAG | qRT-PCR |
| P4 (15q) | AACCCTAACCACATGAGCAACG | qRT-PCR |
| P3 (Xp/Yp) | GCAAAGAGTGAAAGAACGAAGCTT | qRT-PCR |
| P4 (Xp/Yp) | CCCTCTGAAAGTGGACCAATCA | qRT-PCR |
| P3 (U6) | GGAATCTAGAACATATACTAAAATTGGAAC | qRT-PCR |
| P4 (U6) | GGAACTCGAGTTTGCGTGTCATCCTTGCGC | qRT-PCR |
| P3 (eGFP) | TATCATGGCCGACAAGCAGAAGAAC | qRT-PCR |
| P4 (eGFP) | TTTGCTCAGGGCGGACTGGGTGCTC | qRT-PCR |
| P3 (GAPDH) | TCAACGACCACTTTGTCAAGC | qRT-PCR |
| P4 (GAPDH) | CCAGGGGTCTTACTCCTTGG | qRT-PCR |
| GAPDH | CATGAGTCCTTCCACGATACC | RNase H |
| o1 (TelBam3.4) | TAAAAATGTTTCCCGGTTGC | RT-PCR |
| o2 (TelBam3.4) | CACCCTCACCCTAAGCACAT | RT-PCR |
| o1 (TelSau2.0) | CCGTTTGCTGCCCTGAATAA | RT-PCR |
| o2 (TelSau2.0) | TCTGACGCTGCACTTGAACC | RT-PCR & RNase H |
| oR1 (TelBam3.4) | GTTTTCCGCACTGAACCGCTCTAACTG | 5' RACE |
| oR2 (TelBam3.4) | CTCACCCTAAGCACATGAGGAATGTGG | 5' RACE |
| Telorette 4 | TGCTCCGTGCATCTGGCATCCTAACCC | STELA |
| Teltail | TGCTCCGTGCATCTGGCATC | STELA |
| ALU probe | GTGATCCGCCCGCCTCGGCCTCCCAAAGTG | DNA hybridization |
| TS | AATCCGTCGAGCAGAGTT | TRAP |
| ACX | GCGCGGCTTACCCTTACCCTTACCCTAACC | TRAP |
| NT | ATCGCTTCTCGGCCTTTT | TRAP IC |
| TSNT | AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT | TRAP IC |

**Table 1: Oligonucleotide sequences.**
RT: reverse transcription; qRT-PCR: quantitative reverse transcription PCR; RACE: rapid amplification of cDNA ends; STELA: single telomere length analysis; TRAP: telomere repeat amplification protocol.

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1. **Farnung BO**[*], Brun CM[*], Arora R, Lorenzi LE, Azzalin CM (2012) Telomerase Efficiently Elongates Highly Transcribing Telomeres in Human Cancer Cells. PLoS ONE 7(4): e35714.

2. **Farnung BO**, Giulotto E, Azzalin CM (2010) Promoting Transcription of Chromosome Ends. Transcription 1(3):140-143.

3. Nergadze SG[*], **Farnung BO**[*], Wischnewski H, Khoriauli L, Vitelli V, Chawla R, Giulotto E, Azzalin CM (2009) CpG-island Promoters Drive Transcription of Human Telomeres. RNA 15(12):2186-94.

# Curriculum Vitae

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# 9. Peer reviewed articles

In the following the peer reviewed articles are attached, where most of the data presented in this dissertation was published:

1. **Farnung BO**[*], Brun CM[*], Arora R, Lorenzi LE, Azzalin CM (2012) Telomerase Efficiently Elongates Highly Transcribing Telomeres in Human Cancer Cells. PLoS ONE 7(4): e35714.

2. **Farnung BO**, Giulotto E, Azzalin CM (2010) Promoting Transcription of Chromosome Ends. Transcription 1(3):140-143.

3. Nergadze SG[*], **Farnung BO**[*], Wischnewski H, Khoriauli L, Vitelli V, Chawla R, Giulotto E, Azzalin CM (2009) CpG-island Promoters Drive Transcription of Human Telomeres. RNA 15(12):2186-94.

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# Peer reviewed articles

# Telomerase Efficiently Elongates Highly Transcribing Telomeres in Human Cancer Cells

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## Abstract

RNA polymerase II transcribes the physical ends of linear eukaryotic chromosomes into a variety of long non-coding RNA molecules including telomeric repeat-containing RNA (TERRA). Since TERRA discovery, advances have been made in the characterization of TERRA biogenesis and regulation; on the contrary its associated functions remain elusive. Most of the biological roles so far proposed for TERRA are indeed based on *in vitro* experiments carried out using short TERRA-like RNA oligonucleotides. In particular, it has been suggested that TERRA inhibits telomerase activity. We have exploited two alternative cellular systems to test whether TERRA and/or telomere transcription influence telomerase-mediated telomere elongation in human cancer cells. In cells lacking the two DNA methyltransferases DNMT1 and DNMT3b, TERRA transcription and steady-state levels are greatly increased while telomerase is able to elongate telomeres normally. Similarly, telomerase can efficiently elongate transgenic inducible telomeres whose transcription has been experimentally augmented. Our data challenge the current hypothesis that TERRA functions as a general inhibitor of telomerase and suggest that telomere length homeostasis is maintained independently of TERRA and telomere transcription.

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## Introduction

The physical ends of linear eukaryotic chromosomes are transcribed into a variety of non-coding RNA (ncRNA) species constituting the 'telomeric transcriptome'. Among these species, the long ncRNA TERRA (telomeric repeat-containing RNA) was first discovered in mammalian cells and successively described in non-mammalian eukaryotes including zebrafish, *Arabidopsis thaliana*, the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* [1–7]. TERRA molecules are transcribed from the regions immediately preceding the telomeres -the subtelomeres- towards the end of the chromosomes primarily by the DNA-dependent RNA polymerase II (RNAPII), which uses the telomeric C-rich strand as a template. Hence, individual TERRA molecules comprise a subtelomeric tract and G-rich telomeric repeats (5′-UUAGGG-3′ in mammals). TERRA remains associated to telomeres post-transcriptionally suggesting that TERRA is a constitutive component of telomeric heterochromatin [1,3,4,6]. Other RNA species transcribed from chromosome ends comprise ARIA, a C-rich telomeric RNA so far identified only in fission yeast and plants, and two complementary subtelomeric transcripts devoid of detectable telomeric repeats named ARRET, identified in budding and fission yeasts, and αARRET, identified only in fission yeast [1,4,5,7].

Subtelomeric promoters driving the transcription of TERRA have been identified in human cells and comprise CpG dinucleotide-rich islands composed of stretches of 29 and 37 bp tandem repeats (29–37 repeats). 29–37 repeats are preceded by tandemly repeated 61 bp units that are dispensable for promoter activity and of so far uncharacterized function [8,9]. CpG dinucleotides within TERRA promoters are heavily methylated in different cancer and primary cells [9]. In human colorectal carcinoma HCT116 cells knocked out for the two DNA methyltransferase enzymes DNMT1 and DNMT3b (DKO cells), TERRA promoter methylation is completely abolished and RNAPII binding to TERRA promoters and TERRA steady-state levels are markedly augmented [9]. Thus, the concerted activity of DNMT1 and 3b restricts TERRA promoter transcriptional activity, at least in HCT116 cells. Similarly, decreased subtelomeric CpG methylation is accompanied by increased TERRA cellular levels in cells derived from human patients affected by immunodeficiency, centromeric region instability, facial anomalies (ICF), a recessive syndrome deriving from germline mutations in the DNMT3b gene [10]. Strangely, TERRA abundance is reduced in mouse cells deficient for DNMT1 and DNMT3a/b, although global methylation of subtelomeres is compromised, suggesting species-specific mechanisms of TERRA regulation mediated by DNMTs [6,11]. Indeed, TERRA promoters are still to be characterized in mouse cells and it remains possible that murine TERRA promoters are not regulated through CpG methylation.

While TERRA biogenesis and regulation have been extensively studied, a lack of reproducible experimental tools to alter TERRA cellular levels accounts for the sparse knowledge of TERRA-

associated functions. Most of the putative roles so far ascribed to TERRA were deduced from *in vitro* experiments where short TERRA-like RNA oligonucleotides were employed. Such *in vitro* experiments have suggested that TERRA might regulate telomere length homeostasis, telomere replication and telomeric DNA condensation [6,12–14]. In particular, TERRA-like oligonucleotides strongly inhibited telomerase activity in telomeric repeat amplification protocol (TRAP) and telomerase direct assays [6,14]. Therefore, it is generally assumed that TERRA acts as a general inhibitor of telomerase-mediated telomere elongation and a few indirect *in vivo* evidences apparently support this assumption. A budding yeast telomere artificially forced to transcribe underwent shortening, while the length of the remaining telomeres was unaffected [15]. Yeast mutants with compromised Rat1p RNA exonuclease activity bear higher amounts of TERRA molecules and shorter telomeres as compared to wild type counterparts [16]. Finally, telomeres are shorter in cells established from ICF patients than in cells from healthy donors [10]. Nevertheless, it has not been directly tested whether the telomere shortening observed in these different cellular systems truly derives from telomerase inhibition. Thus, the actual biological relevance of the ability of TERRA-like oligonucleotides to inhibit telomerase activity *in vitro* still remains to be assessed.

In order to determine whether TERRA and telomere transcription affect telomerase activity *in vivo*, we have exploited two alternative human cellular systems where TERRA transcription is augmented. We show that DKO cells infected with retroviruses expressing the catalytic subunit of telomerase elongate their telomeres as efficiently as parental cells with intact DNMT activities. Consistently, telomerase biochemical activity appears not to be affected in the same cells. We also present the development of a cellular system where transcription of unique 'transcriptionally inducible telomeres' (tiTELs) can be controlled at will. Transcription induction of tiTELs does not affect their homeostatic length, nor does it prevent telomerase-mediated re-elongation upon shortening induced by telomerase inhibitors. Altogether our results challenge the commonly accepted notion that TERRA is a cellular inhibitor of telomerase and beg for exploring alternative functions exerted by TERRA and telomere transcription. In addition, our results imply that the telomere shortening observed in systems where TERRA was aberrantly elevated [10,15,16] likely derives from compromised telomere integrity rather than telomerase inhibition.

## Results and Discussion

### TERRA steady-state levels are maintained independently of telomere length in human cancer cells

The proposed role for TERRA in inhibiting telomerase activity has suggested a model where long telomeres accumulate or produce more TERRA, thereby preventing telomerase to further extend them [6,14,17]. Correlative evidence using non-isogenic mammalian cells lines of different origins seems to support this hypothesis [6]. On the contrary, recent work in budding yeast has shown that experimentally induced over-elongation or shortening of telomeres does not alter TERRA levels and RNAPII occupancy at chromosome ends [18]. We therefore set up to test whether a correlation exists between TERRA levels and telomere length in isogenic human cells with telomeres of different lengths.

We stably infected cervical cancer HeLa cells with retroviruses expressing the catalytic subunit of human telomerase (hTERT). As a control, we infected the same cells with empty vector (ev) retroviruses or with retroviruses expressing hTERT C-terminally-fused to an influenza hemagglutinin epitope tag (hTERT-HA).

hTERT-HA displays normal catalytic activity *in vitro* but fails to elongate telomeres *in vivo*, likely due to impaired recruitment to telomeres [19]. Stably infected populations were selected and maintained in culture for several population doublings (PDs). Telomere restriction fragment (TRF) analysis of genomic DNA indicated that at the time when the experiments were performed hTERT infection had led to a substantial elongation of bulk telomeres: TRFs were comprised between 2 and 5 kb in ev-infected cells and between 5 and more than 15 kb in hTERT-infected cells (Figure 1A). As anticipated, hTERT-HA expression did not alter telomere length, although it was expressed at levels comparable to hTERT (Figure 1A and E). We then digested genomic DNA with *Msp*I or with its CpG methylation sensitive isoschizomer *Hpa*II and hybridized it to radiolabelled probes corresponding to the 29–37 repeats found within TERRA promoters. Consistent with what we have previously reported [9], we found that 29–37 repeats are largely methylated in HeLa cells. The restriction pattern detected with 29–37 repeat probes was not altered in hTERT-expressing cells as compared to ev and hTERT-HA control cells, indicating that telomere elongation does not affect the methylation state of TERRA promoters (Figure 1B). Northern blot analysis of total RNA using telomeric probes did not disclose any substantial changes in total TERRA cellular levels in hTERT-infected cells as compared to ev- or hTERT-HA-infected controls (Figure 1C). A size shift of TERRA molecules towards higher molecular weights was evident in cells with elongated telomeres, suggesting that at least in these cell lines telomere length might influence the length of TERRA molecules (Figure 1C). Consistent with the northern blot analysis, quantitative real-time PCR (qRT-PCR) measurements of TERRA molecules transcribed from chromosome arms 10q, 15q and XpYp revealed no statistical significant change in TERRA steady-state levels upon telomere elongation (Figure 1D). We also performed analogous experiments in primary human lung fibroblasts (HLF) and, as for HeLa cells, telomere elongation did not significantly alter the methylation state of TERRA promoters and cellular TERRA levels (Figure S1).

In a complementary approach, we cultured HeLa cells in presence of the telomerase inhibitor BIBR1532 [20] for approximately 19 weeks. BIBR1532 treatment led to a substantial shortening of bulk telomeres: telomere length was mostly comprised between 2 and 5 kb in untreated control cells and between 1 and 3 kb in BIBR1532-treated cells (Figure 2A). Treated cells divided similarly to untreated cells indicating that telomeres were long enough to sustain normal cell growth (data not shown). Telomere shortening did not induce any substantial change in the methylation state of 29–37 repeats nor in total or chromosome specific TERRA steady-state levels (Figure 2B–D). Altogether, these experiments indicate that, as recently shown for budding yeast [18], telomere length alterations do not affect TERRA promoter methylation or TERRA cellular levels in human cells. We conclude that TERRA transcription is not regulated by telomere length, at least in the cell types that we have analyzed.

