Regulation of telomere length and homeostasis by telomerase enzyme processivity

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Summary

Telomerase is a ribonucleoprotein consisting of a catalytic subunit, the telomerase reverse transcriptase (TERT), and an integrally associated RNA that contains a template for the synthesis of short repetitive G-rich DNA sequences at the ends of telomeres. Telomerase can repetitively reverse transcribe its short RNA template, acting processively to add multiple telomeric repeats onto the same DNA substrate. The contribution of enzyme processivity to telomere length regulation in human cells is not well characterized. In cancer cells, under homeostatic telomere length-maintenance conditions, telomerase acts processively, whereas under nonequilibrium conditions, telomerase acts distributively on the shortest telomeres. To investigate the role of increased telomerase processivity on telomere length regulation in human cells with limited lifespan that are dependent on human TERT for lifespan extension and immortalization, we mutated the leucine at position 866 in the reverse transcriptase C motif of human TERT to a tyrosine (L866Y), which is the amino acid found at the equivalent position in HIV-1 reverse transcriptase. We report that, similar to the previously reported gain-of-function *Tetrahymena* telomerase mutant (L813Y), the human telomerase variant displays increased processivity. Human TERT-L866Y, like wild-type human TERT, can immortalize and extend the lifespan of limited-lifespan cells. Moreover, cells expressing human TERT-L866Y display heterogenous telomere lengths, telomere elongation, multiple telomeric signals indicative of fragile sites and replicative stress, and an increase in short telomeres, which is accompanied by telomere trimming events. Our results suggest that telomere length and homeostasis in human cells may be regulated by telomerase enzyme processivity.

Key words: Telomerase, Telomere, Processivity, Trimming, Fragile sites

Introduction

Telomeres are nucleoprotein structures found at the ends of eukaryotic chromosomes. In humans, telomeric DNA consists of a tandem array of the hexameric sequence TTAGGG that varies in length from 4–14 kb (Blackburn, 1991; de Lange, 2004; Moyzis et al., 1988; Smogorzewska and de Lange, 2004; Zakian, 1989; Zakian, 1995). The repetitive sequences terminate in a single-stranded G-rich overhang at the 3′ end of the chromosome (Blackburn, 2001; Wright et al., 1997), which is sequestered within the intra-telomeric duplex DNA, forming a protective structure known as the telomere loop (t-loop) (Cesare and Griffith, 2004; Griffith et al., 1999). T-loops have been observed in purified telomeric DNA from human, mouse, plant and protozoa (Cesare et al., 2003; Griffith et al., 1999; Muñoz-Jordán et al., 2001; Murti and Prescott, 1999) and in native telomeric chromatin from mouse and chicken cells (Nikitina and Woodcock, 2004). The t-loop prevents the chromosome end from being recognized as a double-stranded break, from end-to-end fusions, abnormal recombination and degradation (de Lange, 2002).

In a population of cells, there is a distribution of telomere lengths for each chromosome end (Hemann et al., 2001). In 90% of cancers, the maintenance of this telomere length equilibrium depends on telomerase, a specialized reverse transcriptase (Kim et al., 1994). In the remaining cancerous human cells, maintenance occurs through a less well understood mechanism termed alternative lengthening of telomeres (ALT) (Dunham et al., 2000). Physical markers of ALT cells include long, heterogeneous telomeres (Bryan et al., 1997), extrachromosomal telomeric repeat (ECTR) DNA of both linear and circular forms (Cesare and Griffith, 2004; Nabetani and Ishikawa, 2009; Ogino et al., 1998; Tokutake et al., 1998) and frequent reciprocal sister chromatid exchanges at telomeres (T-SCEs) (Bechter et al., 2004; Londoño-Vallejo et al., 2004). Copying of a tag sequence within a telomere or from one telomere to other telomeres (Dunham et al., 2000; Muntoni et al., 2009) suggests the involvement of homologous recombination (HR) in ALT. In ~5 to 30% of interphase nuclei of ALT cells, a fraction of telomeric DNA colocalizes with promyelocytic leukemia bodies, forming ALT-associated promyelocytic leukemia bodies (APBs) (Yeager et al., 1999).

Telomerase is capable of *de novo* addition of G rich repeats to the 3′ end of the chromosome (Greider and Blackburn, 1985; Greider and Blackburn, 1987; Yu et al., 1990). The telomerase holoenzyme is composed of an RNA and a protein subunit. The RNA component, TR, contains the template for telomere repeat synthesis. The protein component, telomerase reverse transcriptase (TERT), contains motifs common to all reverse transcriptases (RT) (Counter et al., 1997; Harrington et al., 1997;

Lingner et al., 1997; Meyerson et al., 1997; Nakamura et al., 1997). Alteration of conserved sequences in these RT motifs leads to the inactivation of telomerase activity and reduced telomere length (Counter et al., 1997; Harrington et al., 1997; Lingner et al., 1997; Lundblad and Szostak, 1989; Meyerson et al., 1997; Nakamura et al., 1997). A unique feature of telomerase, which distinguishes it from other RTs, is its ability to repetitively reverse transcribe its relatively short RNA template, a process known as repeat addition processivity. For processive DNA synthesis by telomerase to occur, the 3′ end of the single-stranded DNA substrate must first base pair with the telomerase RNA template, forming a stable RNA-DNA hybrid. Next, the RNA template is reverse transcribed for synthesis of one telomeric repeat onto the 3′ end of the DNA primer. Once the 5′ boundary of the template is encountered, the RNA–DNA hybrid is dissociated from the active site; however, the DNA substrate remains associated with TERT to allow for repeated DNA synthesis on the same substrate. Realignment of the template and the new 3′ end of the DNA substrate then occurs, followed by repositioning of the new RNA-DNA hybrid in the active site, which is now poised for another round of elongation (Autexier and Lue, 2006; Brault et al., 2008; Lue, 2004; Qi et al., 2012). Retroviral RTs are capable of continuous and processive nucleotide addition along a template; however, catalysis does not involve stable complex formation with the RNA template or a translocation step (Ricchetti and Buc, 1996).

The structural determinants of processivity found within telomerase are numerous and include several domains in TERT, secondary structures in TR (for a review, see Lue, 2004) and regions that bind telomerase-associated processivity factors (Eugster et al., 2006; Latrick and Cech, 2010; Zaug et al., 2010; Zhang et al., 2006). It is presumed that TERT's contribution to processivity is dependent on amino acids specific to TERT that are not found in other RTs. For example, in motif C, the catalytic center of the enzyme, the two aspartic acid residues [DD] that are directly involved in phosphoryl transfer are preceded by a tyrosine [YMDD] in retroviral RTs, while it is a leucine, valine or phenylalanine in TERTs (Nakamura et al., 1997; Xiong and Eickbush, 1990). Substitution of the leucine with a tyrosine at this position in *Tetrahymena* TERT (L813Y) results in an enzyme with increased processivity (Bryan et al., 2000). This study was the first to report a mutation in TERT leading to an increase in processivity, and implies that it may be advantageous for telomerase to synthesize a smaller number of repeats in a single enzymatic reaction (Bryan et al., 2000). A more recent study showed that mutations in the newly identified motif 3 of human TERT (V658A and D684A) can also increase the processivity of the enzyme (Xie et al., 2010). However, neither of these studies addressed the impact of this increase in enzyme processivity on telomere length.

Mutational studies in yeast have shown that telomerase processivity is a major determinant of telomere length *in vivo*. Converting the amino acids found in the Est2p E motif to those found at similar positions in human TERT (hTERT) and HIV-1 RT resulted in an enzyme with slightly increased processivity and longer telomeres (Peng et al., 2001). However, telomerases isolated from yeast are less processive *in vitro* compared to telomerases from ciliated protozoa and humans (Cohn and Blackburn, 1995; Fulton and Blackburn, 1998; Lue and Peng, 1997; Lue and Xia, 1998), which are capable of adding hundreds of nucleotides to the starting primer without dissociating (Bryan et al., 2000; Greider, 1991; Morin, 1989). Furthermore, telomere length maintenance is regulated differently in yeast compared to ciliates and humans. Although yeast telomerase acts largely nonprocessively *in vivo*, it processively elongates the shortest telomeres (Chang et al., 2007; Teixeira et al., 2004). In mammalian cells, several studies also indicate that telomerase elongates the shortest telomeres (Britt-Compton et al., 2009; Hemann et al., 2001; Liu et al., 2002; Ouellette et al., 2000; Samper et al., 2001). However, a recent study using cancer cell lines has shown that under telomere-length maintenance conditions, every telomere is elongated by a single, processive telomerase molecule (Zhao et al., 2011). Under nonequilibrium conditions, telomerase acts distributively on the shortest telomeres. Evidence from two additional studies suggests that telomerase processivity is important in regulating telomere maintenance in human cells. First, BIBR1532, a non-nucleoside telomerase inhibitor, acts by reducing the processivity of human telomerase and induces progressive telomere shortening and cellular senescence in telomerase-positive cancer cells (Damm et al., 2001; Pascolo et al., 2002). Secondly, an ancestral mutation in telomerase (V791I-V867M) found in patients with familial pulmonary fibrosis causes defects in processivity and is associated with telomere shortening (Alder et al., 2011).

