Aging Cell (2012) **11,** pp482–490 Doi: 10.1111/j.1474-9726.2012.00810.x

hTERT mutations associated with idiopathic pulmonary fibrosis affect telomerase activity, telomere length, and cell growth by distinct mechanisms

Allison R. Tsang,[1,2] Haley D.M. Wyatt,[1,2,3] Nicholas S.Y. Ting[1,2] and Tara L. Beattie[1,2,4]

[1]Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, AB T2N-4N1, Canada
[2]Southern Alberta Cancer Research Institute, University of Calgary, Calgary, AB T2N-4N1, Canada
[3]London Research Institute, Cancer Research UK, Clare Hall Laboratories, South Mimms EN6 3LD, UK
[4]Department of Oncology, University of Calgary, Calgary, AB T2N-4N1, Canada

Summary

Telomerase is a ribonucleoprotein reverse transcriptase (RT) that synthesizes specific DNA repeats, or telomeric DNA, at the ends of chromosomes. Telomerase is minimally composed of a protein subunit, TERT, and an RNA component, TR. Aberrant telomerase activity has been associated with most human cancers and several premature aging diseases, such as idiopathic pulmonary fibrosis (IPF), a chronic, progressive, and fatal lung disease characterized by alveolar epithelial cell damage and fibrosis. Our study focuses on three *hTERT* mutations that were identified in a subset of patients with IPF, in which these patients also exhibited shorter telomeres compared with age-matched controls. We characterized how three IPF-associated hTERT mutations, V144M, R865C, and R865H, affected telomerase function both *in vitro* and in human cells. We demonstrated that the R865 residue is crucial for repeat addition processivity and thus telomere synthesis in telomerase-positive 293 cells and telomerase-negative BJ cells, consistent with its location in the hTERT nucleotide-binding motif. In contrast, while the V144M mutant did not exhibit any biochemical defects, this mutant was unable to elongate telomeres in human cells. As a result, our studies have identified hTERT V144 and R865 as two critical residues required for proper telomerase function in cells. Together, this may explain how inherited *hTERT* mutations can lead to shortened telomeres in patients with IPF and, thus, provide further insight into the role of naturally occurring telomerase mutations in the pathophysiology of certain age-related disease states.

Key words: telomerase; telomeres; pulmonary fibrosis; premature aging.

Introduction

Telomeres are DNA–protein structures found at the ends of linear chromosomes. They help maintain genomic stability by forming a special cap structure that protects chromosome ends from degradation, end-to-end fusions, and translocations (Bailey & Murnane, 2006). Mammalian telomeres are comprised of telomeric DNA [both single-stranded (ss) and double-stranded (ds) DNA] and a variety of proteins, the core of which are called the shelterin complex (Palm & de Lange, 2008). In humans, telomeric DNA consists of a tandem array of TTAGGG repeats that range from 10 to 15 kb at birth. In normal cells, telomeres shorten after each round of cell division because conventional DNA polymerases are unable to replicate the ends of linear DNA (Olovnikov, 1973; Harley *et al.*, 1990). Telomeres are considered to be the molecular clock that limits the proliferative capacity of cells because short telomeres have been shown to induce replicative senescence (Counter, 1996). Furthermore, telomere shortening has been associated with organismal aging and several age-related human diseases (Garcia *et al.*, 2007). Mechanisms that can maintain telomere length and overall telomere structure can lead to prolonged cellular proliferation and thus lifespan extension (Bodnar *et al.*, 1998; Vaziri & Benchimol, 1998; Shay & Wright, 2007; Palm & de Lange, 2008).

The mechanism by which most eukaryotes synthesize and maintain their telomeres is through the enzyme telomerase, a ribonucleoprotein RT. Telomerase minimally consists of a catalytic protein subunit, the telomerase reverse transcriptase (TERT), and an RNA molecule, the telomerase RNA (TR) (Greider & Blackburn, 1985, 1989). To synthesize telomeric repeats *de novo*, TERT reverse transcribes an RNA template within TR onto the 3′-ends of ssDNA (Autexier & Lue, 2006). One unique feature of telomerase is its ability to catalyze multiple rounds of telomere synthesis without dissociating from the DNA substrate, a characteristic known as repeat addition processivity (RAP) (Greider, 1991).