### DNA methyltransferase-deficient cells have normal telomerase activity

The elevated TERRA levels present in DKO cells make them a suitable cellular system to test the effects exerted by TERRA on telomerase activity. We stably infected DKO and HCT116 parental (par) cells with hTERT or ev retroviruses and selected stable populations. Possibly due to the different transcriptional state of the two cell lines, we repeatedly found that over-expressed hTERT protein levels were more than two-fold higher in par than in DKO cells (Figure 3A). As shown by qRT-PCR and northern
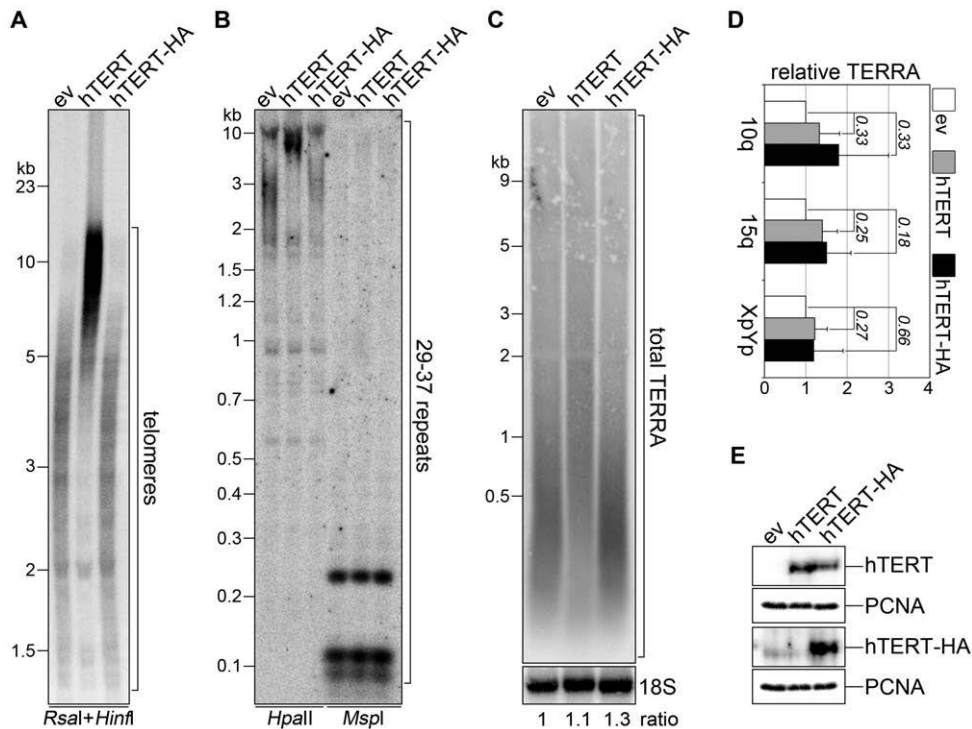
**Figure 1. TERRA steady-state levels are not affected by telomere elongation in HeLa cells.** (**A**) TRF analysis of HeLa cells infected with empty vector (ev), hTERT or hTERT-HA retroviruses. DNA was digested with *Rsa*I and *Hinf*I restriction enzymes and hybridized with telomeric probes. (**B**) The same DNA as in **A** was digested with *Hpa*II (methylation sensitive) or *Msp*I (methylation insensitive) restriction nucleases and hybridized with a probe detecting the 29–37 bp repeats of TERRA promoters. (**C**) Nuclear RNA was hybridized using telomeric probes to detect total TERRA and successively with 18S rRNA probes to control for loading. Numbers at the bottom are the ratios between TERRA and 18S signal expressed as fold increase over ev-infected samples. Molecular weights are on the left in kilobases. (**D**) qRT-PCR analysis of the steady-state levels of TERRA transcripts originating from 10q, 15q and Xp/Yp chromosome ends. Bars are averages from three independent experiments expressed as fold increase over ev-infected samples. Error bars and numbers are standard deviations and P-values, respectively. (**E**) Western blot analysis of infected cells using anti-hTERT (to detect all hTERT molecules), anti-HA (to detect hTERT-HA) and anti-PCNA (loading control) antibodies.
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blot, the steady-state levels of TERRA molecules transcribed from 61-29-37 repeat-containing 10q and 15q chromosome ends were dramatically increased in DKO cells (Figures 3B and S2A) and, consistently with the results above described for HeLa and HLF cells, hTERT stable expression did not alter TERRA amounts in either cell line (Figure 3B). We prepared protein extracts and analyzed telomerase activity using quantitative TRAP assays. No significant difference in telomerase activity was measured in ev-infected par and DKO cells, while hTERT infection led to a substantial increase in telomerase activity in both cell lines (Figure 3C and D). TRAP activity was slightly higher in hTERT-infected par cells than in hTERT-infected DKO cells and we ascribe this difference to the lower amounts of total hTERT protein expressed in the DNMT-deficient cell line (Figure 3A, C and D). RNA isolated from TRAP extracts contained 10q and 15q TERRA molecules, although a large fraction of them (approximately 90 and 80% for par and DKO cells, respectively) remained in the cell pellets left after extraction (Figure 3E), likely because the majority of TERRA is chromatin associated [1,3,6]. Still, considering that 10q and 15q TERRA molecules are ~300-fold more abundant in DKO than in par cells (Figure 3B), we estimate that DKO TRAP extracts contained ~600-fold more TERRA molecules than par extracts. In conclusion, we report that TRAP activity is similar in cells with drastically different TERRA levels. This is in stark contrast with results obtained *in vitro* using short TERRA-like oligonucleotides [6,14]. We believe that the short RNA oligonucleotides employed

*in vitro* do not behave as natural, long TERRA molecules, possibly due to their use in high, non-physiological concentrations.

## Efficient telomerase-mediated telomere elongation in DKO cells

To monitor how telomerase elongates telomeres in par and DKO cells we prepared genomic DNA from infected cells 2, 5 and 9 days after infection. We then performed TRF analysis using probes specifically detecting chromosome 10q subtelomeric sequences or telomeric repeat probes detecting bulk telomeres. Although 10q and bulk TRFs were substantially shorter in DKO than in parental cells, they both underwent progressive lengthening throughout the chosen time-course in cells infected with hTERT but not in ev control cells (Figure 4A). Thus, telomerase is able to elongate telomeres both in par and DKO cells including 10q telomeres, which are heavily transcribed in DKO cells (Figures 3B and S2A). To gain more quantitative data, we made use of single telomere length analysis (STELA), a PCR-based approach that allows to precisely measure the length of individual telomeres [21]. STELA of 15q telomeres confirmed that the average telomere length in DKO cells is lower than in parental cells (5.9 kb and 3.8 kb in par and DKO cells, respectively; Figure 4B and C). Nevertheless, as already suggested by TRF analysis, 15q telomeres were efficiently elongated in both cell lines infected with hTERT retroviruses (Figure 4D). By quantitative analysis of STELA products and normalization through the population doubling (PD) time calculated for the two cell lines
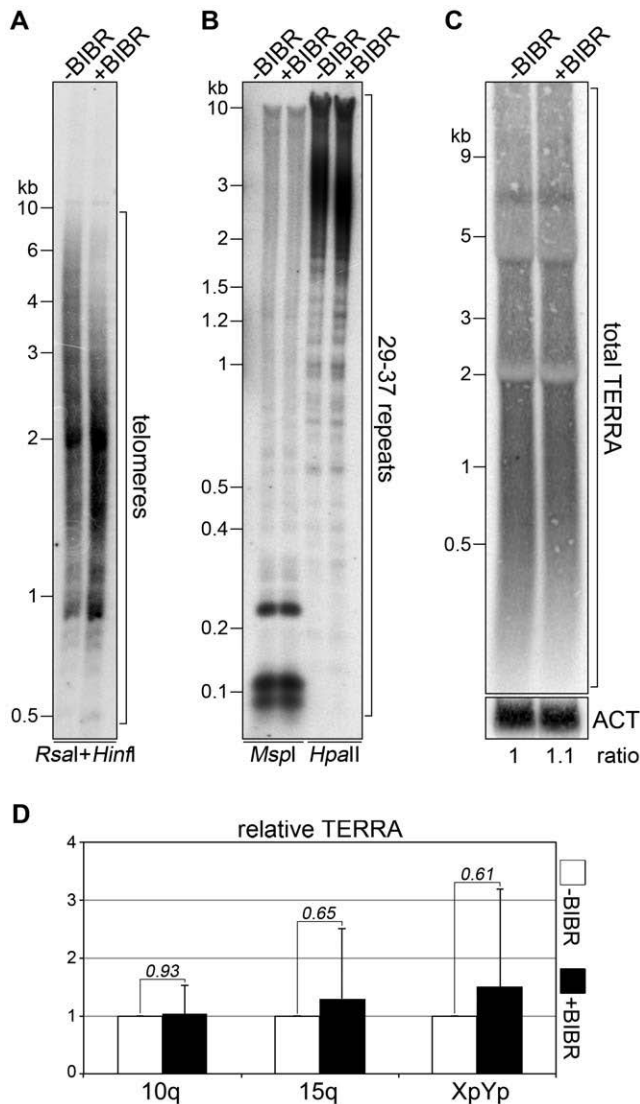
**Figure 2. TERRA steady-state levels are not affected by telomere shortening in HeLa cells. (A–D)** HeLa cells were treated with the telomerase inhibitor BIBR1532 (+BIBR) for 19 weeks or left untreated (−BIBR). Telomere length, TERRA promoter methylation and total or chromosome specific TERRA steady-state levels were analyzed as in Figure 1. Total RNA was used for all experiments. For northern blot analysis of total TERRA (**C**) beta-actin (ACT) was used as a normalization control. Molecular weights are on the left in kilobases.
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(17.7 h and 29.6 h for par hTERT and DKO hTERT cells, respectively) we estimated that telomerase elongation rates were approximately ~204 bp/PD in par cells and ~186 bp/PD in DKO cells (Figure 4C and D). This slight difference in telomerase elongation rates does not correlate with the enormously increased levels of 15q TERRA in DKO cells (Figure 3B) and is likely to be ascribed to the different hTERT protein levels detected in infected par and DKO cells (Figure 3A). Trypan blue staining and fluorescence-activated cell sorter (FACS) analysis of Annexin V and propidium iodide-stained cells did not reveal any major difference in the fraction of dead or apoptotic cells in the different cell lines nor did it show substantial changes in the distribution of cells among the different phases of the cell cycle (Figure S2B). This indicates that the higher PD time measured for DKO cells is likely

attributable to a longer cycling time of these cells, a notion further corroborated by the evident delay in cell cycle progression observed for synchronized DKO cells released from a nocodazole block (Figure S3A).

Normal telomere elongation in hTERT infected DKO cells does not support the notion that TERRA functions as a telomerase inhibitor *in vivo*. Still, we considered the possibility that TERRA transcripts in DKO cells are unable to inhibit telomerase either because they do not properly localize to telomeres or because they are drastically down-regulated during S-phase, when telomerase is acting [17,22]. We combined indirect immunofluorescence with RNA fluorescence *in situ* hybridization (IF/RNA-FISH) to simultaneously detect the telomeric factor TRF2 and TERRA molecules. DKO cells contained more and brighter TERRA foci than parental cells and hTERT infections did not alter the overall pattern of TERRA hybridization. Approximately 20 and 30% of TRF2 foci co-localized with TERRA foci in par and DKO cells, respectively (Figure S2C and D), most likely reflecting the fact that the increased levels of TERRA in DKO cells facilitated the detection of TERRA foci. On the other hand, the fraction of TERRA foci co-localizing with telomeres was similar in both cellular backgrounds, indicating that the intrinsic ability of TERRA to remain associated to telomeric heterochromatin does not require intact DNMT activities (Figure S2C and D). Importantly, hTERT expression did not alter the co-localization rates in either cell line. Hence, the fraction of TERRA associated to telomeres is similar in par and DKO cells, and telomere elongation does not noticeably affect TERRA localization. We then blocked par and DKO cells in G2/M phase and released them synchronously into the cell cycle for a time sufficient to progress through S-phase (Figure S3A). We prepared total RNA at different time points and dot-blot hybridized it to TERRA and actin probes. TERRA levels remained stable throughout the entire time course in both cell lines (Figure S3B and C). Altogether, these observations disclose that telomerase-mediated telomere elongation is not substantially impaired in DKO cells, where TERRA transcription rates and steady-state levels are dramatically increased [9]. This lack of robust inhibition does not stem from mislocalization of TERRA or from its down-regulation during S-phase. This is well in line with our observations that telomerase activity is unaffected in the same cell lines.

## Generation of human cancer cell lines carrying transcriptionally inducible telomeres

DNMT1/3b deletion leads to global transcriptional changes in DKO cells [23]. To obtain a cellular system in which transcription of unique telomeres could be altered, we generated stable cell lines carrying transgenic telomeres that can be transcribed in an inducible fashion ('transcriptionally inducible telomeres'; tiTELs). We combined the telomere seeding phenomenon with the inducible gene expression Tet-ON technology [24–26] and engineered a telomere seeding vector comprising a hygromycin resistance cassette and a doxycycline (DOX)-inducible minimal CMV promoter placed upstream of a (TTAGGG)n telomeric array (Figure 5A). A ~1.6 kb unique sequence referred to as 'transcriptionally inducible subtelomere' (tiSUBTEL) separates the telomeric sequence from the inducible promoter (Figure 5A). The seeding plasmid was linearized by *Apa*I digestion in order to expose the telomeric tract at its 3′ end and transfected into a Tet repressor-expressing cell line derived from HeLa cells (T-Rex-HeLa). Independent hygromycin-resistant clones were selected and tested for tiTEL seeding by Southern blot analysis. Genomic DNA was digested with *Xho*I in order to release tiTEL TRFs and hybridized using radiolabeled probes corresponding to tiSUBTEL
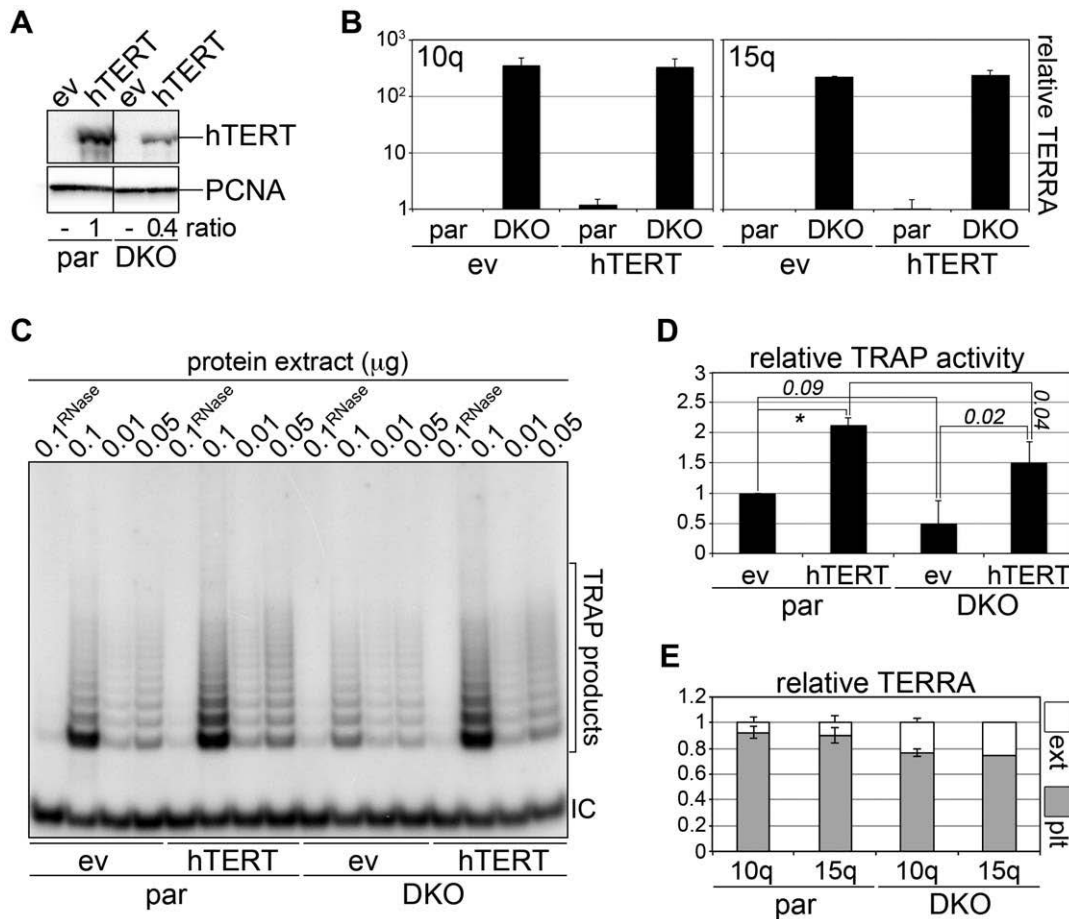
**Figure 3. Cells deficient for DNMT1 and 3b display normal telomerase activity.** (**A**) Western blot analysis of hTERT-expression in HCT116 parental (par) or DNMT1 and 3b double KO (DKO) infected cells. Numbers at the bottom are the ratios between hTERT and PCNA (loading control) signals, expressed as fold increase over hTERT-infected parental cells. (**B**) qRT-PCR analysis of the steady-state levels of TERRA transcripts originating from 10q and 15q chromosome ends expressed as fold increase over ev-infected par cells. Bars and error bars are averages and standard deviations from three independent experiments. (**C**) Analysis of TRAP amplification products from the indicated cells lines. Three different amounts of total proteins were used for each cell line and to control for specificity one sample was pre-treated with RNase A. Control primers amplifying an internal control (IC) were included in all reactions. (**D**) Quantification of telomerase activity in the indicated samples using qRT-PCR-based TRAP assays. Bars and error bars are averages and standard deviations from three independent experiments, after normalization through ev-infected parental cell samples. Numbers indicate P-values (*: $P < 0.01$). (**E**) qRT-PCR-based quantification of 10q and 15q TERRA transcripts in TRAP extracts (ext) or in cell pellets left after extraction (plt). Relative TERRA amounts are expressed as fractions of total TERRA molecules from pellet plus extract. Bars and error bars are averages and standard deviations from three independent experiments.
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sequences (SBP in Figure 5A). Two clones (cl12 and cl17) showed the typical smearing hybridization pattern expected for newly seeded tiTELs (Figure 5B) and were chosen for further characterization. In both clones, tiTEL TRFs were mostly comprised between 3 and 6 kb. Because *Xho*I cuts approximately 2.4 kb upstream of the first telomeric repeat on the seeding plasmid (Figure 5A), tiTELs telomeric tracts were stabilized at approximately 0.6 to 3.4 kb, a size range comparable to the one of bulk telomeres in parental and in clonal cells (Figure S4A). We further confirmed tiTEL seeding in clones 12 and 17 using additional experimental approaches. We stably infected both clonal cell lines with hTERT-expressing retroviruses and confirmed that both tiTELs and natural telomeres were readily elongated upon hTERT infection (Figures 5B and S4A). STELA with oligonucleotides corresponding to tiSUBTEL sequences produced amplification products hybridizing to both SBP and telomeric probes only when we used genomic DNA from cl12 and cl17 cells but not from parental cells (Figure S4B). DNA FISH of

metaphase chromosomes prepared from cl12 and cl17 cells using fluorescently labeled seeding plasmids devoid of telomeric repeats revealed that newly seeded tiTELs were integrated at one and two chromosome ends in clone 17 and in clone 12 cells, respectively (Figure 5C). Finally, dot blot hybridization of genomic DNA digested with the BAL31 exonuclease revealed a progressive disappearance of tiTEL hybridization signals over time (Figure S4C).