To investigate the role of increased telomerase processivity on telomere length regulation in human cells with limited lifespan that are dependent on TERT for lifespan extension and immortalization, we mutated the leucine at position 866 in the C motif of human TERT to a tyrosine (L866Y), which is the amino acid found at the equivalent position in HIV-1 RT. We determined the mutant's levels of activity and processivity and studied its effect on telomere length. We report that, similar to the *Tetrahymena* telomerase mutant (L813Y), the human telomerase variant displays an increase in processivity. hTERT-L866Y, like wild-type hTERT, can immortalize and extend the lifespan of limited lifespan cells. Moreover, hTERT-L866Y expressing cells display heterogenous telomere lengths, telomere elongation, multiple telomeric signals indicative of fragile sites and replicative stress, and an increase in short telomeres, which is accompanied by telomere trimming events. Our results suggest that telomere length and homeostasis may be regulated by telomerase enzyme processivity.

Results

hTERT-L866Y and wild-type hTERT reconstituted *in vitro* and in cells display similar levels of telomerase activity

Alignment of TERT motif C from diverse organisms indicates that this region is well conserved within the TERT family (Bryan et al., 2000; Gillis et al., 2008; Mitchell et al., 2010; Peng et al., 2001). Six TERT sequences and the HIV-1 RT sequence are shown aligned using Clustal W (Fig. 1A). The amino acid in hTERT corresponding to the *Tetrahymena* L813 residue was mutated to a tyrosine (L866Y), the corresponding amino acid in HIV-1 RT. *In vitro* transcription and translation expressed *Tetrahymena* TERT-L813Y reconstitutes levels of telomerase activity equal to wild-type expressed enzyme as assessed using a primer extension assay (Bryan et al., 2000). To determine their activities, hTERT-L886Y and wild-type hTERT were expressed in rabbit reticulocyte lysate (RRL) in the presence of [^{35}S] methionine. A major band of ~130 kDa was observed for both proteins, corresponding to full-length protein (Fig. 1B). The mutant and wild-type hTERT proteins were expressed in the

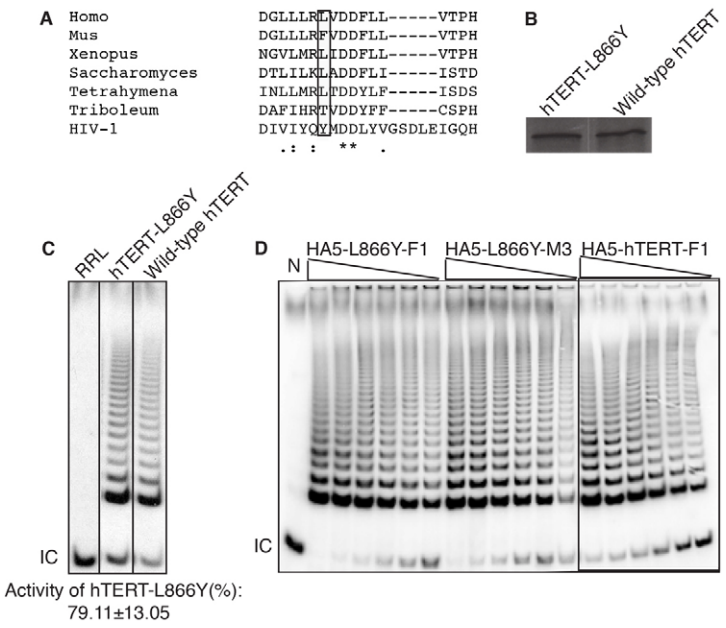


Fig. 1. Location of C motif mutation and activity of reconstituted mutant L866Y-hTERT and wild-type telomerases. (**A**) Alignment of TERTs from Homo sapiens (Homo), *Mus musculus* (Mus), *Xenopus laevis* (Xenopus), *Saccharomyces cerevisiae* (Saccharomyces), *Tetrahymena thermophila (*Tetrahymena), *Triboleum castaneum* (Triboleum) and reverse transcriptase from HIV-1 (HIV-1) was performed using Clustal W. The point (.) symbol indicates conservation between groups of weakly similar properties; colon (:) indicates conservation between groups of strongly similar properties; and * indicates a single, fully conserved residue. The box indicates the leucine at position 866 in hTERT and the corresponding residues at equivalent positions within the other TERTs and HIV-1 RT. (**B**) *In vitro* synthesized [^{35}S] methionine-labeled hTERT-L866Y and wild-type hTERT were analyzed by SDS-PAGE. (**C**) Telomerase mutant hTERT-L866Y and wild-type complexes were reconstituted in rabbit reticulocyte lysate and activity assayed using TRAP. A 1:50 dilution of lysate that reconstitutes telomerase activity in the linear range was quantified. IC indicates the internal control. RRL indicates rabbit reticulocyte lysate not expressing any protein. Boxes indicate removal of intervening lanes from the original gel. Activity levels corresponding to each complex were quantified from $n=3$ experiments. (**D**) Telomerase activity was assayed for clones F1 and M3 hTERT-L866Y-expressing HA5 cells as described for C. Activity of HA5 cells expressing wild-type telomerase (HA5-hTERT-F1) is shown for comparison. N, No cell lysate. Box indicates presence of data from two different gels. 1 μg, 0.5 μg, 0.25 μg, 0.125 μg, 62.5 ng, and 31.25 ng of total protein were assayed in the final reaction.

presence of *in vitro* transcribed telomerase RNA, and telomerase activity was assayed over a wide range of protein dilutions using a PCR-based telomerase (TRAP) assay to determine which concentration of protein generates telomerase activity in the linear range (data not shown). Quantification of TRAP activity was performed for the 1 in 50 dilution, which generates telomerase activity in the linear range of activity (Fig. 1C). hTERT-L866Y-reconstituted enzyme displayed levels of activity comparable to wild-type-reconstituted telomerase (79%±13.05). Levels of telomerase activity of the L866Y enzyme were also similar to wild-type enzyme activity levels when telomerase was reconstituted in HA5 cells, a human embryonic kidney cell line with limited lifespan that expresses SV40 large T and small t antigens (Stewart and Bacchetti, 1991) and lacks hTERT expression (Fig. 1D). Therefore, we concluded that hTERT-L866Y exhibits levels of activity comparable to wild-type hTERT enzyme.

hTERT-L866Y reconstituted *in vitro* and in cells display increased processivity

The *Tetrahymena* TERT-L813Y mutant synthesized in RRL was twice as processive as wild-type (Bryan et al., 2000). To determine if the equivalent human mutant was also more processive, wild-type hTERT and mutant hTERT-L866Y were expressed in RRL with *in vitro* synthesized hTR. The reconstituted enzymes were incubated with biotinylated $(T_2AG_3)_3$ substrate primer and the extension products were analyzed on denaturing gels. Longer extension products were generated by hTERT-L866Y and are clearly visible at the top of the gel (Fig. 2A). To confirm this finding, the proteins were also co-expressed with hTR in 293 cells and cell extracts prepared from these cells were used in the primer extension assay. Similar to RRL-synthesized and reconstituted hTERT-L866Y, the mutant enzyme produced in 293 cells generated longer extension products compared to wild-type enzyme (Fig. 2B). Quantification of processivity indicates that, similar to the results for *Tetrahymena* telomerase (Bryan et al., 2000), *in vitro* reconstituted hTERT-L866Y enzyme is twice as processive as wild-type enzyme (Fig. 2B). Interestingly, hTERT-L866Y from 293 cells is three times more processive than wild-type. The reason for the difference in processivity of telomerase reconstituted in RRL and in cells may be due to the presence of processivity factors in cell extracts that are missing in RRL. *Tetrahymena* telomerase immunopurified from cell extracts has been shown to be more processive than RRL-synthesized telomerase, possibly due to factors in RRL that inhibit processivity (Bryan et al., 2000).