## Transcription induction of tiTELs

To test whether tiTELs responded to transcription induction, we cultured cl12 and cl17 cells in presence or absence of doxycycline (DOX) for 24 hours. We collected total RNA and subjected it to northern blot analysis using SBP probes (Figure 5A). DOX treatment led to the appearance of RNA species, referred to as 'transcriptionally inducible TERRA' (tiTERRA), comprised in length between approximately 0.5 and 6 kb (Figure 5D). Because the inducible promoter sequence is placed approximately 1.6 kb
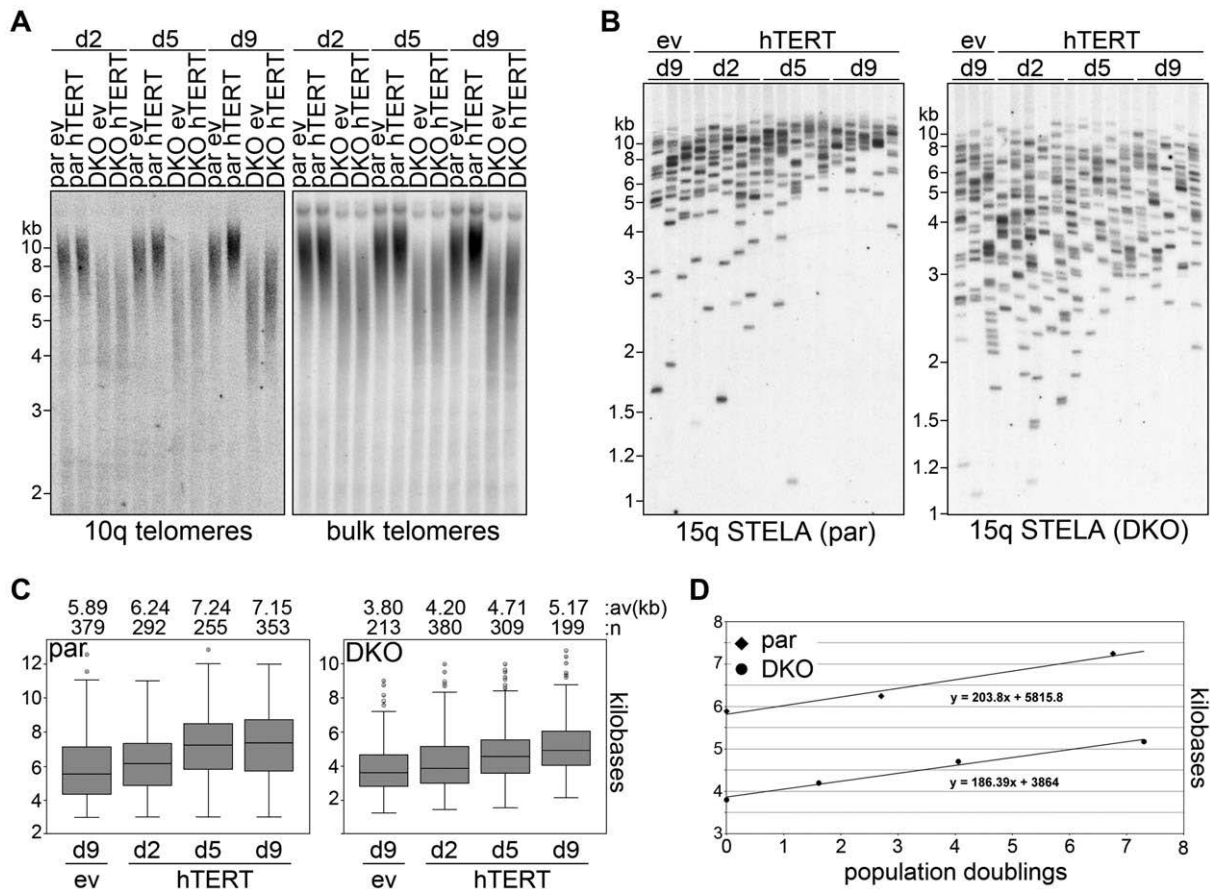
**Figure 4. Efficient telomerase-mediated elongation of telomeres in DKO cells. (A)** TRF analysis of par and DKO cells infected with empty vector (ev) or hTERT retroviruses. Genomic DNA was collected 2, 5 and 9 days (d) after infection, digested with *RsaI* and transferred to a nylon membrane. The same membrane was first hybridized with a probe detecting 10q TRFs and successively with a probe detecting total telomeres. **(B)** STELA of 15q telomere length using the same DNA as in **A**. Marker molecular weights are on the left in kilobases. **(C)** Box plot representation of 15q telomere lengths. Average telomere lengths in kilobases and the number of total telomeres analyzed (n) are indicated for each sample. **(D)** 15q telomere elongation rates expressed as average telomere length at different population doublings. Note that for par cells, only three time points were used because no statistically significant difference in average telomere length was measured between day 5 and day 9. doi:10.1371/journal.pone.0035714.g004

upstream of the telomeric sequence, we conclude that tiTEL transcription can proceed through most of the telomeric tract. We then performed qRT-PCR analysis by reverse transcribing RNA with random hexamers and PCR amplifying the obtained cDNA using SBF and SBR oligonucleotides (Figure 5A). As expected, no tiTERRA was detected in parental cells, confirming the specificity of the RT-PCR approach (Figure 5E). Although at low levels, tiTERRA transcripts were already detected in both uninduced clones, revealing a basal transcriptional activity from the inducible CMV promoter in absence of induction (Figure 5E). Absolute quantifications of tiTERRA molecules indicated that, in uninduced clones, tiTERRA was maintained at levels comparable to the ones of endogenous TERRA molecules transcribed from 10q chromosome ends (Figure S5A and B). This indicates that tiTERRA is maintained at physiological levels in both clonal cell lines. Consistent with the northern blot results, DOX treatment induced a 10-to-20-fold increase in tiTERRA levels in both clones (Figures 5E and S5B). TiTERRA molecules were also detected in PCR experiments performed with cDNA reverse transcribed using C-rich oligonucleotides (TelC) complementary to the telomeric stretch within TERRA molecules (Figure S5A and C). Thus, as

with natural TERRA, individual tiTERRA transcripts contain both telomeric and subtelomeric sequences.

TERRA expression is epigenetically regulated and the histone deacetylase inhibitor trichostatin A (TSA) promotes accumulation of TERRA transcripts in human cancer cells [27]. We treated cl12 and cl17 cells with TSA in presence or absence of DOX and quantified tiTERRA by qRT-PCR. TSA alone already induced mild accumulation of tiTERRA molecules in both cell lines, while concomitant treatments with TSA and DOX led to a 25-to-38-fold increase in tiTERRA molecules as compared to untreated control cells (Figure 5E). Therefore, as with natural telomeres, tiTELs are embedded within heterochromatin and their transcription is repressed through mechanisms that require histone deacetylation. We also measured tiTERRA levels in cl12 and cl17 cells bearing elongated telomeres due to stable infection with hTERT retroviruses and found no statistically significant difference as compared to ev-infected control cells (Figure 5E). This indicates that telomere elongation does not influence tiTERRA expression and suggests that tiTEL transcription, similarly to the one of endogenous telomeres, is not controlled by canonical telomere position effect [28].
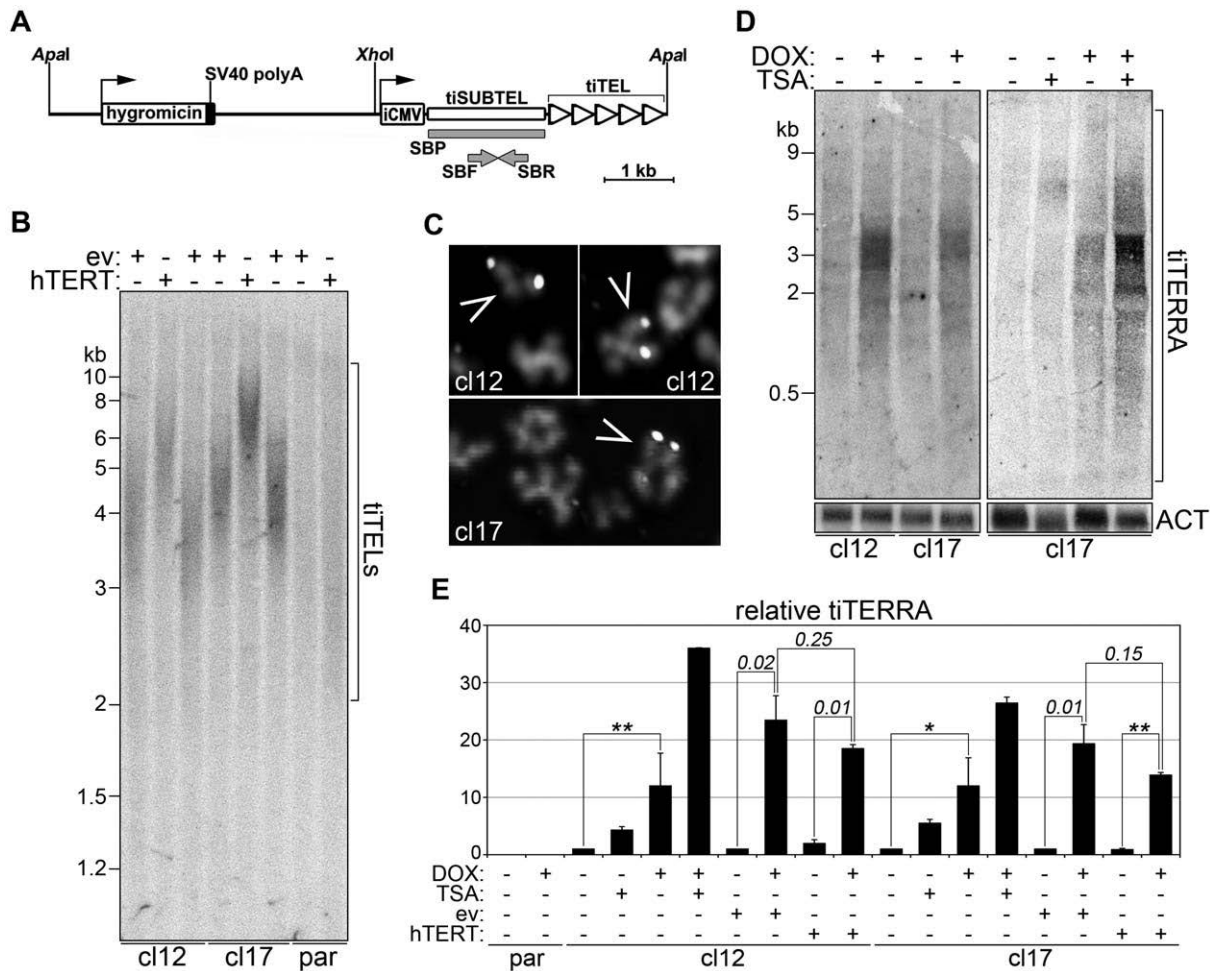
**Figure 5. Generation and characterization of transcriptionally inducible telomeres (tiTELs).** (**A**) Scheme of the tiTEL seeding vector. iCMV: inducible CMV promoter; SBF and SBR: oligonucleotides used in RT-PCR experiments; SBP: probe used in TRF, STELA and northern blot experiments. (**B**) tiTEL TRF analysis of genomic DNA prepared from clone 12 (cl12), clone 17 (cl17) and parental (par) cells infected with hTERT-expressing retroviruses or ev control retroviruses. DNA was hybridized using SBP probes. Marker molecular weights are on the left in kilobases. (**C**) Partial metaphases from cl12 and cl17 hybridized *in situ* to detect tiTELs (arrowheads). (**D**) Northern blot analysis of total RNA from cl12 and cl17 treated for 24 h with combinations of doxycycline (DOX) and trichostatin A (TSA) or left untreated. SBP probes were used to detect tiTERRA. The same membranes were stripped and re-probed to detect beta-actin transcripts (ACT) to control for loading. (**E**) qRT-PCR analysis of tiTERRA steady-levels in the indicated cell lines treated with different combinations of DOX and TSA or left untreated. For each cell line, values are expressed as fold increase over untreated samples. Bars and error bars are averages and standard deviations from 3 to 7 experiments. P values for relevant samples are indicated by numbers or by the asterisks (*: $P < 0.01$; **: $P < 0.001$).
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## Telomerase elongates tiTELs independently of tiTERRA induction

To monitor whether tiTEL length homeostasis was affected by transcription induction, we cultured cl12 and cl17 cells in presence or absence of DOX for approximately 60 PDs. As a control we also treated cells with BIBR1532. Quantitative RT-PCR analysis performed at various time points during the course of the experiment confirmed that that tiTERRA induction was maintained in DOX-treated cells (data not shown). Moreover, the growth rates of DOX-treated and untreated cells were undistinguishable (data not shown). TiTEL STELA analysis revealed that DOX treatment did not induce any significant changes in tiTEL length as compared to untreated controls, while tiTEL shortening was readily detected in BIBR1532-treated cells as expected (Figure 6A). In addition, concomitant treatments with DOX and BIBR1532 shortened tiTELs to extents similar to the ones induced by BIBR1532 only (Figure 6A). Similar results were obtained

when we analyzed the length of endogenous XpYp telomeres from the same cells (Figure 6B). These results indicate that tiTERRA transcription induction does not affect tiTEL length in a detectable manner, even upon prolonged treatments.