Processivity implies that telomerase is capable of extending one DNA end during the course of one DNA binding event. However, telomerase can also act distributively, which occurs when telomerase extends a DNA primer, dissociates and binds a new DNA end for elongation. Mutant *Tetrahymena* TERT-L813Y was found to act processively on DNA ends (Bryan et al.,

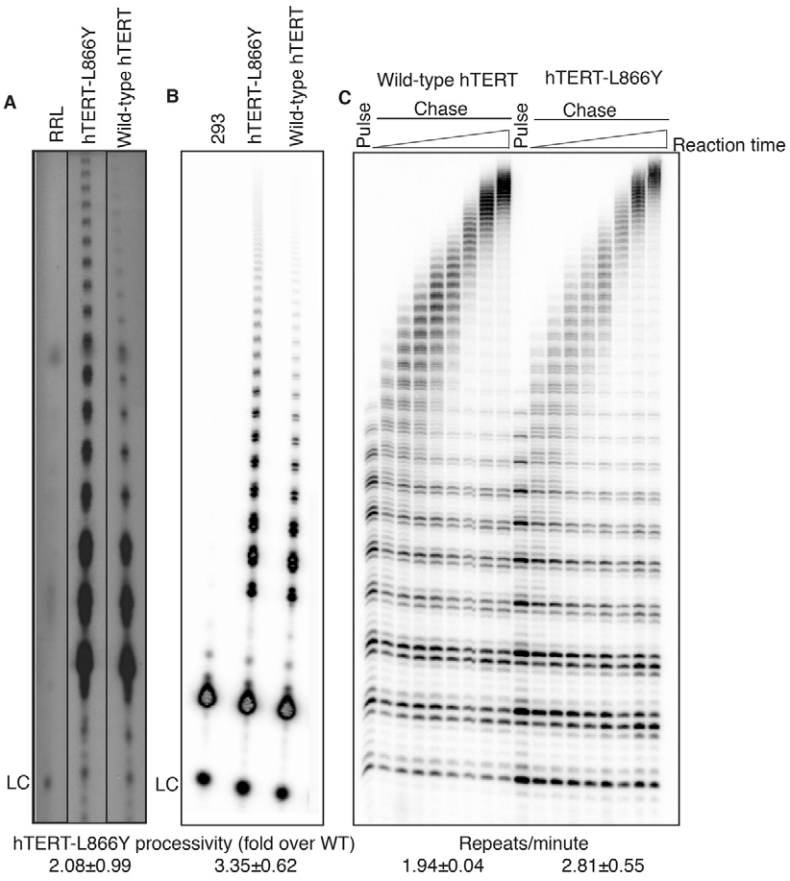


Fig. 2. Mutant hTERT-L866Y reconstituted in RRL and HA5 cells displays higher levels of processivity than wild-type telomerase. (**A**) Mutant hTERT-L866Y and wild-type telomerases were reconstituted in rabbit reticulocyte lysate and their processivities assessed via the direct primer extension assay using a biotinylated telomeric $(T_2AG_3)_3$ primer. Processivities were quantified from $n=3$ experiments; mutant hTERT-L866Y telomerase reconstituted in RRL was 2.08±0.99-fold more processive than wild-type telomerase. RRL indicates rabbit reticulocyte lysate not expressing any protein. Boxes indicate removal of intervening lanes from the original gel. (**B**) Mutant hTERT-L866Y and wild-type telomerase complexes were reconstituted in 293 cells and their processivities assayed by direct primer extension as for A. The mutant hTERT-L866Y telomerase complex was found to be 3.35±0.62-fold more processive than wild-type telomerase ($n=3$), (**C**) A pulse-chase analysis was conducted to determine the mode of action of hTERT-L866Y. Mutant hTERT-L866Y and wild-type telomerases reconstituted in 293 cells were incubated in a pulse reaction for 5 minutes, during which [α^{32}P] dGTP is incorporated into any new telomeric repeats synthesized on the end of the telomeric $(T_2AG_3)_3$ primer. After the pulse, nonradioactive dGTP was added to initiate several chase reactions lasting 2, 4, 6, 8, 10, 15, 20 or 30 minutes. The extension rate for each telomerase complex is indicated ($n=4$). LC, loading control.

2000). Therefore, a pulse-chase assay was performed using telomerase complexes reconstituted in 293 cells to determine human TERT-L866Y's mode of action. The pulse chase assay tracks only telomere products extended through continuous translocation events. The initially labeled telomeric products continue to be extended by the same telomerase enzyme during the chase reaction. During the chase reaction, the primers that are extended by any enzymes dissociated from the pulsed telomere products are not seen (Xie et al., 2010). A 5-minute pulse using telomeric $(T_2AG_3)_3$ primer and [α-^{32}P] dGTP was followed by a chase with 50-fold excess of non-radioactive dGTP. Long extension products observed in the pulse chase experiment indicates that hTERT-L866Y telomerase acts processively. The rate of extension was found to be 2.81±0.55 repeats/minute for hTERT-L866Y and 1.94±0.04 repeats/minute for wild-type telomerase ($n=4$) (Fig. 2C).

HA5 cells expressing hTERT-L866Y or wild-type hTERT exhibit similar growth rates

The *in vivo* effects of the increased processivity resulting from the tyrosine mutation at amino acid position 813 of *Tetrahymena* TERT are unknown. Therefore, the human mutant and wild-type TERTs were each expressed in HA5 cells. The ability of wild-type hTERT to maintain telomeres and immortalize HA5 cells has been previously described (Armbruster et al., 2001; Counter et al., 1998; Moriarty et al., 2005). Two clones of mutant and wild-type telomerase-expressing cells were selected and passaged for ~200 population doublings (PD) (Fig. 3A). One HA5-hTERT-L866Y clone, F1, grew as well as the two HA5-wild-type hTERT clones. The other HA5-hTERT-L866Y clone, M3, grew at a slightly slower rate. Previous studies have shown that hTERT levels are limiting in telomerase-positive human cell lines and that increasing hTERT levels results in increased telomerase activity and telomere lengthening (Cristofari and Lingner, 2006; Pickett et al., 2009). Thus it was also important to confirm that the expression and activity of hTERT-L866Y and hTERT-WT in limited lifespan HA5 cells were similar. Western blots were performed using an anti-hTERT antibody to assess whether hTERT protein levels fluctuated during culture or differed between hTERT-L866Y and hTERT-WT expressing cells. hTERT protein levels remained relatively unchanged during passage (Fig. 3B). More importantly, the levels of hTERT-L866Y expressed by the HA5 cells in both clones were never higher than the levels of wild-type hTERT in the two clones. TRAP assays were also performed to confirm that the mutant and wild-type proteins reconstituted similar levels of telomerase activity (Fig. 1D).

hTERT-L866Y-expressing clones display heterogenous telomere lengths and increased frequency of short telomeres

To examine the possible effects of hTERT-L866Y processivity on telomere length, cells expressing mutant or wild-type telomerase were passaged for 200 PD. Genomic DNA was extracted and metaphase spreads were prepared from early, middle and late passage cells. The average telomere lengths of HA5 clones hTERT-L866Y-F1, hTERT-L866Y-M3, hTERT-WT-F1 and hTERT-WT-M2 were measured by telomere

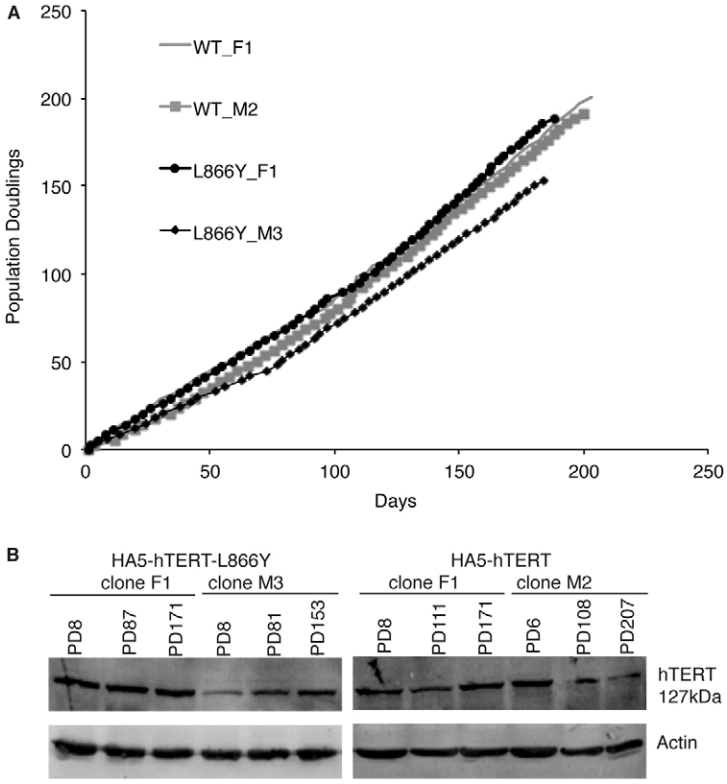


Fig. 3. HA5 cells expressing hTERT-L866Y or wild-type hTERT exhibit similar growth rates. (**A**) Growth curves of HA5 cells infected with retroviruses expressing hTERT-L866Y or wild-type hTERT. Two clones each of HA5 cells expressing hTERT-L866Y (L886Y_F1 and L886Y_M3) and wild-type hTERT (WT_F1 and WT_M2) were selected for passage and monitored for ~200 days. (**B**) HA5 cells expressing hTERT-L866Y or wild-type hTERT were evaluated for protein expression by western blot analysis at early, middle and late passages using an anti-hTERT antibody. Actin expression is also shown to confirm equal loading. Population doubling (PD) is shown above each lane.

restriction fragment (TRF) analysis (Fig. 4A). Telomere lengths for both hTERT-L866Y clones were relatively homogenous at early population doublings, and progressively became longer but also more heterogeneous, with shorter telomeres clearly visible. The telomere lengths of the HA5-hTERT-WT-M2 clone fluctuated in length with increasing passage from longer to shorter to intermediate to a final TRF length of ~3.2 kb. The average telomere lengths of the HA5-hTERT-WT-F1 clone were shorter than the average telomere lengths of HA5-hTERT-WT-M2 and did not fluctuate as extensively as for the HA5-hTERT-WT-M2 clone.

To obtain a distribution of relative telomere lengths, telomere lengths of individual chromosomes for the four HA5 clones expressing hTERT-L866Y-F1, hTERT-L866Y-M3, hTERT-WT-F1 or hTERT-WT-M2 were measured using quantitative fluorescence *in situ* hybridization (Q-FISH) (Fig. 4B). At early passage, HA5-hTERT-L866Y clone F1 displayed telomere lengths mainly ranging from 100–1000 arbitrary fluorescence units (a.f.u.); however, by middle passage, the longer telomeres (400–1200 a.f.u.) disappeared and the number of shorter (100 a.f.u.) telomeres increased. By late passage, telomeres were elongated again, with an increase in the number of telomeres ranging in size from 200–1500 a.f.u. (Fig. 5A). The HA5-hTERT-L866Y M3 clone's telomeres displayed slightly different elongation and shortening patterns, presumably due to slight differences in hTERT-L866Y expression, as evidenced by the difference in growth rate. At early passage, most telomeres were relatively short, ranging in size from 100–600 a.f.u., but included some longer telomeres ranging in size from 800–1200 a.f.u. Longer telomeres (400–2000 a.f.u.) appeared by middle passage and many of the shorter telomeres (100–300 a.f.u.) disappeared. Interestingly, at late passage, telomeres of short and intermediate length (100–700 a.f.u.) disappeared and the number of long telomeres (800–2500 a.f.u.) continued to increase (Fig. 5B). Thus for both hTERT-L866Y clones, the distribution of telomere lengths shifted from short telomeres present at early or middle passage to longer telomeres by late passage. In contrast, telomere lengths of the HA5-hTERT-WTF1 and M2 clones (Fig. 5C,D) fluctuated from longer to shorter but were maintained short into late passage.

The appearance of heterogeneous telomeres evident by TRF analysis and the eventual long telomere length distribution evident by Q-FISH analysis of late passage HA5-L866Y clones compared to the wild-type hTERT clones suggests that the telomerase mutant's increase in processivity may contribute to the lengthening of the cells' telomeres.

hTERT-L866Y expressing cells contain extrachromosomal telomeric DNA

The heterogeneity of the telomere length of the hTERT-L866Y expressing cells was reminiscent of cells that maintain their telomeres by recombination. One characteristic of ALT cells is the presence of extrachromosomal telomeric DNA, including t-circles, which are likely the result of telomere trimming events that counteract extensive recombination-based telomere lengthening (Pickett and Reddel, 2012). However, the generation of t-circles by trimming also occurs in telomerase-positive cells to negatively regulate experimentally induced telomere elongation (Pickett et al., 2009), in response to experimentally induced telomere deprotection (Wang et al., 2004) and in normal mammalian cells to apparently set an upper limit on telomere length (Pickett et al., 2011). To determine if the long and heterogeneous telomeres of the HA5-hTERT-L866Y clones could be substrates for telomere trimming that might account for the increased frequency of shorter

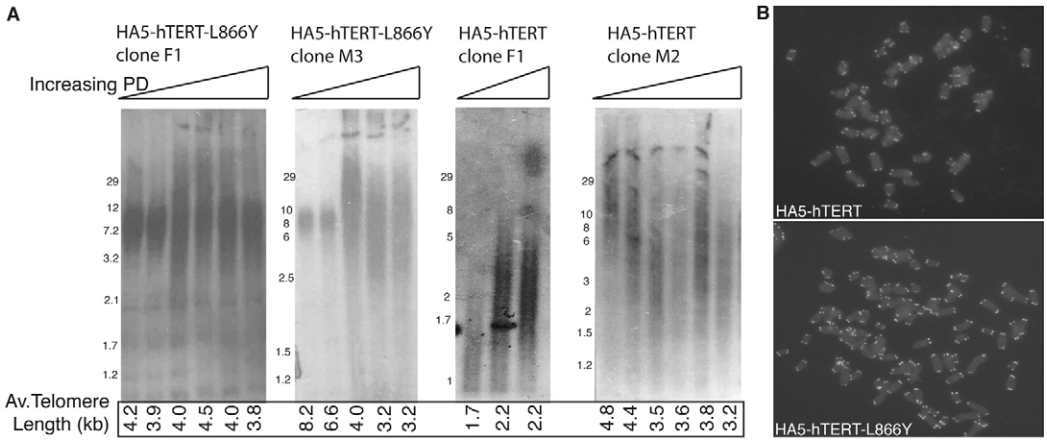


Fig. 4. HA5 cells expressing hTERT-L866Y display heterogenous telomere lengths. (**A**) TRF analysis of genomic DNA digested with *Hin*fI and *Rsa*I from HA5 clones expressing hTERT-L866Y or wild-type hTERT at increasing passage. Selected marker sizes (kb) are shown to the left of each TRF. Average telomere length at each passage is shown below each TRF in kb. (**B**) Representative images of metaphase spreads of HA5 cells expressing hTERT-L866Y or wild-type hTERT that have undergone Q-FISH analysis using a peptide nucleic acid probe against the G-rich telomere DNA strand. Chromosomes were counterstained with DAPI. Magnification 63×.