We reasoned that our analysis of tiTEL length homeostasis in cells treated with DOX could be biased by the fact that telomeres were already at their homeostatic length when we first induced tiTERRA transcription, while it is possible that TERRA-mediated inhibition of telomerase could only be revealed when telomerase is vigorously elongating telomeres. Therefore, we cultured cl12 and cl17 cells in presence of BIBR1532 for about 60 PDs in order to induce a substantial telomere shortening. At this point, no obvious impairment of cell division rates was observed indicating that telomeres were still long enough to sustain proper cell division (data not shown). We then washed off the telomerase inhibitor and cultured cells either in presence or absence of DOX over a time course of 27 days. STELA analysis revealed that tiTELs were re-
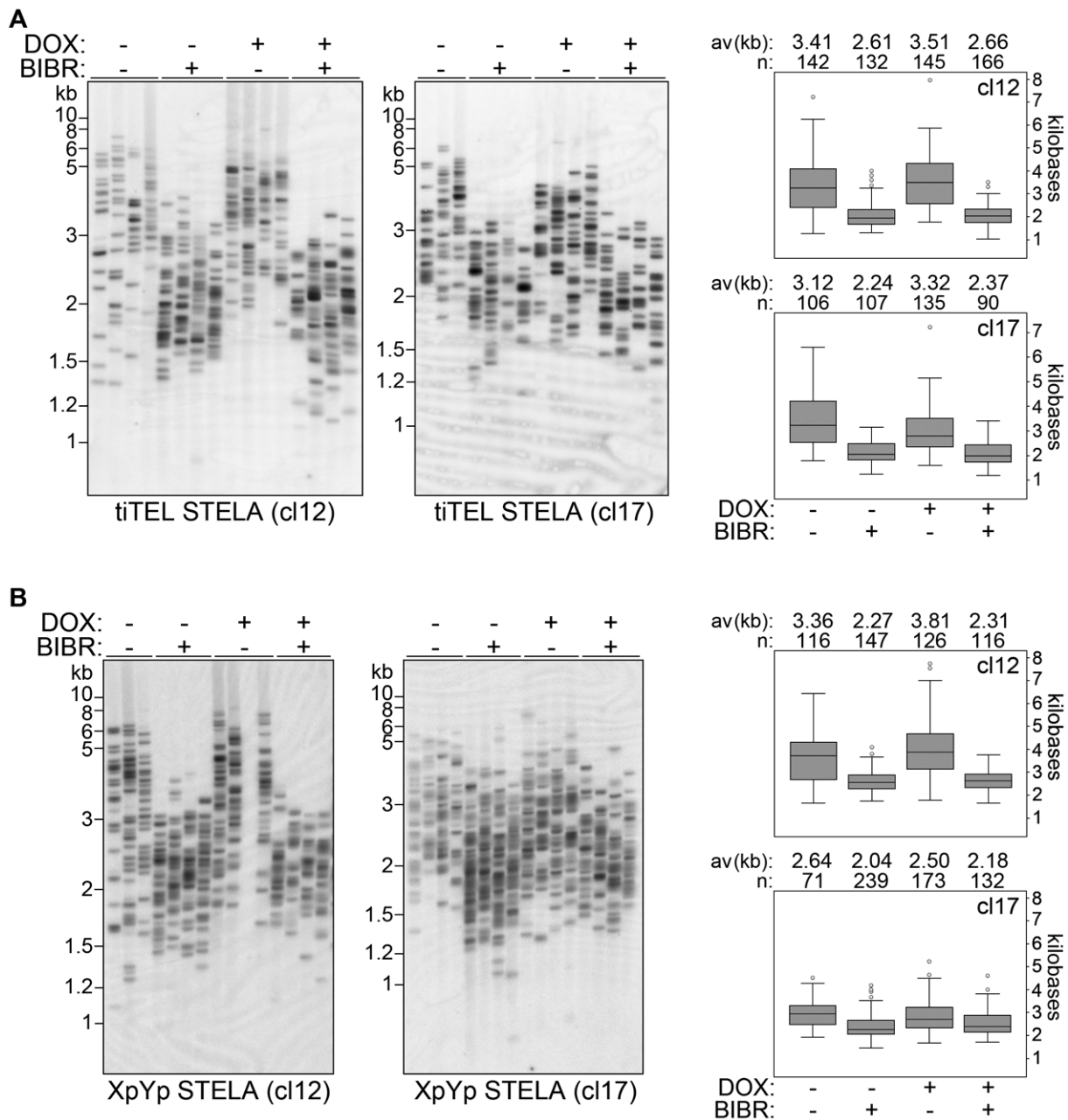
**Figure 6. TiTEL length homeostasis is maintained upon prolonged tiTEL transcription induction.** Cl12 and cl17 cells were treated with combinations of DOX and BIBR1532 (BIBR) for 60 population doublings. Genomic DNA was collected and STELA was performed for tiTELs (**A**) and XpYp telomeres (**B**). Marker molecular weights are on the left in kilobases. The Whisker-boxplots on the left represent STELA quantifications. Average (av) telomere lengths in kilobases and the number of total telomeres analyzed (n) are indicated for each sample.
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elongated over the chosen time course with similar kinetics in DOX-treated and untreated samples. TiTEL elongation rates were: 67 and 71 bp/day for cl12 with and without DOX, respectively, $P > 0.1$ for treated vs untreated samples at each analyzed time point; 58 and 61 bp/day for cl17 with and without DOX, respectively, $P > 0.1$ (Figure 7A and B). We therefore conclude that tiTERRA transcription does not affect telomerase-dependent tiTEL lengthening in our clones. As previously explained for DKO cells, it remained possible that tiTERRA levels could be down-regulated during S-phase, when telomerase acts. Dot blot hybridization of RNA prepared from synchronized cl12 and cl17 cells confirmed that tiTERRA was maintained at

higher levels in DOX-treated cells than in untreated controls during S-phase, thus disproving the above mentioned hypothesis (Figure S6). Our data clearly suggest that telomerase elongates tiTELs through pathways that are not regulated by tiTERRA or its transcription.

## Conclusions

By using independent yet complementary cellular systems, we have shown that telomerase-mediated telomere elongation is not substantially affected by the transcriptional activity associated to telomeres in human cancer cells. These observations argue against
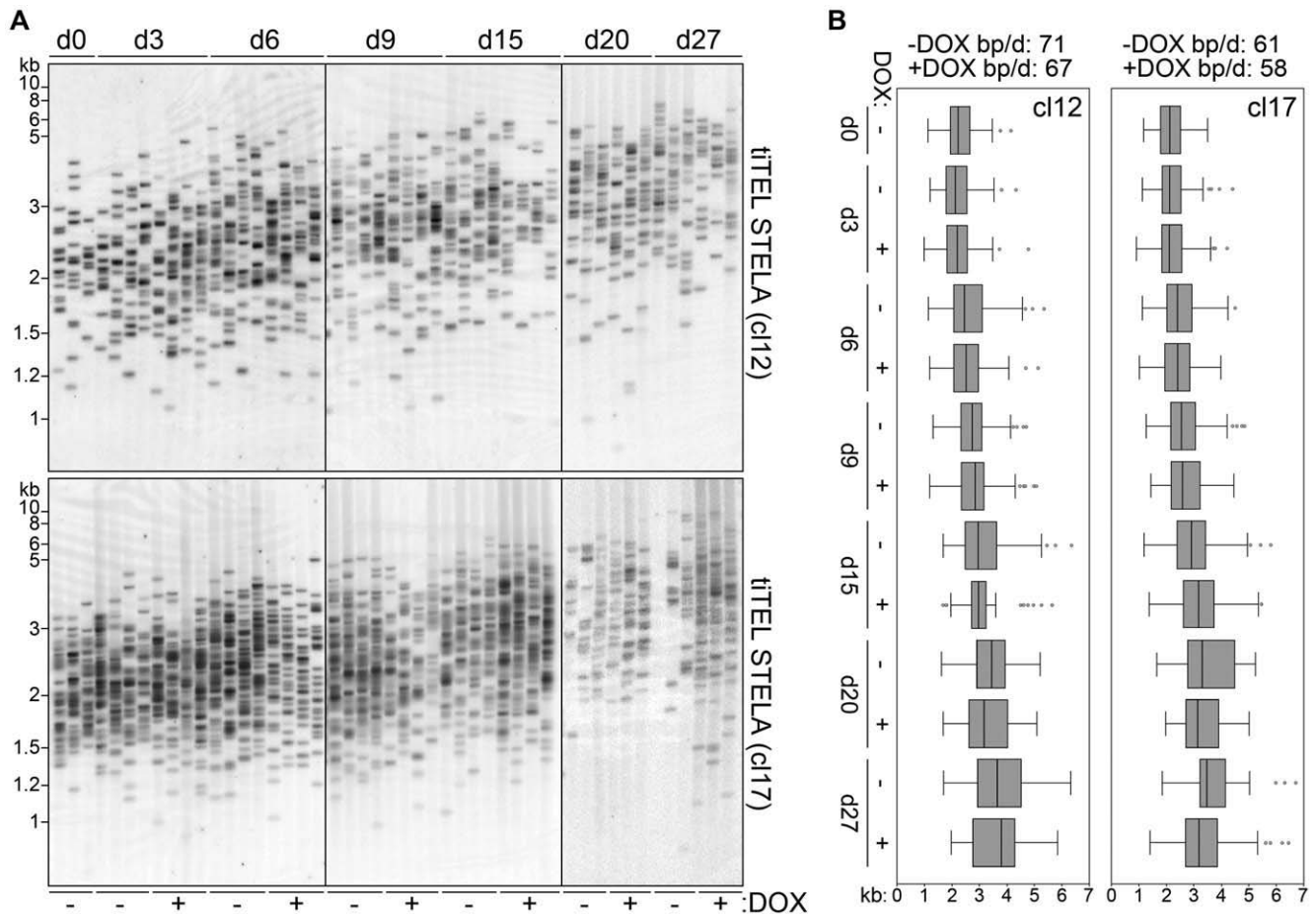
**Figure 7. Telomerase elongates tiTELs independently of their transcription induction. (A)** Cl12 and cl17 cells were treated with BIBR1532 for 60 population doublings and released into normal medium in presence or absence of DOX. STELA analysis of tiTELs was performed 0, 3, 6, 9, 15, 20 and 27 days (d) after release. Marker molecular weights are on the left in kilobases. **(B)** Whisker-boxplots represent STELA quantifications for each sample. Telomere elongation rates are expressed in base pairs per day (bp/d). All P-values between induced and uninduced samples were >0.1 and therefore are not indicated. For each condition, 77 to 400 telomeres were analyzed.
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the currently accepted notion that TERRA might function as a general telomerase inhibitor, thereby contributing to maintain telomere length homeostasis. It still remains possible that TERRA could restrict telomerase activity in some particular contexts, for example in a specific set of tissues or during development. Our observations also indicate that the telomere shortening observed in cells with augmented TERRA levels, such as yeast cells carrying inducible telomeres, Rat1-deficient yeast cells, DKO cells and cells from ICF patients [4,10,15], is unlikely to derive from telomerase inhibition. One possible explanation is that the local increase of TERRA molecules at hyper-transcribing telomeres could harm their integrity for example by impairing replication. Supporting this model, human cancer cells depleted for the RNA surveillance factor UPF1 are characterized by aberrant accumulation of TERRA molecules at telomeric heterochromatin and sudden loss of telomeric sequences due to incomplete replication of the leading strand telomere [3,29,30]. Our data also highlight that the *in vitro* set up assembled using short TERRA-like oligonucleotides might not recapitulate accurately the biological scenario where TERRA is acting. Our tiTEL system could be used to carefully test to what extent the results so far obtained *in vitro* underscore the *in vivo* functions associated to TERRA and/or telomere transcription.

## Materials and Methods

### Cell culture procedures

HeLa, HEK293T, HLF, HCT116 par, HCT116 DKO and T-Rex-HeLa (Invitrogen) cells were cultured in high-glucose D-MEM (Gibco Life Technologies) supplemented with 10% of fetal calf serum or tetracycline-free fetal calf serum for T-Rex-HeLa cells, nonessential amino acids (Invitrogen), and penicillin/streptomycin (Invitrogen). Where indicated, 1 μg/ml doxycycline (Sigma-Aldrich), 200 ng/ml trichostatin A (Sigma-Aldrich), or 1 mM BIBR1532 (Tocris Bioscience) were added to the medium. Plasmid transfections were performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Retroviruses were produced in HEK 293T cells according to standard procedures. Positively transfected or infected cells were selected in medium containing 200 mg/mL hygromycin (Fluka) or 1.5 μg/ml Puromycin (Sigma-Aldrich). For cell synchronizations, cl12 and cl17 cells were blocked in G1/S phase with 2 μg/ml aphidicolin (Sigma-Aldrich) for 20 h and released into fresh medium after washing them twice in 1× PBS. HCT116 par and DKO cells were blocked in G2/M phase with 60 ng/ml nocodazole (Sigma-Aldrich) for 14–18 h. Mitotic cells were collected by shake-off, washed three times in culture medium

and released into fresh medium. Cell samples were collected at different time points after release and cell cycle progression was monitored by fluorescence-activated cell sorter (FACS) analysis of propidium iodide-stained cells using a BD FACSCalibur flow cytometer and the FlowJo software. To analyze cell viability, cells were stained with 0.2% trypan blue (Sigma) and counted using the Cellometer Auto T4 instrument (Nexcelom Bioscience). Apoptotic cells were scored using the Annexin V-FITC Apoptosis Detection Kit (eBioscience Diagnostics) according to manufacturers instructions.

## Plasmids

TiTEL seeding plasmids were constructed by inserting a 1.6-kb-long inverted luciferase cDNA downstream of the strong human cytomegalovirus (CMV) immediate-early promoter followed by two tetracycline operator sequences. A 1.2-kb-long (TTAGGG)n stretch was excised from the plasmid pCMVTelo (kind gift from Eric Gilson) and cloned immediately downstream of the Luciferase sequence. Retroviral plasmids pBABE-puro-hTERT and pBABE-puro-hTERT-HA were from Bob Weinberg's laboratory and were purchased from Addgene (plasmids 1771 and 1772). hTERT expression was monitored by standard western blot analysis of total protein extracts using rat monoclonal anti-hTERT antibodies for total hTERT (kind gift from Elena Giulotto), rabbit polyclonal anti-HA-tag antibodies for hTERT-HA (C29F4, Cell Signaling) and mouse monoclonal anti-PCNA antibodies to control for loading (sc-56, SantaCruz Biotechnology).

## Telomere length analysis

Total genomic DNA was collected by standard phenol/chloroform extraction or using the Wizard Genomic DNA Purification Kit (Promega). For telomere restriction fragment (TRF) analysis, 5 μg of genomic DNA were digested with RsaI and/or HinfI. For tiTEL TRF analysis, 15 μg of genomic DNA were digested with XhoI and SspI. For TERRA promoter methylation analysis, 10 μg of DNA were digested either with MspI or HpaII. Restriction fragments were electrophoresed in 0.7% (for TRF analysis) or 1.2% agarose gels (for methylation analysis) and transferred to nylon membranes (GE Osmonics). Alternatively, gels were dried for in gel hybridization. Hybridizations were performed for 16 h at 50–64°C using radiolableled DNA probes corresponding to a mixture of 1–5-kb-long telomeric DNA fragments (teloA probe, to detect bulk telomeres), to tiSUBTELs (SBP probe, to detect tiTELs), to 10q subtelomeric sequences (10q probe, to detect 10q TRFs), or to 29–37 repeats (29–37 probes, to detect TERRA promoter sequences). Post-hybridization washes were done at 50–64°C in 0.2–0.5× SSC, 0.2% SDS. Radioactive signals were detected using a Typhoon FLA 9000 phosphoimager (GE Healthcare). For STELA, 2 μg of DNA were digested with XhoI or EcoRI and purified with the GeneClean Turbo Kit (MP Biomedicals). 1 μl of 10 μM telorette 3 or telorette 4 oligonucleotides was mixed with 20 ng of digested DNA, incubated at 60°C for 10 min and cooled down to RT before adding 0.1 μl of 100 mM ATP, 0.1 μl of 400 U/μl T4 DNA ligase, 1 μl of 10× T4 DNA ligase buffer (New England Biolabs) and water up to 20 μl. Reactions were incubated at 35°C for 12 h and at 70°C for 15 min. 400 pg of ligated DNA were PCR amplified in 15 μl reactions containing 0.5 μM TelTail reverse primer, 0.5 μM forward primer (tiTEL-STELA, Xp/Yp-STELA, or 15qF), 0.3 mM dNTPs, 5 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 0.5 mM $MgCl_2$, 1.5 U of Taq Thermoprime Polymerase (ABgene) and 0.15 U of Pwo polymerase (Roche). PCR cycling was as follows: 15 sec at 94°C, 30 sec at 65°C, 10 min at 68°C for 25 cycles. Amplified fragments

were separated in 0.8% agarose gels and hybridized in gel using radiolabeled TeloA probe, SBP probe or a probe corresponding to 15q subtelomeres (15q probe). Hybridizations and signal detection were as for TRF analysis. For STELA analysis we used QuantityOne and R software. For each gel lane the fragment with the lowest intensity was considered as a single telomere and the corresponding signal was used to normalize all other fragments in the same lane. Molecular sizes were calculated using DNA molecular weight markers run in the same gels. Calculated telomere lengths were cumulated and used to compute average telomere length. Data were represented in Box-Whisker-plots showing the lowest value excluding outliers (data points that are less than 1.5 times the lower quartile range), the lower quartile, the median, the upper quartile and the highest value excluding outliers (data points that are more than 1.5 times the upper quartile). Outliers are represented by dots. For BAL31 experiments 20 μg of DNA were digested with 6 U of BAL31 (New England Biolabs) and 2 μg aliquots were collected at the indicated time points, dot blotted on nylon membranes and successively hybridized using SBP, TeloA and Alu repeat probes.

## RNA isolation and analysis

Total and nuclear RNA was prepared and digested with rDNase using the NucleoSpin RNAII Kit (Macherey-Nagel). RNA was additionally digested once for northern blot and dot blot analysis or twice for qRT-PCR analysis using DNaseI (Qiagen). For northern blotting, 10–15 μg of total or nuclear RNA were separated in 1.2% agrose formaldehyde gels and transferred to nylon membranes. For dot-blotting 1 μg of total RNA was spotted to nylon membranes. Membranes were hybridized with SBP, TeloA, 18S or β-actin radiolabeled probes as for genomic DNA hybridizations. Radioactive signals were detected using the Typhoon FLA 9000 phosphoimager and quantified using ImageJ or Quantity One software (BioRad). For qRT-PCR analysis 2–5 μg of total RNA were reverse-transcribed with Superscript II or III reverse transcriptases (Invitrogen) using random hexamers (New England Biolabs) or TeloC oligonucleotides and PCR amplified using the LightCycler 480 SYBR Green I Master mix (Roche) and the oligonucleotides listed in Table S1. PCR cycling was 10 sec at 98°C and 30 sec at 60°C for 45 cycles using the Rotor-Gene Q instrument (Qiagen). Averages, standard deviations, and P-values (two-tailed Student's t-test) were calculated with Microsoft Excel software. For absolute quantifications we generated standard curves using known amounts of plasmid DNA containing tiTEL or 10q subtelomeric sequences.