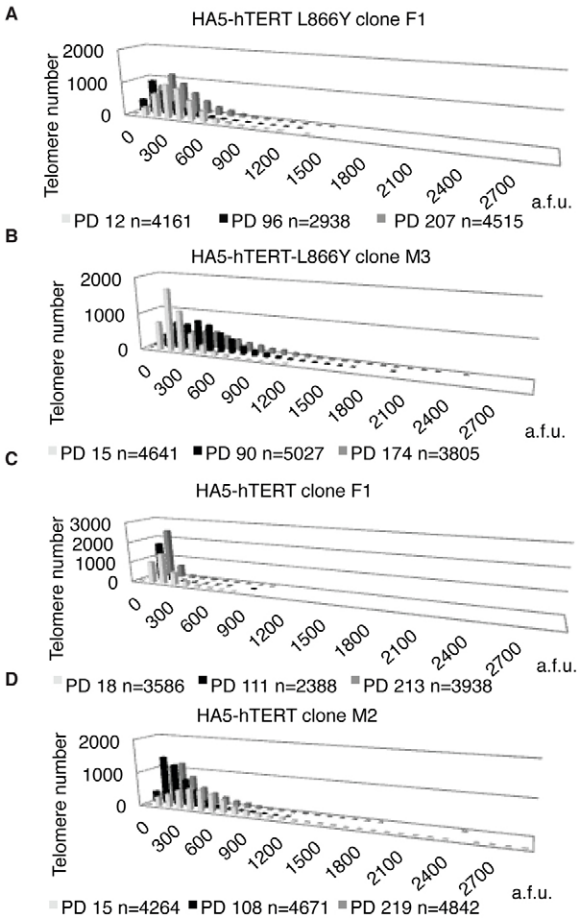


Fig. 5. hTERT-L866Y-expressing clones display an increased frequency of short telomeres. (A–D) The distribution of relative telomere lengths from HA5-hTERT-L866Y clones F1 (A) and M3 (B) and HA5-hTERT clones F1 (C) and M2 (D) obtained by Q-FISH analysis. White, early passages; black, middle passages; gray, late passages; PD, population doubling. n, number of chromosomes analyzed; a.f.u., arbitrary fluorescence units.

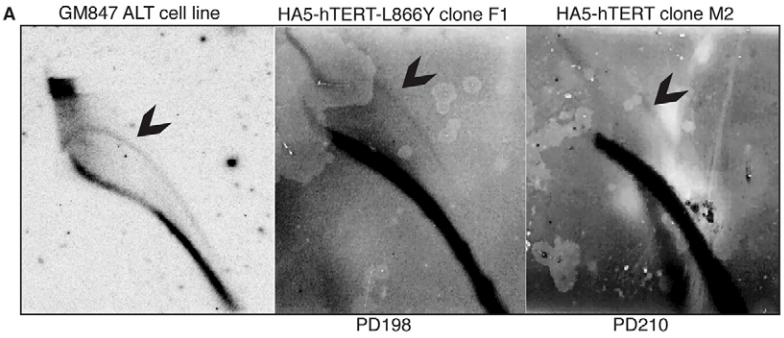
telomere lengths, we assessed for the presence of t-circles using 2D gel electrophoresis. The prominent arc present in the 2D gels represents double-stranded linear telomeric DNA. An arc of open-form double-stranded DNA circles containing telomeric sequences was present in HA5-hTERT-L866Y clone F1 and HA5-hTERT clone M2 at late passage (Fig. 6A). These results demonstrate that the expression of a telomerase enzyme with increased processivity may lead to elongated telomeres and to the generation of t-circles.

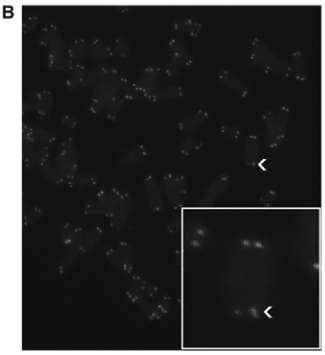
Chromosomes from hTERT-L866Y-expressing cells contain fragile sites

The telomeric Q-FISH signal at individual chromosome arms is normally represented as a single signal with an intensity that is roughly equal to the telomeric signal of the sister chromatid. Upon deregulation of normal telomere metabolism, aberant telomeric signals, such as doublets or strings of fragmented telomeric signals, can appear. They represent areas of single-stranded DNA that are not packaged properly due to incomplete replication or improper processing of stalled forks and are referred to as fragile sites (Sfeir et al., 2009). Following expression of the mutant L866Y telomerase with increased processivity, a large percentage of chromosome arms displayed a fragile telomere phenotype (Fig. 6B). The frequency of fragile telomeres displayed by HA5-hTERT-L866Y clone M3 at middle and late passages were 2.57% and 1.34%, respectively. HA5-hTERT-L866Y clone F1 displayed an increased frequency of fragile telomeres at late passage (2%), whereas fragile sites were less frequent in wild-type telomerase-expressing cells (Fig. 6C). The identification of multiple telomeric signals in the hTERT-L866Y-expressing cells indicates that increased telomere length possibly resulting from increased telomerase processivity imparts telomeric replication stress on the cell.

Discussion

Human telomerase reverse transcribes its intrinsic RNA template to synthesize DNA on the ends of chromosomes. This enzymatic function maintains telomere length in 90% of cancer cells. By mutating a leucine within the C motif of human TERT to a tyrosine, the processivity of human telomerase increased by





C

Passage	Fragile telomeres (%) Early	Middle	Late
HA5-hTERT-L866Y clone F1	1.26	0.46	1.99
HA5-hTERT-L866Y clone M3	0.9	2.57	1.87
HA5-hTERT clone F1	0.36	0.34	0.23
HA5-hTERT clone M2	1.34	0.62	0.45

Fig. 6. Expression of telomerase with increased processivity results in t-circle accumulation and telomere fragility. (**A**) Two-dimensional pulsed field gel electrophoresis of telomere restriction fragments from HA5 cells expressing hTERT-L866Y or wild-type hTERT at late passage. Gels were hybridized with a C-rich telomere DNA probe $(C_3TA_2)_6$. GM847 ALT cells were used as a positive control. Black arrowheads indicate t-circles. (**B**) Representative image of metaphase spread from HA5 cells expressing mutant L866Y hTERT that has undergone Q-FISH analysis. Magnification 63×. White arrowhead indicates fragile site. The inset box illustrates the same fragile site enlarged for clarity. (**C**) Quantification of fragile sites for each hTERT-L866Y- and wild-type hTERT-expressing clone at early, middle and late passage expressed as a percentage (%) of the number of chromosomes scored.

2- and 3-fold when reconstituted in RRL and 293 cells, respectively. The activity of the L866Y telomerase mutant did not change despite this increase in processivity. The increase in processivity may relate to the amino acid side chains present in tyrosine. The comparable residues L813 in *Tetrahymena* TERT and Y183 in HIV-1 RT are well characterized. Mutation of leucine 813 in *Tetrahymena* TERT to a tyrosine resulted in higher processivity. Mutation to a phenylalanine resulted in intermediate processivity compared to wild-type, and an alanine substitution resulted in reduced processivity compared to wild-type, indicating that the side chains present in tyrosine may be responsible for the changes in processivity (Bryan et al., 2000). Furthermore, according to the crystal structure of HIV-1 RT complexed with template-primer, hydrogen bonding between amino acid side chains and nucleotides of the template-primer impacts processivity (Ding et al., 1998). It is possible that mutation of leucine to a tyrosine, which contains an aromatic ring and a hydroxyl group, allows further hydrogen bonding with the primer to occur, thus increasing the affinity of the enzyme for the primer. Accordingly, Bryan et al. found that a 2-fold increase in processivity correlated with a 3-fold slower dissociation rate of primer from the *Tetrahymena* L813Y mutant compared to wild-type telomerase (Bryan et al., 2000).

To date, many studies have identified hTERT residues that regulate the intrinsic processivity of telomerase but few have demonstrated a relationship between processivity and telomere length in human cells. Studies in yeast have demonstrated that there is a good correlation between the level of telomerase processivity and changes in telomere length (Peng et al., 2001). In contrast to our results and those obtained for the *Tetrahymena* TERT mutant, a double-substitution Est2p mutant converting the leucine and alanine residues preceding the two aspartic residues to tyrosine and threonine (LA658YT) resulted in reduced processivity and greatly shortened telomeres (Peng et al., 2001). While evidence suggests that telomere length regulation differs in yeast compared to humans, this result may instead reflect a destabilization caused by the second mutation. The equivalent position of Est2p A659 in Triboleum castaneum TERT is valine 342. Its crystal structure recently revealed that V342's side chains help form the hydrophobic dNTP binding pocket (Gillis et al., 2008; Mitchell et al., 2010). The A659T mutation in yeast TERT may have caused a destabilization of the pocket, leading to a reduced nucleotide binding efficiency. Furthermore, it has been shown that tyrosine 183 and methione 184, the equivalent residues preceding the two aspartic residues in HIV-1 RT, work in conjuction within the dNTP binding pocket to regulate processivity (Harris et al., 1998).

We observed an increase in telomere length upon expression of a human telomerase enzyme with increased processivity. Despite continued expression of this mutant, the increase in telomere length could not consistently be maintained. Telomeres became heterogenous in length, with detectable short telomeres at early passage for clone L866Y-M3 and at intermediate passage for clone L866Y-F1. We speculate that the longer telomeres could not be maintained because their increased lengths were problematic for the replication machinery. Telomeres are known to be challenging for the replication machinery because they form secondary structures such as t-loops and G quadruplexes which can cause increased replication fork pausing (Crabbe et al., 2004; Fouché et al., 2006; Gilson and Géli, 2007; Ivessa et al., 2002; Makovets et al., 2004; Miller et al., 2006; Ohki and Ishikawa, 2004). A variety of proteins, such as TRF1, ATR and the RecQ helicases RTEL and BLM work together to prevent replication fork stalling. Their deletion in mammalian cells results in telomeres with aberrant structures during metaphase (Martinez et al., 2009; McNees et al., 2010; Okamoto et al., 2008; Sfeir et al., 2009). These structures, referred to as fragile sites, range from telomere signal doublets to long strings of fragmented telomeric signals. They represent areas of single-stranded DNA that are not packaged properly due

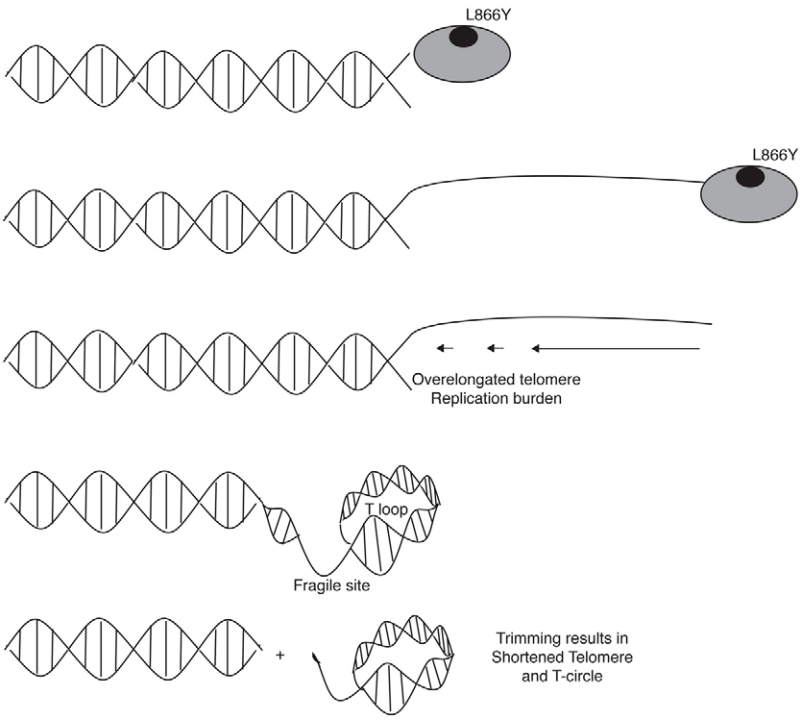


Fig. 7. Human telomerase processivity regulates telomere length and homeostasis. Model illustrating a telomerase enzyme with increased processivity imparted by the L866Y mutation (shown by a black circle) overelongating a telomere. The overlengthened telomere poses replication problems because the additional telomeric repeats are burdensome for the replication machinery, resulting in a fragile site. Following replication, the t-loop forms. However, the telomere is trimmed due to the instability of the telomeric fragile site, resulting in a shortened telomere and a t-circle.

to incomplete replication or improper processing of stalled forks (Sfeir et al., 2009). The identification of multiple telomeric signals in the hTERT-L866Y-expressing cells, similar to those reported in the above studies, indicates that increased telomere length possibly due to increased telomerase processivity imparts telomeric replication stress on the cell. Regulation of telomere length through control of telomerase processivity may therefore be important to avoid replication stress.

Telomeres form a t-loop in which the 3′ single-stranded overhang is tucked into the double-stranded telomeric DNA (Cesare and Griffith, 2004; Griffith et al., 1999; Wang et al., 2004). The t-loop resembles a stabilized homologous recombination intermediate (de Lange, 2004; de Lange and Petrini, 2000; Griffith et al., 1999; Lustig, 2003; Wang et al., 2004). Recombination of the t-loop is normally repressed, however its resolution results in a circular telomeric molecule, the t-circle, and a terminally deleted telomere (Lustig, 2003; Pickett et al., 2009; Pickett et al., 2011; Wang et al., 2004). These circles are thought to form when the branch point of the t-loop migrates, forming a Holliday junction that is then resolved by XRCC3 (Pickett et al., 2011; Wang et al., 2004). It has been proposed that telomere elongation beyond a threshold length due to increased telomerase activity or overexpression triggers a trimming event involving resolution of the t-loop (Pickett et al., 2009; Pickett et al., 2011). Telomere lengths become heterogenous with increasing passage and extrachromosomal telomeric repeats, including t-circles, appear. Similarly in our study, telomeres became heterogenous in length and we detected t-circles in the hTERT-L866Y-expressing cells. Telomere trimming also appears to be a normal part of telomere length regulation. The occurrence of t-circles in several eukaryotic species reflects the fact that they are a common by-product of telomere maintenance processes and may prevent telomere overlengthening (Tomaska et al., 2009). T-circles have been observed in mutant strains of *Kluyveromyces lactis* (Cesare et al., 2008; Groff-Vindman et al., 2005), *Caenorhabditis elegans* (Raices et al., 2008), at low levels in the plant *Arabidopsis thaliana* (Zellinger et al., 2007), *Xenopus laevis* during development (Cohen and Méchali, 2002) and in mammalian ALT cells (Cesare and Griffith, 2004; Nabetani and Ishikawa, 2009; Wang et al., 2004). Telomere trimming also occurs at low levels in human telomerase positive cells such as Hela, HT1080, BJ cells expressing hTERT, and normal human cells (Pickett et al., 2009; Pickett et al., 2011; Wang et al., 2004). Considering the conservation of this mechanism across many eukaryotic species, it is not surprising that we also detected t-circles in the HA5 cells expressing wild-type hTERT that had the longer telomeres (clone M2).

However, unlike wild-type hTERT-expressing cells, both clones of the hTERT-L866Y-expressing cells displayed a striking telomere length heterogeneity clearly evident by TRF analysis, and long telomere length distribution evident by Q-FISH analysis of late passage cells. These results, the presence of t-circles and the higher levels of fragile sites in the hTERT-L866Y expressing cells compared to the wild-type hTERT-expressing cells support a role for telomerase processivity in the regulation of telomere length and homeostasis. While heterogenous telomere length and the presence of ECTR are reminiscent of the ALT phenotype, cells expressing increased telomerase activity do not display other ALT-associated properties, such as T-SCEs, homologous recombination and telomere dysfunction-induced foci (Pickett et al., 2009). A small 3% increase in APBs, also a feature of ALT cells, was reported in telomerase-positive cells undergoing telomere trimming (Pickett et al., 2009). We did not observe an increase in the number of APBs in HA5 cells expressing telomerase with increased processivity compared to wild-type hTERT-expressing cells (data not shown). Our results are consistent with a previous study that observed telomeric circles regardless of APB presence within the cell (Cesare and Griffith, 2004).

It is rare for a site-directed mutation to 'improve' an enzyme that has undergone natural selection over time (Bryan et al., 2000). We have demonstrated that telomere length and homeostasis may be regulated by telomerase processivity. We speculate that the natural processivity of telomerase is limited by the presence of amino acid side chains that interact with the primer to prevent increases in telomere length above a pre-set threshold level. The presence of fragile sites at telomeres upon expression of a telomerase enzyme with increased processivity indicates that the cells with longer telomeres experience telomere replication stress. We also show that telomeres undergo trimming when telomerase with increased processivity is introduced. Recombination involving the t-loop occurs when the cell is under selective pressure for viability (Lundblad, 2002). We postulate that telomeres undergo trimming to bypass telomere replication difficulty (Fig. 7). Therefore, regulation of a maximal telomere length by controlled levels of telomerase processivity may be important not only to reduce the total amount of DNA synthesis, but also to inhibit recombination.