## Telomere repeat amplification protocol (TRAP) assays

For standard TRAP assays, $1\times10^6$ cells were collected by trypsinization and lysed for 20 minutes on ice in TRAP extraction buffer (0.25 mM sodium deoxocholate, 1% Nonidet P-40, 150 mM NaCl, 10 mM Tris HCl pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1 mM DTT, 1× protease inhibitor complex). Protein concentrations were determined using Bradford assays and 0.1, 0.05 and 0.01 μg of total proteins were used for TRAP reactions carried out as previously described [31]. Radioactive signals were detected and analyzed as above. qRT-PCR-based TRAP assays were performed using the Quantitative Telomerase Detection Kit (Allied Biotech Inc.) according to the manufacturer's instructions and the Rotor-Gene Q instrument (Qiagen).

## DNA and RNA fluorescence in situ hybridization (FISH)

DNA and RNA FISH experiments were carried out as previously described with a few modifications [24,29,32]. For

DNA FISH, metaphase chromosome spreads were prepared from cl12 and cl17 cells and hybridized *in situ* using nick translation-labeled biotinylated probes corresponding to the entire seeding plasmid devoid of the telomeric tract. Before hybridization, chromosomes were treated with 10 μg/ml RNaseA for 30 min at 37°C and 0.005% pepsin in 0.01 N HCl for 20 minutes at 37°C. Chromosomes were then fixed in 4% paraformaldehyde diluted in 1× PBS. Each slide was hybridized with 300 ng of nick-translated probe. Slides were than washed three times in 50% formamide, 2× SSC at 42°C and three times in 2× SSC at 42°C. For probe detection, slides were washed in 4× SSC, 0.05% Tween-20, blocked in 5% milk in 4× SSC, 0.05% Tween-20 for 30 minutes at RT and incubated with 5 μg/ml avidin-fluoresceine (Vector Laboratories) in the same solution for 45 minutes at RT. Slides were washed three times in 4× SSC, 0.05% Tween-20 for 10 min at RT and then incubated with 5 μg/ml goat biotinylated anti-avidin antibodies (Vector Laboratories) for 45 min at RT. After three washes in 4× SSC, 0.05% Tween-20, we performed a second incubation with avidin-fluorescine for 45 minutes at RT and washes. DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI) and images were acquired using the Deltavision multi-plexed system (Applied Precision). For RNA FISH cells plated on coverslips were first incubated with mouse monoclonal anti-human TRF2 primary antibodies (4A794, Millipore) and succes-sively with donkey anti-mouse secondary antibody conjugated with Alexa 488 (Invitrogen). Cells were fixed in 4% paraformaldehyde and subjected to RNA FISH using TeloA probes labeled with Cy3-dCTP (Perkin Elmer). Images were taken as 0.2 μm Z-stacks using the Deltavision Multiplexed system (Applied Precision). TRF2 and TERRA foci were analyzed using the spot detection and co-localization applications of the Imaris software (Bitplane). Foci were estimated to have an average diameter of 0.7 μm and they were considered to be co-localizing when found to be 0.6 μm or less apart from each other.

## Supporting Information

**Figure S1   Shows that TERRA steady-state levels are not affected by telomere elongation in human primary fibroblasts.** (**A**) TRF analysis of human lung primary fibroblasts (HLF) infected with empty vector (ev), hTERT or hTERT-HA retroviruses. DNA was digested with *Rsa*I and *Hinf*I restriction enzymes and hybridized with telomeric probes. (**B**) The same DNA as in **A** was digested with *Hpa*II (methylation sensitive) or *Msp*I (methylation insensitive) restriction nucleases and hybridized with a probe detecting the 29–37 bp repeats of TERRA promoters. (**C**) Total RNA was hybridized using telomeric probes to detect total TERRA and successively with beta-actin (ACT) probes to control for loading. Numbers at the bottom are the ratios between TERRA and actin signals expressed as fold increase over ev-infected samples. Molecular weights are on the left in kilobases. (**D**) qRT-PCR analysis of the steady-state levels of TERRA transcripts originating from 10q, 15q and Xp/Yp chromosome ends. Bars are averages from three independent experiments expressed as fold increase over ev-infected samples. Error bars and numbers are standard deviations and P-values, respectively. *: P<0.01. (**E**) Western blot analysis of infected cells using anti-hTERT (to detect all hTERT molecules), anti-HA (to detect hTERT-HA) and anti-PCNA (loading control) antibodies.
(TIF)

**Figure S2   Shows 10q TERRA steady-state levels, cellu-lar state and TERRA localization in par and DKO cells upon telomere elongation.** (**A**) Total RNA was hybridized using probes to detect TERRA molecules transcribed from 10q

subtelomeres in the indicated cell lines. The same membrane was stripped and hybridized with 18S rRNA probes to control for loading. Molecular weights are on the left in kilobases. (**B**) Top: examples of FACS analysis of Annexin V and propidium iodide stained cells. Bottom: quantifications of alive cells (trypan blue negative cells), apoptotic cells (Annexin V – AV – positive cells) and cells in the different phases of the cell cycle as judged by propidium iodide (PI) staining. Values are averages and standard deviations form three independent experiments. (**C**) Examples of anti-TRF2 indirect immunofluorescence combined with TERRA RNA FISH in the par and DKO cells infected with hTERT or empty vector (ev) retroviruses. In the merge panels TRF2 is in green, TERRA in red and DAPI-stained DNA in blue. (**D**) Quantification of co-localization of TRF2 and TERRA foci. Bars and error bars are averages and standard deviations of co-localization events per nucleus. For each condition 50 nuclei were analyzed.
(TIF)

**Figure S3   Shows TERRA steady-state levels in synchro-nized par and DKO cells.** (**A**) FACS profiles of propidium iodine-stained cells blocked in G2/M using nocodazole and released into the cell cycle for the indicated hours. (**B**) Dot-blot analysis of total RNA isolated at indicated hours after release. The same membranes were first hybridized with telomeric probes (to detect TERRA), stripped and re-hybridized with beta-actin probes to control for loading. (**C**) Quantification of dot blots as in **B**. TERRA values were normalized through the corresponding actin values and expressed as fold increase over par cells at time 0. Bars and error bars represent averages and standard deviations from two independent experiments.
(TIF)

**Figure S4   Shows the characterization of tiTELs in cl12 and cl17 cells.** (**A**) TRF analysis of bulk telomeres using *Hinf*I and *Rsa*I-digested DNA hybridized to telomeric probes. (**B**) Control experiments demonstrating specificity of tiTEL STELAs. Nt: no template control. The same membrane was first hybridized using SBP probes, stripped and re-hybridized using telomeric probes. (**C**) Dot blot hybridization of BAL31-digested genomic DNA from parental (par), cl12 and cl17 cells. The same membrane was hybridized successively to detect tiTEL, telomeric repeat and alu repeat DNA. Radioactive signals associated to the three hybridizations are quantified below. Note that alu signals remain constant throughout the time course, while telomeric and tiTEL signals gradually diminish in both clonal cell lines.
(TIF)

**Figure S5   Shows the characterization of tiTEL tran-scription induction in cl12 and cl17 cells.** (**A**) Sketch of oligonucleotides used in RT-PCR experiments. TelC oligonucle-otides were used for RT, SBF and SBR oligonucleotides were used for PCR. (**B**) Absolute quantification of tiTERRA (ti) and natural TERRA transcribed from 10q chromosome ends in cl12 and cl17 cells treated or not with DOX. (**C**) Agarose gel analysis of tiTERRA RT-PCR products from cl12 and cl17 cells treated or not with DOX.
(TIF)

**Figure S6   Shows tiTERRA and total TERRA steady-state levels in synchronized cl12 and cl17 cells.** (**A**) FACS profiles of propidium iodine-stained cells blocked in G1/S using aphidicolin and released into the cell cycle for the indicated hours in presence or absence of DOX. (**B**) Dot blot analysis of total RNA isolated at indicated hours after release. The same membranes were hybridized successively with SBP (to detect tiTERRA),

telomeric (to detect total TERRA) and beta-actin (loading control) probes. (**C**) Quantification of dot-blots as in **B**. TERRA values were normalized through the corresponding actin values and expressed as fold increase over unsynchronized, untreated cells. Points and error bars represent averages and standard deviations from two independent experiments.
(TIF)

**Table S1  Shows the oligonucleotides used in this study.**
(DOCX)

## Author Contributions

Conceived and designed the experiments: CMA CMB BOF. Performed the experiments: CMB BOF RA LEL. Analyzed the data: CMA CMB BOF RA LEL. Wrote the paper: CMA CMB BOF.

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# Promoting transcription of chromosome ends

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We recently identified CpG island promoters driving transcription of human telomeric repeat-containing RNA (TERRA). This discovery has shaped a new concept in telomere biology, where TERRA promoters and downstream telomeric sequences constitute autonomous genic units.

Telomeres are the heterochromatic nucleo-protein complexes located at the termini of linear eukaryotic chromosomes. They allow cells to distinguish between natural chromosome ends and DNA double-stranded breaks.[1,2] Telomeres also set the lifespan of human somatic cells by triggering cellular senescence when they become "critically short" in the absence of lengthening mechanisms upon successive cell division cycles.[1-3] In vertebrates, the DNA component of telomeres comprises variably long tracts of 5'-TTAGGG-3'/3'-AATCCC-5' tandem repeats, with the G-rich strand extending beyond its complement to form a 3' overhang, termed the G-overhang (**Fig. 1A**).[1,2] The core protein component of mammalian telomeres is referred to as shelterin, and in humans it comprises the six polypeptides TRF1, TRF2, POT1, TPP1, TIN2 and hRap1. Shelterin proteins regulate telomere capping and telomere-length homeostasis.[1,2]

The longstanding dogma that telomeres are transcriptionally silent genomic loci was overturned by the discovery that long non-coding (nc) RNA molecules named TERRA emanate from chromosome ends in several eukaryotes, including yeasts, birds, fishes, plants and mammals.[4-8] Mammalian TERRA comprises telomeric UUAGGG repeats, and ranges in length from approximately 100 bases up to more than 9 kilobases (kb). Moreover, mammalian TERRA localizes preferentially to telomeres throughout the entire cell cycle, suggesting the existence of post-transcriptional mechanisms that regulate the positioning of TERRA at telomeres.[4,5] Indeed, depletion of the human proteins UPF1, hEST1A/SMG6 and SMG1, which are effectors of the nonsense mediated mRNA decay (NMD) pathway,[9] causes accumulation of telomere-bound TERRA without affecting its half-life or steady-state levels, implying that these factors actively displace TERRA from telomeres. Depletion of the same proteins also leads to stochastic loss of entire telomeric tracts.[4] These observations suggest that an excess of chromatin-bound TERRA may compromise telomere integrity or that the telomeric DNA-damage induced by SMG protein depletions might increase TERRA binding to telomeres.[4,10] In addition, recent studies show that the shelterin component TRF2 and several heterogeneous ribonucleoproteins (hnRNPs) interact with endogenous TERRA in mammalian protein extracts and promote TERRA association to telomeric heterochromatin in vivo.[11,12]

While TERRA-associated functions continue to elude researchers, discovery has progressed in TERRA biogenesis. The DNA-dependent RNA polymerase II (RNAPII) uses the C-rich telomeric strand as template to produce TERRA, as demonstrated by substantially decreased TERRA steady-state levels measured in mammalian cells treated with RNAPII inhibitors or in RNAPII-deficient yeast mutants.[5,6,13] In addition, RNAPII was found to physically associate with mammalian telomeres in vivo, as well as with
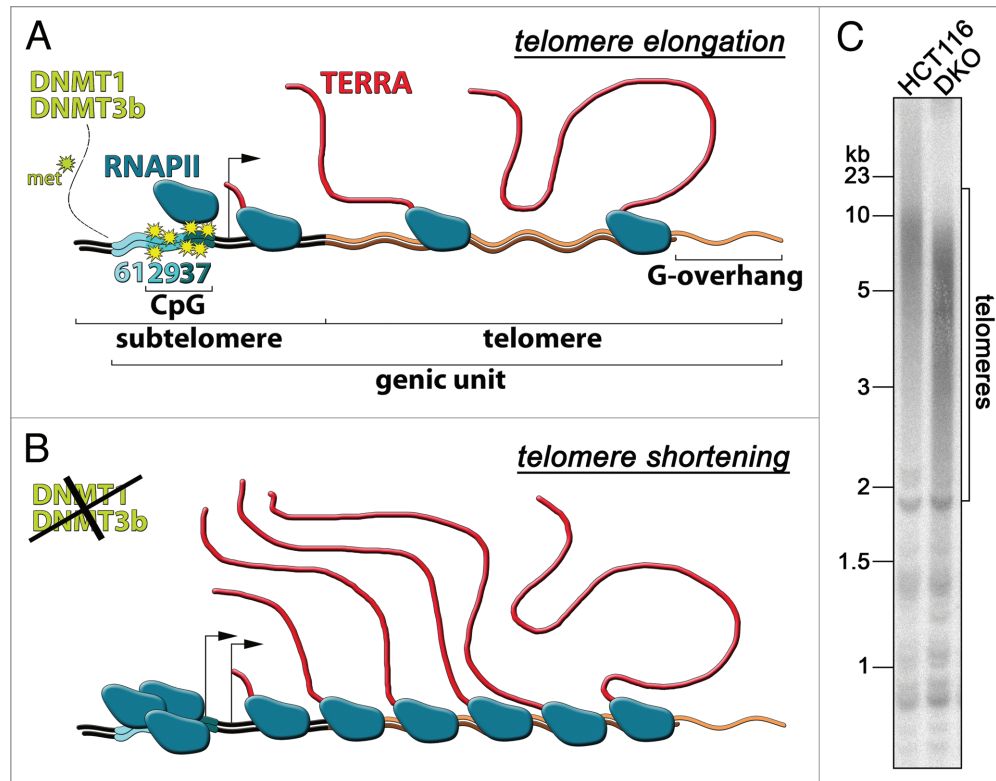
**Figure 1.** (A) Cartoon sketching of a telomeric genic unit composed of a subtelomeric promoter (61-29-37 repeats) and telomeric repeats. The arrow indicates the TERRA transcription start site. The yellow stars represent methyl groups added to promoter cytosines through concerted action of DNMT1 and DNMT3b. In these settings, telomere extension is favored. (B) Lack of DNMT1 and DNMT3b methyl transferase activities leads to increased telomere transcription, appearance of alternative TERRA start sites and telomere shortening. (C) Genomic DNA from HCT116 and DKO cells was digested with *Hinf*I and *Rsa*I, electrophoresed in 0.7% agarose gels and hybridized to radio-labeled telomeric probes according to standard protocols. Molecular weights are indicated on the left. Note that telomeres in DKO cells are shorter than in parental HCT116 cells.

the shelterin component TRF1.[5] Finally, at least a fraction of mammalian and yeast TERRA is 3'-end polyadenylated, as are the majority of RNAPII products.[5,6,13] In budding yeast, the poly-A polymerase Pap1p promotes TERRA polyadenylation and *pap1*-deficient strains show markedly reduced TERRA levels, implying that poly-A tails could stabilize TERRA molecules.[6]

### Discovery of Human TERRA Promoters

When analyzed, individual human TERRA transcripts contained both a telomeric and a subtelomeric RNA tract,[4] suggesting the exciting possibility that TERRA transcription start sites and putative promoters could lie within subtelomeres. With this speculation in mind, we were intrigued by the peculiar sequence organization of the human subtelomeres of chromosomes Xq/Yq and 10q

(originally named TelBam3.4 and TelSau2.0, respectively).[14] These subtelomeres share a conserved repetitive region that comprises, in a centromere to telomere direction, a 61 bp repeat element, a 29 bp repeat element and a 37 bp repeat element, altogether referred to as 61-29-37 repeats.[14,16] The 29 bp and the 37 bp repeats form a DNA island rich in CpG dinucleotides (CpG in **Fig. 1A**), a feature associated with many mammalian RNAPII promoter sequences.[15] Bioinformatics analysis combined with in situ hybridization experiments showed that at least 20 human chromosome ends share 61-29-37 repeats, although different numbers of tandem repeats are present at different subtelomeres.[14,16]

We developed green fluorescent protein (GFP)-based promoter reporter assays using plasmids comprising progressive 5' deletions of a 61-29-37 repeat-containing genomic tract from chromosome XqYq and found that the 29 bp and the 37 bp

repeat elements alone are sufficient to drive reporter gene expression. In addition, 61-29-37 repeats are bound in vivo by phosphorylation-activated RNAPII. Finally, we found that most XqYq TERRA transcription start sites (TSSs) are located 27 nucleotides downstream of the last 37 bp repeat and about 250 bp upstream of the telomeric tract (**Fig. 1A**).[16] Considering that TERRA molecules are up to more than 9 kb long, we concluded that at least some TERRA transcripts are mainly composed of UUA GGG RNA repeats and that transcription can proceed for several kb through the telomeric tract.

Methylation of cytosines within CpG dinucleotides, the only known methylation-based DNA modification occurring in the human genome, influences gene expression and nuclear architecture.[17] CpG methylation is established and maintained by DNA-methyltransferases (DNMTs), and generally leads to transcriptional silencing of genes located within the

methylated loci.[17] Southern blot-based methylation analysis of genomic DNA from different cancerous and primary human cells revealed that 61-29-37 repeats are embedded within heavily methylated genomic loci, with telomerase-positive cancer cell lines displaying higher degrees of methylation as compared to telomerase-negative cancer cell lines and primary fibroblasts.[16]

We then used human colon carcinoma HCT116 cell lines knocked-out for either DNMT1 or DNMT3b alone or concomitantly deleted for both enzymes (double KO; DKO).[18,19] While a single DNMT gene deletion did not substantially affect the methylation state of TERRA promoters, hypomethylation was observed in DKO cells. This was accompanied by a dramatic increase in cellular TERRA levels, by augmented binding of active RNAPII to TERRA promoter and telomeric DNA, and by appearance of alternative transcription start sites as compared to parental cells (**Fig. 1B**).[16] These data strongly suggest that the methylation of 61-29-37 repeats represses RNAPII-dependent transcription of telomeres and that DNMT1 and DNMT3b cooperatively maintain TERRA promoter methylation and TERRA transcription start site usage, at least in HCT116 cells.

Interestingly, DKO cells have overall shorter telomeres than parental cells (**Fig. 1C**), indicating that loss of methylation at subtelomeric 61-29-37 promoters and increased TERRA cellular levels might inhibit telomere elongation. Similarly, it has been reported that DNMT3b-deficient cells derived from human patients affected by ICF (immunodeficiency, centromere instability and facial abnormalities type I) syndrome display markedly diminished subtelomeric CpG methylation, shorter telomeres and higher TERRA levels as compared to control cells from healthy individuals.[20,21] An apparent contradiction to the effects exerted by DNMTs on TERRA and telomere length homeostasis in human cells is posed by the observations that mouse embryonic stem (ES) cells deficient for DNMT1 or for both DNMT3a and DNMT3b, although displaying hypomethylated subtelomeric CpG islands, have lower TERRA levels and longer telomeres as compared to ES

cells derived from wild-type animals.[5,22] It seems thus conceivable that DNMTs and CpG methylation might regulate TERRA and telomere length differently in mouse and human cells. It should also be pointed out that TERRA promoters have not yet been identified in mouse cells, leaving open the possibility that murine TERRA promoters are not regulated through CpG methylation.

Another example linking DNA methylation, TERRA expression and telomere stability is represented by the human ATRX (alpha thalassemia/mental retardation syndrome X-linked) protein, which belongs to the SWI2/SNF2 family of chromatin remodeling factors.[23] Mutations in ATRX give rise to complex trait syndromes, at least in part due to impairment of ATRX-associated functions in gene transcription regulation.[23] ATRX mutations also lead to changes in the CpG methylation pattern of several classes of highly repeated sequences, including subtelomeric repeats corresponding to TelBam3.4 sequences.[24] Independent studies have shown that ATRX deficiencies induce accumulation of DNA damage markers at telomeres, as well as increased TERRA steady-state levels. Nevertheless, no obvious effect was observed on telomere length, possibly due to the short experimental time-courses used by the authors.[25,26]

## Conclusions and Future Directions

The existence of regulated promoters dedicated to the transcription of TERRA from independent chromosome ends defines a novel scenario in telomere biology, where subtelomeric TERRA promoters, together with downstream telomeric tracts, constitute autonomous genic units (**Fig. 1A**).

The identified TERRA promoters could only be mapped to approximately half of the human subtelomeres, while TERRA transcription from chromosome ends apparently devoid of such promoters (for example Xp/Yp and 11q) has been previously documented.[4] It will be essential to test whether TERRA transcription from 61-29-37 promoter-less chromosome ends is mediated by alternative promoter types, or rather by run-on transcription of telomeres from upstream genes as is

the case in budding yeast mutants lacking Rat1p RNA exonuclease activity.[6] Also, the identification of TERRA promoters in non-human organisms will clarify how extensively TERRA biogenesis pathways are conserved throughout evolution.

Another major challenge is the characterization of TERRA-associated functions. Increasing evidence indicates that the vast repertoire of nc transcripts produced by a cell might be involved in regulating epigenetic memory, dosage compensation, heterochromatin formation and gene expression.[27] Indeed, transfection of short interfering RNA (siRNA) molecules directed against the UUAGGG repetitive TERRA sequence into human cancer cells induced downregulation of TERRA steady-state levels, diminished density at telomeres of di- and tri-methylated histone H3 lysine 9 (H3K9), telomeric DNA damage and cell cycle arrest.[12] It is therefore tempting to speculate that TERRA plays fundamental roles in assuring telomere integrity by depositing and/or maintaining telomeric heterochromatin. Nevertheless, it remains to be carefully evaluated to which extent the scored phenotypes derive from TERRA downregulation rather than from DNA damage induced at telomeres by transfection of telomeric siRNA sequences.

As above mentioned, TERRA might also act as a negative regulator of telomere lengthening mechanisms, as suggested by correlative evidence derived from mammalian and yeast systems, including the telomere shortening observed in cells deficient for DNMTs (**Fig. 1C** and refs. 5, 6, 20 and 21). TERRA-like short RNA oligonucleotides are able to inhibit telomerase activity in vitro, by base-pairing with the template region of telomerase RNA moiety.[5,28] Therefore, TERRA might repress telomerase-mediated extension of telomeres in vivo. It is also possible that the physical action of transcription of telomeres could influence telomere length, both in telomerase-positive and -negative cells, by stripping telomere length regulators off the telomeric sequence.

We believe that a mechanistic characterization of the regulatory circuits governing transcription from TERRA promoters will expand our understanding of TERRA functions and clarify if

and how TERRA contributes to hetero-chromatin establishment and telomere integrity. One first effort to be undertaken is a comprehensive isolation of transcription factors (TFs) that specifically regulate TERRA transcription through direct binding to 61-29-37 repeats. Experimental functional impairment of such TFs will help to unravel TERRA-associated functions.

Landing on "TERRA," the Latin noun for planet Earth, has marked the beginning of a new era in telomere biology. New avenues are now open towards the characterization of the complex connection between telomeres and crucial aspects of human biology such as cellular senescence, organismal aging and cancer development. In addition, TERRA de-regulation might at least in part contribute to the development of syndromes such as ICF and alpha thalassemia/mental retardation X-linked. Understanding how TERRA integrates into these phenomena might, in the long term, pave the way for the development of new therapeutic approaches.

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# CpG-island promoters drive transcription of human telomeres

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## ABSTRACT

The longstanding dogma that telomeres, the heterochromatic extremities of linear eukaryotic chromosomes, are transcriptionally silent was overturned by the discovery that DNA-dependent RNA polymerase II (RNAPII) transcribes telomeric DNA into telomeric repeat-containing RNA (TERRA). Here, we show that CpG dinucleotide-rich DNA islands, shared among multiple human chromosome ends, promote transcription of TERRA molecules. TERRA promoters sustain cellular expression of reporter genes, are located immediately upstream of TERRA transcription start sites, and are bound by active RNAPII in vivo. Finally, the identified promoter CpG dinucleotides are methylated in vivo, and cytosine methylation negatively regulates TERRA abundance. The existence of subtelomeric promoters, driving TERRA transcription from independent chromosome ends, supports the idea that TERRA exerts fundamental functions in the context of telomere biology.

Keywords: telomeres; TERRA; promoters; transcription; CpG methylation

## INTRODUCTION

Telomeres are heterochromatic DNA-protein complexes located at the end of linear eukaryotic chromosomes. Telomeres are essential to assure genome stability by allowing cells to distinguish between intrachromosomal DNA double-stranded breaks and natural chromosome ends, thereby preventing inappropriate DNA repair events (Palm and de Lange 2008; Xin et al. 2008). Telomeres also set the life span of normal adult somatic cells (Harley et al. 1990; Allsopp et al. 1992; Allsopp and Harley 1995). The DNA component of mammalian telomeres consists of tandem arrays of duplex 5′-TTAGGG-3′/3′-AATCCC-5′ repeats, with the G-rich strand extending beyond its complement to form a 3′ overhang; the protein component includes several factors, the most prominent of which are grouped together under the name "shelterin," a multiprotein com-

plex involved in telomere length regulation and telomere protection (Palm and de Lange 2008; Xin et al. 2008).

In contrast to the longstanding idea that telomeres are transcriptionally silent, we and others recently discovered that RNA polymerase II (RNAPII) transcribes telomeric DNA into telomeric repeat-containing RNA (TERRA) molecules in a variety of eukaryotes including mammals, zebra fish, and budding yeast (Azzalin et al. 2007; Azzalin and Lingner 2008; Chawla and Azzalin 2008; Ho et al. 2008; Luke et al. 2008; Schoeftner and Blasco 2008). As for the majority of RNAPII products, at least a fraction of TERRA is polyadenylated, and RNAPII associates with telomeres in vivo (Azzalin and Lingner 2008; Schoeftner and Blasco 2008). In mammals, TERRA transcripts contain telomeric 5′-UUAGGG-3′ RNA repeats, range in size from about 100 bases (b) up to more than 9 kilobases (kb), and are detected exclusively in nuclear cellular fractions (Azzalin et al. 2007; Schoeftner and Blasco 2008). Mammalian TERRA forms discrete nuclear foci that localize to telomeres not only in interphase cells but also in transcriptionally inactive metaphase cells (Azzalin et al. 2007; Ho et al. 2008; Schoeftner and Blasco 2008), suggesting the existence of post-transcriptional mechanisms retaining TERRA at telomeres. Interestingly, some human suppressors with morphogenetic defects in genitalia proteins (namely, SMG1, UPF1, and hEST1A/SMG6), which are effectors of an evolutionarily

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conserved RNA quality control pathway known as non-sense-mediated mRNA decay (Isken and Maquat 2008), negatively regulate TERRA localization to telomeres without affecting TERRA degradation rate or total cellular levels (Azzalin et al. 2007). Short hairpin RNA-mediated depletion of SMG1, UPF1, and hEST1A/SMG6 leads to sudden loss of discrete telomeric tracts (Azzalin et al. 2007), suggesting that dysfunctional TERRA localization may hinder telomere integrity.

In humans, transcription of at least a fraction of TERRA molecules starts within different subtelomeres (the genomic regions immediately adjacent to telomeres) and proceeds toward chromosome ends (Azzalin et al. 2007). Nevertheless, the molecular details of TERRA biogenesis remain to be elucidated. Here we report that CpG dinucleotide-rich DNA island promoters, sharing conserved repetitive DNA elements, are located on different human chromosome ends and drive transcription of independent TERRA molecules. TERRA CpG-island promoters are able to sustain expression of reporter genes in human cells, are located immediately upstream of TERRA transcription start sites on different subtelomeres, and are bound by active RNAPII in vivo. We also show that DNA methyltransferase (DNMT) 1- and 3b-mediated cytosine methylation of TERRA promoters negatively correlates with RNAPII binding to TERRA promoters and cellular TERRA abundance, suggesting that cytosine methylation represses TERRA promoter transcriptional activity.

## RESULTS AND DISCUSSION

### Human subtelomeres contain active CpG-island promoters

The pro-terminal DNA sequences associated with the long-arm telomeres of human chromosomes X/Y (Xq/Yq) and 10 (10q) were isolated nearly 20 years ago and named TelBam3.4 and TelSau2.0, respectively (Brown et al. 1990). The two sequences share a conserved repetitive region that extends for ∼1.6 kb (nucleotides 2110–3117) and ∼1.3 kb (nucleotides 408–1789) until ∼280 nucleotides (nt) upstream of the terminal array in TelBam3.4 and TelSau2.0, respectively (Supplemental Fig. S1). This conserved region contains three different repetitive DNA tracts: the most centromere-proximal tract comprises tandemly repeated 61-base-pair (bp) units (five repeats in TelBam3.4 versus six repeats in TelSau2.0); a second, more distal tract comprises 29-bp tandem repeats (nine repeats versus 18 repeats); a third tract comprises five tandemly repeated 37-bp DNA units in both sequences (Supplemental Fig. S1). We refer to the tandem repeat-containing region as "61-29-37 repeats" and to the ∼280 nt comprised between the last 37-bp repeat and the telomeric hexamers as "pre-tel" (Supplemental Fig. S1). BLAST search analysis demonstrated that 61-29-37 repeats are also present at 13 other human subtelomeres

(Supplemental Fig. S2, chromosome arms 1p, 2p, 3q, 4p, 5p, 8p, 9p, 11p, 15q, 16p, 17p, 19p, 21q), although variable numbers of tandem repeats are observed at different loci. Five additional subtelomeres containing TelBam3.4-like sequences (Supplemental Fig. S2, 3q, 6p, 9q, 12p, 20p) were previously identified using in situ hybridization (Brown et al. 1990). One intrachromosomal locus at 2q13 also contains 61-29-37 repeats (Supplemental Fig. S2) and corresponds to an ancestral telomere–telomere fusion point (Ijdo et al. 1991).

61-29-37 repeats possess a remarkably high (67%–86%) overall content in CpG dinucleotides, with a peak corresponding to the 29- and 37-bp repeat tracts (Supplemental Fig. S1). High CpG contents are typical of CpG islands that are found in broad-type promoters of many eukaryotic RNAPII-transcribed genes (Sandelin et al. 2007). Indeed, bioinformatic analysis of TelBam3.4 and TelSau2.0 sequences predicted the existence of CpG-island promoters spanning the 29- and 37-bp repeats (Supplemental Fig. S1). In order to experimentally test the promoter activity of this region, we generated promoter reporter plasmids where progressive 5′ deletions of a ∼3.5-kb subtelomeric DNA tract comprising 61-29-37 repeats were inserted upstream of a green fluorescence protein (eGFP) reporter gene (Fig. 1A). Fluorescence microscopy inspection of human HeLa cells transfected with reporter plasmids revealed that a ∼1-kb sequence comprising the 29- and 37-bp repeat tracts was sufficient to induce cellular eGFP expression (Fig. 1B). Quantification of eGFP fluorescence by densitometric analysis and of eGFP mRNA by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) showed that the identified promoter is approximately five times less efficient than the strong cytomegalovirus (CMV) promoter (Supplemental Fig. S3). Thus, 61-29-37 subtelomeric repeats contain sequences with promoter activity. In agreement with this, active RNAPII is enriched at both 61-29-37 and telomeric repeats over repetitive *Alu* sequences in vivo, as demonstrated by chromatin immunoprecipitation experiments performed on formaldehyde cross-linked chromatin from U2OS cells using independent antibodies against phosphorylation-activated RNAPII large subunit (Fig. 2; Hirose and Ohkuma 2007).