The goal of telomerase inhibition is to prevent cells from maintaining telomere length and ultimately induce cell growth arrest or cell death. However, extensive telomere shortening is normally required before an effect is observed (Harley, 2008). Treatment with an inhibitor that targets domains that regulate telomerase processivity following activation of trimming to rapidly shorten telomeres could reduce this lag phase and induce death more rapidly in cancer cells.

Materials and Methods

Plasmid construction

The pMSCV-puromycin-FLAG-hTERT plasmid was constructed by insertion of *Bgl*II- and *Eco*RI- (New England Biolabs, Pickering Ontario, Canada) digested PCR products encoding FLAG-hTERT into pMSCV-puromycin (gift from Gerardo Ferbeyre). The plasmids pET28b-hTERT (Bachand and Autexier, 1999), pMSCV-puromycin-FLAG-hTERT and pcDNA6/myc-His C-hTERT (Cristofari and Lingner, 2006) were used as templates to generate pET28b-hTERT-L866Y, pMSCV-puromycin-FLAG-hTERT-L866Y and pcDNA6/myc-His C-hTERT-L866Y by site-directed mutagenesis. pBluescript II SK(+)-hTR was a kind gift from Joachim Lingner (Cristofari and Lingner, 2006).

Cell culture, retroviral transfection and transient transfection

Retroviruses were produced by transfecting Phoenix cells grown in alpha-MEM (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% FBS (Wisent, St-Bruno, Quebec, Canada) and 1% antibiotics/antimycotics (Wisent) with pMSCV-puromycin-hTERT, pMSCV-puromycin-hTERT-L866Y or empty vector plasmids. The viral supernatants were recovered, filtered and stored at −80℃ until use. HA5 cells (gift from Silvia Bacchetti) were grown in alpha-MEM (Invitrogen) supplemented with 10% FBS and 1% antibiotics/antimycotics and treated with 4.5 µg/ml polybrene (Sigma-Aldrich, Oakville, Ontario, Canada) and infected with the viral supernatants twice a day for 2 days. 48 hours later the cells were disaggregated and seeded into four 10 cm plates with 0.35 µg/ml puromycin (Invitrogen). Monoclonal colonies were selected and grown in alpha-MEM supplemented with 10% FBS, 1% antibiotics/antimycotics and 0.35 ug/ul puromycin for ~200 population doublings. Counting of population doubling began as soon as monoclonals were transferred to 6-cm plates. Protein and DNA extracts were prepared from cells collected at various passages for Telomere Repeat Amplification Protocol (TRAP) assays, western analysis and telomere restriction fragment (TRF) analysis. To create 'supertelomerase'- and 'supertelomerase-L866Y'-expressing cells, pcDNA6/myc-His C-hTERT and pBluescript II SK(+)-hTR or pcDNA6/myc-His C-hTERT-L866Y and pBluescript II SK(+)-hTR were transiently transfected into 293 cells using lipofectamine 2000 transfection reagent (Invitrogen). Cell extracts were collected in 300 µl 1× Trapeze CHAPs buffer (Millipore, Billerica, Massachusetts) containing 40 units (U) of RNase out/ml (Invitrogen).

In vitro transcription and translation, SDS-PAGE and TRAP assay

In vitro transcription and translation were performed using the TNT T7-coupled transcription/translation rabbit reticulocyte lysate (RRL) system (Promega, Madison, Wisconsin) as per the manufacturer's instructions. Full-length hTERT or hTERT-L866Y were synthesized in RRL in the presence of 300 ng pET28b-hTERT or pET28b-hTERT-L866Y, 300 ng purified hTR and 0.8 µCi of L-[^{35}S] methionine (Perkin Elmer, Waltham, Massachusetts). hTR was synthesized from FspI-linearized phTR+1 (Autexier et al., 1996). The reactions were incubated at 30℃ for 90 minutes. 1 µl of the RRL reaction was separated by SDS-PAGE. The remaining RRL was diluted in TRAP lysis buffer (10 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 150 mM NaCl) at 1:50, 1:65, 1:100, 1:200, 1:250 and 1:400. 1 µl of these dilutions was then used in a two-step TRAP assay. Reaction conditions for the first step were 20 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 1 mM EGTA, 0.005% Tween-20 (Bio-Rad, Hercules, California), 0.1 mg/ml BSA (New England Biolabs), 40 pmol TS primer (IDT, Coralville, Iowa), 20 pmol NT primer (IDT), 1×10^{13} M TSNT primer (IDT) and dNTPs in 50 µl reaction, incubated at 30℃ for 30 minutes. For the second step, 20 pmol of ACX primer (IDT), 0.5 µl of [α-^{32}P] dGTP (3000 Ci/mmol, Perkin Elmer) and 2 U Taq (Invitrogen) were added to the tube and the reaction was amplified for 30 cycles at 94℃ for 30 seconds, 60℃ for 30 seconds and 72℃ for 30 seconds. The products were separated on a 10% acrylamide gel in 0.6× TBE, dried at 80℃ for 45 minutes and autoradiographed on a phosphorimager cassette overnight and scanned using the Storm 840 (GE Healthcare, Mississauga, Ontario, Canada). Quantification was carried out as previously described (Moriarty et al., 2002).

The level of telomerase activity in cell extracts from HA5 cells expressing pMSCV-puromycin-hTERT, pMSCV-puromycin-hTERT-L866Y and empty vector was evaluated using the TRAP assay as described in Hrdlicková et al. (Hrdlicková et al., 2006) with a few exceptions. Differing amounts of protein extract in TRAP lysis buffer (0.625 µg, 1.25 µg, 2.5 µg, 5 µg, 10 µg, 20 µg of total protein) were first incubated with 0.5 µg of the TS primer and 2.5 mM each dNTP in 1× TRAP reaction buffer (see above) containing 0.8 mM spermidine (Bioshop, Burlington, Ontario, Canada) and 5 mM β-mercaptoethanol (Sigma-Aldrich) in a total reaction volume of 50 µl for 30 minutes at 37℃. The reactions were stopped by incubation at 94℃ for 2 minutes. Aliquots of synthesis (2.5 µl, thus containing 31.25 ng, 62.5 ng, 0.125 µg, 0.25 µg, 0.5 µg, 1 µg of protein) were then PCR amplified with 0.1 µg unlabeled TS primer, 20 µM NT, 20 µM ACX, 1×10^{13} M TSNT, 0.5 µl of [α-^{32}P] dGTP (3000 Ci/mmol) and 2 U of Taq. PCR conditions were 94℃ for 2 minutes followed by 36 cycles of 30 seconds at 94℃, 30 seconds at 50℃ and 1 minute at 72℃. The TRAP PCR products were then separated on 7.5% polyacrylamide gels in 0.5× TBE.

Primer extension assay using RRL and 293 cell extracts

Primer extension assays using equal amounts of hTERT or hTERT-L866Y proteins expressed in RRL in the presence of hTR (20 µl of RRL sample) were assayed for telomerase activity in a 40 µl final reaction volume, as previously described (Huard et al., 2003) but with minor changes. Standard reaction conditions were 50 mM Tris–HCl pH 8.3, 50 mM KOAc, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM spermidine, 2.5 µM HPLC-purified 5′-biotinylated $(T_2AG_3)_3$ primer (Operon, Huntsville, Alabama, USA), 4 µl of [α-^{32}P] dGTP (3000 Ci/mmol, Perkin Elmer), 1 mM dATP and 1 mM dTTP. The reaction mix was incubated at 30℃ for 2 hours. The elongation step was terminated by the addition of 150 µl of RNase stop buffer [10 mM EDTA, 2 M NaCl, 0.1 mg/ml RNase A (Fermentas, Burlington, Ontario, Canada)] and incubation at 37℃ for 10 minutes. Following the addition of 150 µl of proteinase K solution [10 mM Tris–HCl pH 7.5, 100 mM NaCl, 25 mM EDTA, 0.5% SDS and 0.47 mg/ml proteinase K (Roche Applied Science, Laval, Quebec, Canada)] and a 10 minutes incubation at 37℃, pre-washed Streptavidin MagnaSphere® Paramagnetic beads (Promega) and a 5′ labeled T_2AG_3 oligonucleotide in 10 mM Tris–HCl pH 7.5, 1 M NaCl and 0.5 mM EDTA were added. The elongation products were immobilized on magnetic beads at room temperature for 30 minutes. Bead–elongation product complexes were washed once with buffer A (10 mM Tris–HCl pH 7.5, 1 M NaCl, 0.5 mM EDTA), twice with buffer B (10 mM Tris–HCl pH 7.5), resuspended in 10 µl of formamide buffer [95% deionized formamide (Invitrogen), 10 mM EDTA, 1 mM Tris pH 8.3] and boiled for 30 minutes. Telomerase reaction products were resolved on 8% polyacrylamide–8.3 M urea gels. Gels were dried (Bio-Rad) at 80℃ for 2 hours and exposed to X-ray film (Kodak, Rochester, New York, USA).