### Subtelomeric CpG-island promoters drive transcription of TERRA molecules

To determine whether TelBam3.4 and TelSau2.0 subtelomeres are transcribed in vivo, we performed Northern blot analysis of nuclear RNA extracted from human telomerase-negative HLF primary fibroblasts and U2OS tumor cells as well as telomerase-positive HeLa and HEK293T tumor cells, using strand-specific DNA probes, which correspond to TelBam3.4 and TelSau2.0 pre-tel sequences (Fig. 3A). The TelBam3.4 probe is 100% identical to Xq/Yq pre-tels; 92%–98% identical to 8p, 9p, 15q, and 19p pre-tels; and
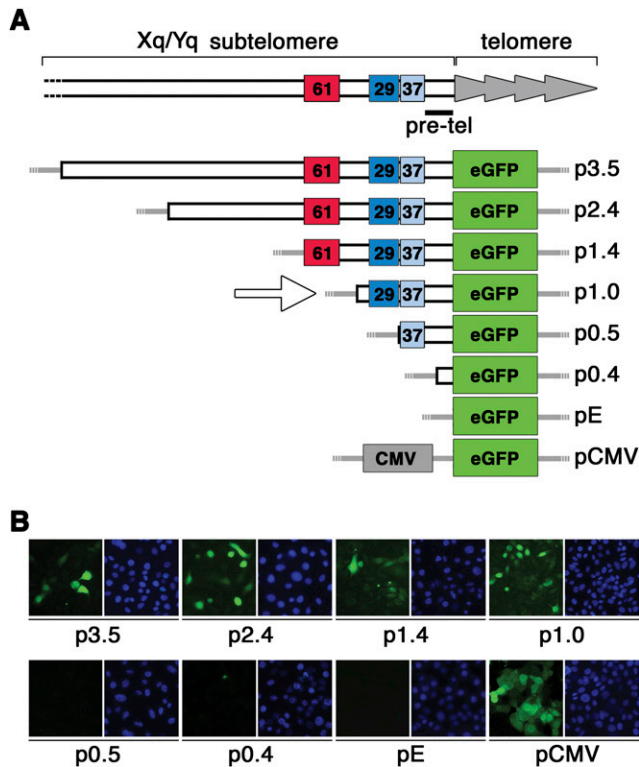
**FIGURE 1.** Promoter reporter assays. (*A*) A ~3.5-kb DNA fragment from Xq/Yq subtelomeres was cloned upstream of an eGFP cDNA (green box). The fragment contains five 61-bp tandem repeats (red box), ten 29-bp repeats (dark blue box), and five 37-bp repeats (light blue box). The original reporter plasmid was digested with restriction enzymes in order to generate plasmids containing serial 5′ deletions. Plasmid names are given on the *right*. pE: promoter-less eGFP negative control plasmid; pCMV: cytomegalovirus promoter eGFP positive control plasmid. Gray lines indicate plasmid backbone sequences. The arrow points to the reporter plasmid containing the minimal DNA tract sufficient to induce eGFP expression. (*B*) Reporter plasmids containing the puromycin resistance gene were transfected into HeLa cells. Puromycin was added 24 h after transfection, and 4 d later cells were fixed and imaged by fluorescence microscopy to detect eGFP expression (green, *left* panels) and DAPI-stained DNA (blue, *right* panels).

90%–91% identical to 10q and 21q pre-tels. The TelSau2.0 probe is 100% identical to 10q pre-tel sequences and 89%–95% identical to 8p, 9p, 16p, 15q, 19p, and 21q pre-tels. The two probes are therefore expected to detect partially overlapping families of transcripts originating from a multitude of chromosome ends. After high-stringency washes, both probes detected RNA species varying in length from ~500 b up to more than 5 kb in the different cell lines tested (Fig. 3B; Supplemental Fig. S4). The radioactive signals were completely abolished upon ribonuclease A treatment (data not shown), confirming that they originated from RNA. We obtained smeary patterns of hybridization also when we hybridized the same filters with telomeric probes, although the telomeric signal extended to molecular weights lower than 100 b (Fig. 3B; Supplemental Fig. S4). These results

suggest that transcripts originating from TelBam3.4 and TelSau2.0 subtelomeres may constitute a fraction of TERRA molecules. It is probable that total cellular TERRA also comprises transcripts deriving from subtelomeres devoid of TelBam3.4 and TelSau2.0 sequences, as well as from 5′ processing of TelBam3.4 and TelSau2.0 RNA molecules. Interestingly, while the signal detected with TelBam3.4 and TelSau2.0 probes remains fairly constant throughout all tested cell lines, the signal detected using telomeric probes reveals a two- to eightfold increase in total TERRA levels in U2OS cells, suggesting that in these cells a substantial fraction of TERRA transcripts does not contain TelBam3.4 and TelSau2.0 sequences. Although the molecular mechanisms leading to increased TERRA levels in U2OS remain unclear, a connection might exist between TERRA cellular expression and the homologous recombination-based processes maintaining telomere length in U2OS cells (Royle et al. 2008).

To verify whether TelBam3.4 and TelSau2.0 subtelomeres are indeed transcribed into TERRA molecules, we employed an RT-PCR protocol previously developed to detect TERRA transcripts (Azzalin et al. 2007). We reverse transcribed nuclear RNA using a telomeric $5'\text{-}(CCCTAA)_5\text{-}3'$ oligonucleotide (Fig. 3A, oC; Supplemental Table S1), complementary to the $5'\text{-}(UUAGGG)_n\text{-}3'$ sequence present in TERRA molecules. We then PCR-amplified the obtained cDNA with unique primer pairs corresponding to TelBam3.4 and TelSau2.0 pre-tel sequences immediately adjacent to the telomeric tracts in the genome (Fig. 3A, o1, o2; Supplemental Table S1). We obtained amplicons matching in size (Fig. 3C) and sequence (data not shown) the ones obtained in PCR reactions performed on genomic DNA, thus proving that the tested subtelomeres are indeed transcribed and that transcription continues into the
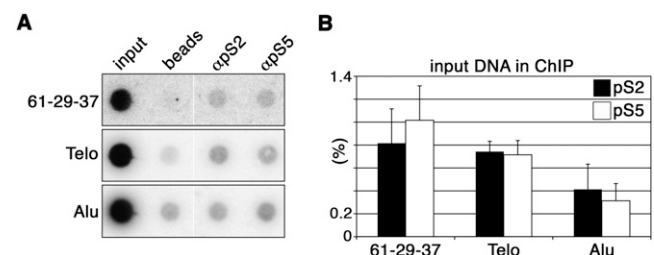


**FIGURE 2.** RNAPII binds to 61-29-37 repeats in vivo. (*A*) Formaldehyde cross-linked chromatin from U2OS cells was immunoprecipitated using antibodies raised against phosphorylated serine 2 (pS2) and serine 5 (pS5) from the C-terminal repeat of human RNAPII. Immunoprecipitated DNA was dot-blotted and hybridized with radioactive DNA probes detecting 61-29-37 repeats. The same blot was stripped and re-hybridized sequentially to detect repetitive telomeric (positive control) and *Alu* sequences. (*B*) The bar graph shows the fraction of input DNA immunoprecipitated in the different samples, after subtraction of the background signal measured for control reactions performed using only beads. Bars and error bars represent averages and standard deviations from three independent immunoprecipitations.
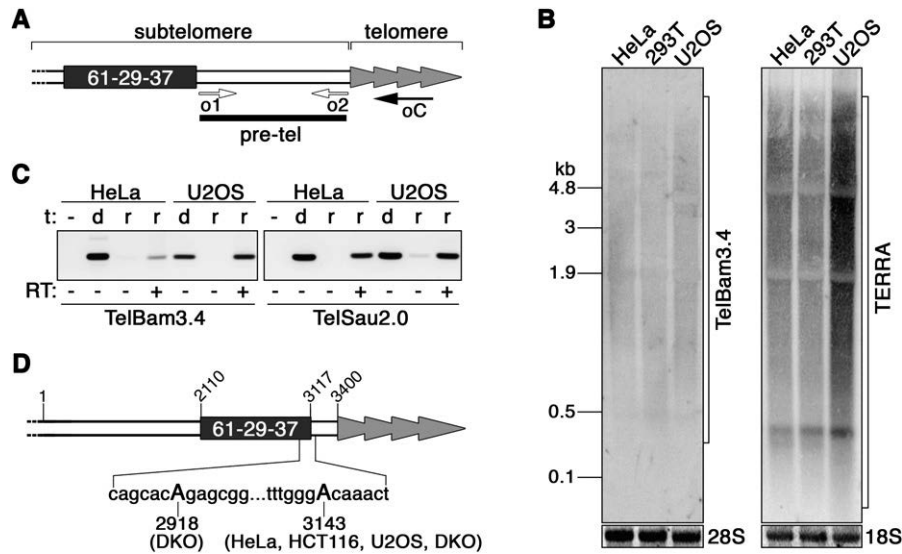
**FIGURE 3.** TERRA transcription from TelBam3.4 and TelSau2.0 subtelomeres. (*A*) Schematic representation of a 61-29-37 repeat-containing chromosome end. Note that the sketch is not to scale. (*B*) Nuclear RNA extracted from the indicated cell lines was electrophoresed and hybridized with radioactive strand-specific probes corresponding to TelBam3.4 pre-tel sequences (black bar in [*A*]). The same blot was stripped and probed for total TERRA. Ethidium bromide-stained 28S and 18S rRNA transcripts are shown to confirm equal RNA loading. Standard molecular weights are on the *left* in kilobases (kb). (*C*) Nuclear RNA (r) from the indicated cell lines was reverse transcribed (RT) using oC oligonucleotides, and cDNA was PCR amplified using o1 and o2 oligonucleotides specific for TelBam3.4 or TelSau2.0 pre-tel sequences (oligonucleotide positions are indicated by arrows in *A*). PCR products were run in agarose gels and stained. Control PCR reactions were performed using genomic DNA (d) as template (t). (*D*) RACE experiments performed on nuclear RNA from HeLa, U2OS, and HCT116 cells and from HCT116-derived cells knocked-out for DNA methyltransferases 1 and 3b (double KO, DKO) identified the capitalized adenines as transcription start sites at TelBam3.4 subtelomeres. Numbers indicate nucleotide positions as they appear in the database entry M57752.1.

telomeric tract. To reinforce these findings, we annealed nuclear RNA prepared from telomerase positive HCT116 human cancer cells with a TelSau2.0 DNA oligonucleotide adjacent to the telomeric tracts in the genome (Fig. 3A, o2; Supplemental Table S1). The oligonucleotide is 100% and 70% complementary to sequences from TelSau2.0 and TelBam3.4 pre-tel transcripts, respectively. We then treated the RNA with RNase H, which digests specifically RNA molecules engaged in DNA/RNA hybrids, and subjected the digested RNA to Northern blot analysis with radioactive telomeric probes. Quantitative analysis of the obtained hybridization profiles reproducibly revealed that the RNase H treatment led to a shift of the telomeric hybridization profile toward lower molecular weights (Fig. 4). Altogether, these results indicate that TERRA molecules originating from TelBam3.4 and TelSau2.0 subtelomeres exist in different human cell lines and constitute a substantial fraction of total TERRA.

We then used rapid amplification of cDNA ends (RACE) to isolate intact 5′ ends of TelBam3.4 transcripts from cDNA obtained by reverse transcription of HeLa, HLF, HCT116, and U2OS nuclear RNA with the telomeric oC oligonucleotide. Our RACE protocol allows amplification of RNA molecules with a methylated 5′ cap only, thereby preventing detection of uncapped and degraded RNA species. In all cell lines, we identified one unique transcription start site located 27 nt downstream from the last 37-bp

repeat (Fig. 3D) and conserved on chromosomes 8p, 9p, 15q, 16p, and 19p, and Xq/Yq subtelomeres. Although our extensive analysis of RACE products revealed only one transcription start site, we cannot exclude the possibility that alternative—perhaps more rare—start sites might exist in the same cells. In addition, as described below, usage of alternative transcription start sites can occur in DNA methyltransferase-deficient cells. Nevertheless, transcription of a fraction of TERRA molecules begins immediately downstream from 61-29-37 repeats and continues toward chromosome ends. Because the physical distance between the identified transcription start site and the telomeric tract is ~250 nt (Fig. 3D), while the length of TelBam3.4 transcripts visualized by Northern blotting varies between ~500 and >5000 b (Fig. 3B), we infer that transcription can proceed through the telomeric tract for several kilobases.

## TERRA promoters are methylated at CpG dinucleotides and cytosine methylation regulates TERRA cellular levels

DNA methyltransferase (DNMT)-mediated methylation of cytosines at promoter CpG dinucleotides negatively regulates transcriptional activity (Esteller 2007; Suzuki and Bird 2008). Furthermore, DNMT3b-deficient human cell lines established from patients affected by the immunodeficiency,

centromere instability, and facial anomalies (ICF) syndrome are characterized by hypomethylated subtelomeric DNA and increased TERRA expression, compared to normal cells from healthy individuals (Yehezkel et al. 2008). Promoter CpG methylation may thus regulate TERRA expression. We digested HLF, HeLa, and U2OS genomic DNA with the methylation-sensitive HpaII restriction enzyme or with its methylation-insensitive isoschizomer MspI and performed Southern blot hybridizations with radiolabeled DNA probes, corresponding to 61-29-37 repeats. While MspI digestion generated prominent hybridization bands ranging in size between 100 and 500 bp, additional bands of higher molecular weight (up to more than 23 kb) appeared in HpaII-digested samples (Fig. 5A). This indicates that 61-29-37 repeats are methylated in vivo. 61-29-37 repeat methylation appears to be nearly complete in HeLa cells, while a lower degree of methylation is observed in HLF and U2OS cells, as demonstrated by increased size heterogeneity of the hybridization bands in HpaII-digested samples (Fig. 5A). This is consistent with recent observations showing that telomerase-positive human cancer cells carry hypermethylated subtelomeric DNA compared to telomerase-negative cancer or primary human cells (Vera et al. 2008; Ng et al. 2009; Tilman et al. 2009). We then analyzed the methylation state of 61-29-37 repeats in human HCT116 cells knocked-out for DNMT1 ($DNMT1^{-/-}$), for DNMT3b ($DNMT3b^{-/-}$) or for both (double KO [DKO]) methyltransferases (Rhee et al. 2000; Rhee et al. 2002). Concomitant disruption of $DNMT1$ and $DNMT3b$ genes completely abolished methylation at 61-29-37 repeat CpG dinucleotides, while only modest changes were observed in single KO cells (Fig. 5B), implying that DNMT1 and DNMT3b enzymes cooperatively maintain DNA methylation at 61-29-37 repeats in HCT116 cells. Northern blot analysis disclosed dramatically increased steady-state levels of TelBam3.4, TelSau2.0, and total TERRA transcripts in DKO cells compared to parental, $DNMT1^{-/-}$, and $DNMT3b^{-/-}$ cells (Figs. 4, 6A; Supplemental Fig. S5). Similarly, treatments of HeLa cells with the DNMT inhibitor 5-azacytidine (5aza) induced an approximately threefold increase in TelBam3.4 and TelSau2.0 RNA species (Supplemental Fig. S6). The extra TelBam3.4 and TelSau2.0 transcripts detected in DKO cells (Figs. 4, 6A) are, at least in part, true TERRA molecules for the following reasons: first, RT-PCR experiments using telomeric oC oligonucleotides for reverse transcription produced TelBam3.4 and TelSau2.0 amplicons matching the corresponding genomic sequences both in parental and DKO cells (Supplemental Fig. S5). Second, RACE experiments performed on nuclear RNA prepared from DKO cells identified the same TelBam3.4 transcription start site as for parental HCT116 cells (see Fig. 3D, nucleotide 3143) in 31 out of the 41 analyzed positive RACE plasmid clones and a second transcription start site within the first 37-bp repeat (see Fig. 3D, nucleotide 2918) in the remaining 10

clones. Third, RNase H digestion of DKO nuclear RNA, previously annealed with TelSau2.0 DNA oligonucleotides, induced a substantial shift of the TERRA hybridization profile toward lower molecular weights (Fig. 4). In conclusion, CpG methylation appears to modulate the cellular abundance of TERRA (including TERRA transcribed from TelBam3.4 and TelSau2.0 subtelomeres) as well as the usage of independent transcription start sites. We hypothesize that CpG methylation represses TERRA promoter transcriptional activity. Consistent with this hypothesis, ChIP experiments revealed increased levels of active
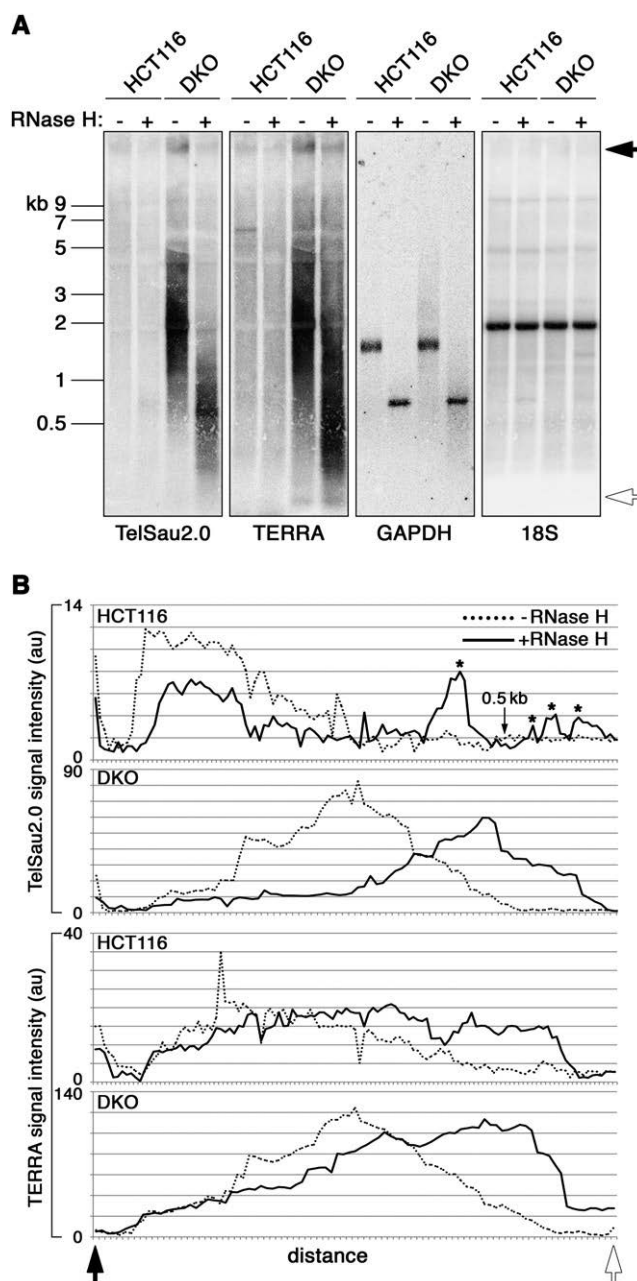


**FIGURE 4.** (Legend on next page)

RNAPII binding to 61-29-37 and telomeric repeat DNA in DKO cells compared to parental HCT116 cells (Fig. 6B). However, we cannot exclude the possibility that, in DKO cells, impaired degradation of TERRA transcripts could occur, thereby contributing to their increased steady-state levels.

## CONCLUDING REMARKS

Aberrant cellular accumulation of TERRA transcripts has been reported in *Saccharomyces cerevisiae* yeast mutants knocked out for the 5′–3′ RNA exonuclease Rat1p, which is thought to degrade RNA species derived from transcription occurring past poly(A) cleavage sites in RNAPII-transcribed genes (Rosonina et al. 2006; Luke et al. 2008). One could therefore speculate that TERRA molecules originate from inefficient transcription termination of genes placed subtelomerically. On the contrary, we reveal that in human cells, CpG-island promoters, embedded within subtelomeric 61-29-37 repeats, drive transcription of TERRA molecules possibly from up to 20 different subtelomeres, suggesting that common regulatory mechanisms control the biogenesis of TERRA transcripts originating from independent chromosome ends. We were unable to identify 61-29-37-like repeats at the remaining 26 human subtelomeres, including 11q and Xp/Yp subtelomeres, which were previously demonstrated to be transcribed (Azzalin et al. 2007). It is likely that different promoter types contribute to the biogenesis of total human TERRA, although we cannot exclude the possibility that ill-defined subtelomeric sequences available in the databases (Riethman et al. 2004; Riethman 2008) might have led us to underestimate the actual number of human subtelomeres carrying 61-29-37 repeats.



**FIGURE 5.** Cytosine methylation at 61-29-37 repeats. (*A,B*) Genomic DNA extracted from the indicated cell lines was digested with the methylation-sensitive HpaII restriction enzyme or with its methylation-insensitive isoschizomer MspI. Digested DNA was electrophoresed, blotted, and hybridized with radioactive DNA probes detecting 61-29-37 repeats. *BDNMT1$^{-/-}$* and *DNMT3b$^{-/-}$* are HCT116-derived clonal cell lines knocked-out for DNA methyltransferases 1 and 3b, respectively. Double KO (DKO−) are HCT116-derived clonal cell lines knocked-out for both methyltransferases. Standard molecular weights are shown on the *left* of each blot in kilobases (kb).

Our discoveries define human telomeres as components of integral "genic" units and make TERRA rise above the transcriptional noise associated with the human genome, supporting the idea that TERRA might exert important functions in telomere biology. It has been proposed that TERRA could repress telomerase activity at chromosome ends by base-pairing with the template sequence in the telomerase RNA moiety (Luke et al. 2008; Schoeftner and Blasco 2008). In this scenario, methylation-mediated transcriptional repression of TERRA CpG-island promoters may be part of an epigenetic tumor suppressor gene-silencing program accompanying cell transformation (Esteller 2007; Suzuki and Bird 2008).

## MATERIALS AND METHODS

### DNA sequence analysis

We aligned the TelBam3.4 and TelSau2.0 sequences with the entire human genome using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). To analyze CpG dinucleotide contents and predict CpG islands, we utilized the CpGPlot/CpGReport at the European molecular biology open software suite program (EMBOSS; http://www.ebi.ac.uk/Tools/emboss/cpgplot/) (Rice et al. 2000).

**FIGURE 4.** Transcripts from TelSau2.0 and TelBam3.4 subtelomeres constitute a substantial fraction of total TERRA molecules. (*A*) Nuclear RNA from the indicated cell lines was annealed to a DNA oligonucleotide 100% and 70% complementary to TelSau2.0 and TelBam3.4 pre-tel transcripts, respectively. The same RNA was also annealed to a DNA oligonucleotide complementary to GAPDH mRNA. RNA was incubated with RNase H or left untreated, electrophoresed, and hybridized with radioactive strand-specific probes corresponding to TelSau2.0 pre-tel sequences. The same blot was stripped and probed sequentially to detect total TERRA, GAPDH (positive control for RNAse H reaction), and 18S (control for RNase H specificity and loading control) RNA sequences. Standard molecular weights are shown on the *left* in kilobases (kb). (*B*) Charts showing the distribution of signal intensity for the Northern blots shown in (*A*). The asterisks indicate RNase H-induced peaks in the TelSau2.0 hybridization profile of HCT116 RNA. The peaks are likely to correspond to TelSau2.0-like pre-tel sequences from which the telomeric RNA tracts have been removed by the RNase H treatment. The black and white arrows indicate the lane area used for signal quantifications. Similar shifts in the telomeric profiles were obtained when RNA was incubated only with TelSau2.0 oligonucleotides, omitting the GAPDH oligonucleotides from the reactions (data not shown).
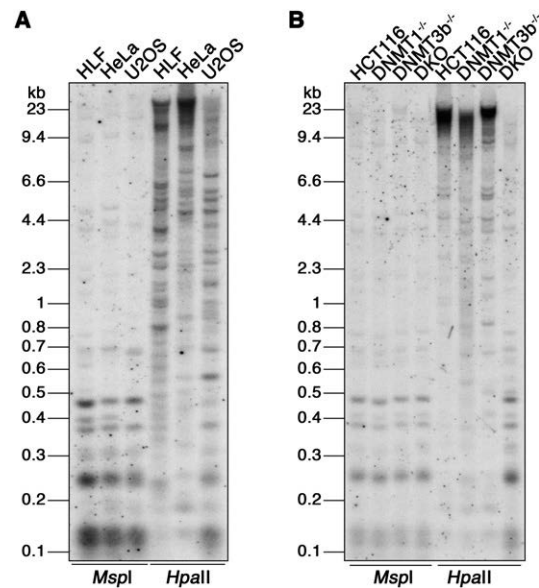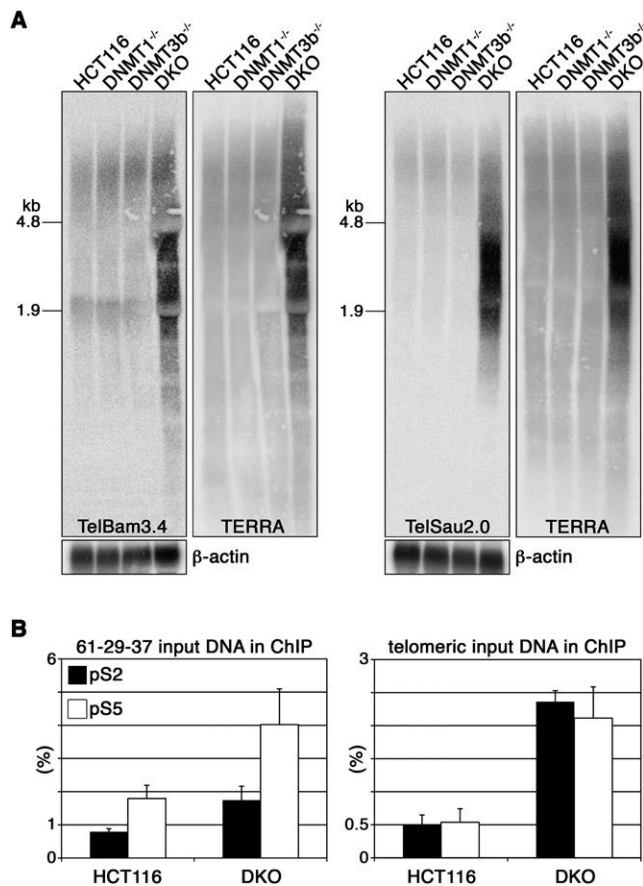
**FIGURE 6.** DNMT1 and DNMT3b enzymes cooperatively repress TERRA cellular levels. (*A*) Nuclear RNA was extracted from the indicated cell lines, electrophoresed, blotted, and hybridized using strand-specific TelBam3.4 or TelSau2.0 radioactive probes. The same blots were stripped and re-probed sequentially for total TERRA and β-actin. Molecular weights of 28S (4.8-kb) and 18S (1.9-kb) rRNA are shown on the *left*. (*B*) Formaldehyde cross-linked chromatin from the indicated cell lines was immunoprecipitated using antibodies raised against phosphorylated serine 2 (pS2) and serine 5 (pS5) from the C-terminal repeat of human RNAPII. Immunoprecipitated DNA was dot-blotted and hybridized sequentially with radioactive DNA probes detecting 61-29-37 and telomeric repeats. Graphs show the fraction of input DNA immunoprecipitated in the different samples, after subtraction of the background signal measured for control reactions performed using only beads. Bars and error bars represent averages and standard deviations from three independent immunoprecipitations.

## Plasmid constructions

Since TelBam3.4 and TelSau2.0 plasmids (Brown et al. 1990) were no longer available, we subcloned a ~3-kb *Bam*HI-*Eco*RI human genomic DNA fragment from the bacterial artificial chromosome clone RP11-34P13. This fragment is identical to a portion of the TelBam3.4 sequence and contains 61-, 29-, and 37-bp repeats. The pre-tel fragment comprised between the last 37-bp repeat and the telomeric repeats was obtained by PCR on human genomic DNA using primers constructed on the TelBam3.4 sequence. We constructed a plasmid containing the eGFP cDNA, under the CMV promoter, and the puromycin resistance gene using the

pEGFN1 and pPUR plasmids (Clontech). We then substituted the CMV promoter with the ~3.5-kb TelBam3.4 fragment and obtained deletions using appropriate restriction enzymes or substituting the CMV promoter with PCR amplified fragments. We checked all constructs by sequencing.

## Cell lines and tissue culture procedures

We cultured HLF (human lung fibroblasts), HeLa (human cervical carcinoma), HEK293T (human embryonic kidney), U2OS (human osteosarcoma), and HCT116 (human colon carcinoma) cells in high-glucose D-MEM supplemented with 10% fetal calf serum, nonessential amino acids, and penicillin-streptomycin (Invitrogen). Where indicated, we treated cells with 10 μM 5-azacytidine (Sigma Aldrich). For promoter reporter assays, we transfected HeLa cells using the Lipofectamine 2000 reagent (Invitrogen). Twenty-four hours after transfection, we selected positively transfected cells in medium containing 1 μg/mL puromycin for 4 d.

## RNA preparation and analysis

We prepared RNA from whole cells or nuclei-enriched cellular fractions (Azzalin et al. 2007) using the Nucleospin RNA II kit (Macherey-Nagel). We treated RNA twice with RNase-free DNase I (New England Biolabs) to eliminate any DNA contaminations. For RNase H experiments, we mixed 10 μg of nuclear RNA with 600 pmol of a TelSau2.0 oligonucleotide and 600 pmol of a GAPDH oligonucleotide (see Supplemental Table S1). We incubated the RNA/DNA mix at 65°C for 4 min and at room temperature for 20 min. We then added 1 U of RNase H (New England Biolabs) to the mix and allowed digestion at 37°C for 1 h. For Northern blots, we electrophoresed 10–20 μg of RNA in 1.2% formaldehyde agarose gels and blotted it to nylon membranes. We hybridized membranes for ~18 h in Church buffer containing $^{32}$P-labeled probes at 50°C–64°C. The strand-specific telomeric probe used to detect total TERRA was described previously (Azzalin et al. 2007). The TelBam3.4 and TelSau2.0 probes were genomic PCR products obtained with o1 and o2 oligonucleotides and labeled by primer extension using the o2 oligonucleotide (see Supplemental Table S1). The β-actin, GAPDH, and 18S rRNA probes were 5′ end-labeled DNA oligonucleotides (see Supplemental Table S1). After hybridization, we washed membranes in 0.2–1× SSC, 0.5% SDS at the same temperatures used for hybridizations. We detected radioactive signals using a Storm PhosphorImager (GE Healthcare) and quantified them with Quantity One (Bio-Rad) and with ImageQuant (GE Healthcare) software. For quantitative RT-PCR experiments, we reverse transcribed 1 μg of total RNA with random hexamers using the SUPERSCRIPT III RNase H⁻ reverse transcriptase (Invitrogen). We PCR amplified the obtained cDNA using eGFP and GAPDH oligonucleotide pairs (see Supplemental Table S1) for 10 sec at 95°C, 20 sec at 60°C, and 20 sec at 72°C (45 cycles) using the LightCycler 480 SYBR Green I master mix and instrument (Roche). The RT-PCR-based approach to amplify chromosome-specific TERRA molecules was previously described (Azzalin et al. 2007). We performed 5′ RACE experiments with the FirstChoice RLM-RACE Kit (Ambion) using the oC oligonucleotide for reverse transcription and the TelBam3.4 gene-specific oligonucleotides oR1 and oR2 for PCR (see Supplemental Table S1).

We cloned RACE products into the pDRIVE vector (Qiagen) and sequenced 18 to 41 independent colony plasmids for each cell line.

## DNA methylation analysis

We prepared genomic DNA with the Wizard genomic DNA kit (Promega) and digested it with HpaII (CpG methylation-sensitive) or MspI (CpG methylation-insensitive) restriction enzymes (New England Biolabs). After electrophoresis in 1.2% agarose gels, we denatured DNA, transferred it to nylon membranes, and hybridized it for ~18 h at 64°C with $^{32}$P-labeled probes generated by random primer labeling of a ~1-kb DNA fragment comprising 61-29-37 repeats. We performed post-hybridization stringency washes in 0.2× SSC, 0.5% SDS at 64°C, and detected and quantified radioactive signals as for Northern blots.

## Chromatin immunoprecipitation

We cross-linked cells in 1% formaldehyde for 30 min at room temperature. We resuspended cell pellets in 1% SDS, 10 mM EDTA, and 50 mM Tris-HCl (pH 8); sonicated them using a Bioruptor (Diagenode); and diluted extracts in 150 mM NaCl, 20 mM Tris-HCl (pH 8), 1% Triton X-100, and 2 mM EDTA. We performed immunoprecipitations using rabbit polyclonal antibodies raised against phosphorylated serine S2 or serine S5 (A300-654A and A300-655A, Bethyl Laboratories) from the human RNAPII C-terminal repeat. After isolating immunocomplexes using protein A and G beads and purifying immunoprecipitated DNA with the Wizard SV gel and PCR cleanup system (Promega), we dot-blotted DNA onto nylon membranes and hybridized it with $^{32}$P-labeled 61-29-37 repeat probes as for DNA methylation analysis. After signal detection, we stripped the filters and hybridized them sequentially with $^{32}$P-labeled probes detecting telomeric and *Alu* repeat (see Supplemental Table S1) sequences.

## SUPPLEMENTAL MATERIAL

Supplemental material can be found at http://www.rnajournal.org.

## ACKNOWLEDGMENTS

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