Primer extension assays using 293 cell extracts were carried out as described above, except for a few minor changes. The reactions were carried out in 20 µl volumes using 30 µg of extract prepared using CHAPs buffer. The elongation step was terminated by the addition of 75 µl of RNase stop buffer, followed by 75 µl of proteinase K solution. The gels were dried at 80℃, autoradiographed on a phosphorimager cassette for 72 hours and scanned using the Storm 840 (GE Healthcare).

Quantification of repeat addition processivity was carried out as described in Drosopoulos et al. (Drosopoulos et al., 2005) with a few minor changes. Briefly, processivity was calculated as a processivity index, i.e. the ratio of long products (products present above the half way point) to total products (all products above and below the half way point). All values obtained were corrected for background. Relative processivity values were calculated as the processivity index of L866Y compared to that of wild-type.

Pulse chase time course experiments were performed and extension rate was calculated as previously described (Drosopoulos et al., 2005; Xie et al., 2010).

Briefly, primer-enzyme complex was allowed to form by pre-incubating the supertelomerase cell extracts in the presence of $(T_2AG_3)_3$ for 5 minutes at 30℃. dNTPs and [α^{32}P] dGTP addition initiates the pulse reaction for 5 minutes following the addition of 50-fold excess nonradioactive dGTP to start the chase reaction. The samples were removed at various time points and analyzed by denaturing gel (Xie et al., 2010). For each chase reaction, the modal band was determined by using the intensity of the 10 most intense bands above the longest product in the pulse reaction only. The relative extension rates were calculated by dividing the number of repeats in the modal band by the time (Drosopoulos et al., 2005).

Western blot analysis

Cells were lysed in RIPA buffer (10 mM Tris-HCl, 0.15 M NaCl, 5 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate) supplemented with 40 U/ml RNAguard (Invitrogen), 0.1 µM PMSF (Bioshop), 1 µg/ml pepstatin (Roche) and 5 µg/ml leupeptin (Roche). 50 µg of protein were loaded onto an 8% SDS-PAGE gel and transferred to a PVDF membrane (Millipore). For hTERT detection, membranes were blocked in 1× PBS with 2% milk and 0.05% Tween-20 for 45 minutes. The membranes were then incubated with 1:500 C20 primary anti-hTERT antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 1× PBS containing 1% milk and 0.05% Tween-20 for 1 hour and 30 minutes and transferred to 1:5000 polyclonal rabbit anti-goat (Dako, Markham, Ontario, Canada) in PBS containing 1% milk and 0.05% Tween-20 for 1 hour. Membranes were washed 3× in 1× PBS containing 0.05% Tween-20. For actin detection, membranes were blocked in 1× TBS with 5% milk and 0.05% Tween-20 for 1 hour. The membranes were then incubated with 1:1000 mouse primary anti-actin antibody (Millipore) in 1× TBS containing 1% BSA and 0.05% Tween-20 overnight at 4℃. The membranes were washed 3× for 5 minutes in 1× TBS containing 0.05% Tween-20 and transferred to 1:2000 anti-mouse antibody (Sigma-Aldrich) in 1× TBS containing 5% milk and 0.05% Tween-20 for 40 minutes. Protein bands were developed with ECL Plus Western Blotting Reagents (GE Healthcare) and detected using the Storm 840 (GE Healthcare).

DNA analysis

Genomic DNA was extracted from cells in DNA lysis buffer (10 mM Tris-HCl pH 8.0, 0.1 M NaCl, 25 mM EDTA and 0.5% SDS), digested with 0.1 mg/ml proteinase K (Roche) at 37℃ for 16 hours, then isolated using a glass hook following the addition of isopropanol and 3 M NaAc pH 5.2. The DNA was resuspended in 10 mM Tris pH 8.0, digested with HinfI and RsaI (New England Biolabs) and separated by PFGE (CHEF-DR II System, Bio-Rad) according to Bryan et al. (Bryan et al., 1995) with a few changes. Electrophoresis of the digested DNA was carried out in a 1% agarose gel in 1× TBE at 5 V/cm for 12 hours at 14℃. The gel then was denatured for 30 minutes in 0.5 M NaOH and 1.5 M NaCl and neutralized for 30 minutes in 1 M Tris-HCl pH 7.5 and 1.5 M NaCl. The gel was dried at room temperature for 30 minutes, then at 50℃ for 1 hour. Hybridization was carried out according to Counter et al. (Counter et al., 1992) except that the three washes were carried out in 2× SSC and the gel was autoradiographed on X-ray film (Kodak) or a phosphorimager cassette for 72 hours and scanned using the Storm 840 (GE Healthcare).

Metaphase spreads and Q-FISH analyses were performed as described (Poon and Lansdorp, 2001). $MgCl_2$ buffer was omitted from the hybridization mixture and the Cy3-labelled telomeric probe (Cy3-TelC) was purchased from Cambridge Research Biochemicals (Billingham, Cleveland, UK). Metaphase slides were mounted using DAPI-antifade (Millipore). Images were captured using a Carl Zeiss Axio-Imager M1 (63×) (Toronto, Ontario, Canada). To correct for lamp intensity and alignment, images from fluorospheres (fluorescent beads; Molecular Probes, Burlington, Ontario, Canada) were also captured. Quantitative analysis of telomere fluorescence from at least 2300 chromosome ends per sample was performed with the TFL-Telo v2.0 software kindly provided by Peter Lansdorp (British Columbia Cancer Center, Vancouver, Canada). Statistical analysis of the measured telomere intensities was performed with Microsoft® Excel 2000 (Microsoft Corporation, Redmond, Washington, USA). For fragile sites, metaphases were analyzed in a blinded fashion by superimposing the telomere image on the DAPI image using TFL-Telo v2.0. Fragile sites were expressed as a percentage of the number of chromosomes scored.

T-circle analysis was performed as previously described (Cesare and Griffith, 2004).

Immunofluorescence

To detect APBs, 50,000–70,000 cells were plated on a coverslip in a 6-well plate. The following day, the cells were fixed in 4% formaldehyde for 10 minutes and permeabilized in 0.5% NP-40 in 1× PBS for 10 minutes. The cells were then blocked in PBG (0.2% cold water fish gelatin (Sigma-Aldrich) and 0.5% bovine albumin fraction V dissolved in 1× PBS) for 1 hour at room temperature, followed by incubation with 1:200 rabbit anti-PML (Santa Cruz Biotechnology) in PBG at 37℃ for 2 hours. Coverslips were washed in PBG and incubated with 1:250 FITC-donkey-anti-goat (Jackson ImmunoResearch, West Grove, Pennsylvania) at 37℃ for 1 hour. Slides were washed with PBG, followed by an ethanol series. Coverslips were then subjected to telomere FISH. Coverslips were placed upside down onto slides containing 60 µl drops of hybridizing solution (70% deionized formamide, 0.5% blocking reagent, 10 mM Tris pH 7.2 and 1:500 Cy3-TelC probe) and denatured for 8 minutes at 80℃ on a heat block, then incubated in the dark for 2 hours at room temperature in damp chambers. Coverslips were placed back into clean 6-well plates, washed 2×15 minutes in wash solution (70% formamide, 10 mM Tris pH 7.2), washed 3×5 minutes in 1× PBS and mounted using DAPI-antifade (Millipore). Imaging was carried out using a Wave FX Spinning Disc Confocal (Quorum, Guelph, Ontario, Canada). Deconvolution was carried out by the inverse filter method using Axiovision software (Zeiss). Analysis was performed using Volocity software (Perkin Elmer).

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Author contributions

Y.D'S., T.W.C. and C.A. designed the experiments. Y.D'S. performed most experimental work. C.L. performed most of the analyses for Fig. 5 and Fig. 6C. T.W.C. performed and analyzed the experiments for Fig. 2C. C.A. supervised the project. Y.D'S. and C.A. wrote the manuscript.

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