Defects in the regulation or expression of any telomerase component have been associated with a variety of human pathologies. For example, upregulation of telomerase has been identified in many human cancers (Kim *et al.*, 1994), while decreased telomerase activity and short telomeres are associated with several premature aging diseases (Garcia *et al.*, 2007). Recently, mutations in the *hTERT* and *hTR* genes have been identified in a subset of patients with idiopathic pulmonary fibrosis (IPF) (Armanios *et al.*, 2007; Tsakiri *et al.*, 2007). IPF is a progressive and fatal lung disease characterized by fibrosis and damage to the lung parenchyma, and in particular to the alveolar epithelium (Dempsey, 2006). While the cause of the injury is unknown, sites of active fibrosis reflect improper repair of the damaged alveolar epithelium and the presence of fibroblastic foci and alveolar apoptosis is considered to be hallmarks of IPF (Dempsey, 2006). IPF is a premature aging disease that typically presents after the fifth decade of life, with prevalence increasing with age. The median mortality for the disease is approximately 3 years after initial diagnosis, and currently, there are no definitive treatments. One of the difficulties in developing therapeutic strategies for IPF is the unclear mechanism responsible for the etiology of the disease; therefore, uncovering the molecular mechanisms behind the onset and development of IPF is crucial for the understanding of this disease.

While the majority of IPF cases are considered sporadic, approximately 15–20% of patients with IPF have a family history of the disease, in which the pattern of inheritance is consistent with autosomal dominance with variable penetrance (Marney *et al.*, 2001). In 2007, Tsakiri *et al.* identified several heterozygous *hTERT* germline mutations in subsets of patients with familial IPF. They also determined that carriers of the *hTERT*

Correspondence
Dr Tara L. Beattie, HRIC 2022A, 3330 Hospital Dr NW, Calgary, AB T2N 4N1, Canada. Tel.: 403-220-8328; fax: 403-283-8727; e-mail: tbeattie@ucalgary.ca

Accepted for publication *9 February 2012*

mutations exhibited shorter telomeres compared with age-matched family members who do not harbor the mutations. This suggests that telomerase haploinsufficiency may lead to accelerated telomere shortening and thus contribute to the development of IPF.

With the molecular mechanisms of IPF largely unknown, we investigated how IPF-associated *hTERT* mutations affect telomerase function. Specifically, we have characterized the effects of three previously identified hTERT mutations that correspond to the V144M, R865C, and R865H amino acids substitutions (Tsakiri *et al.*, 2007) on telomerase function. With the V144 residue residing in the TEN domain of the N-terminus and the R865 residue within motif C of the highly conserved RT domain (Fig. 1A), we hypothesized that these IPF-associated hTERT mutations would disrupt telomerase function. Biochemical and cellular techniques were used to determine the effect that these mutations have on RAP, DNA binding and telomeric DNA synthesis and cell growth. These studies have enabled us to identify hTERT V144 and R865 as two residues that play crucial roles in telomerase function, telomere elongation, and cellular immortalization.

Results

Biochemical characterization of IPF-associated hTERT mutants

As an initial characterization of the IPF-associated hTERT mutants on telomerase activity, we used the telomere repeat amplification protocol (TRAP) and found that all three telomerase mutants were catalytically active and exhibited similar levels of activity compared with the wild-type (WT) enzyme (Fig. 1B). Reactions treated with RNAse A abolished catalytic activity, indicating that DNA synthesis was RNA dependent and specific for telomerase.

As the TRAP assay is a PCR-based assay, it can only detect the presence of telomerase activity and cannot be quantified nor yield specific information about total DNA synthesis or RAP. We therefore used the conventional telomerase assay (CTA) to further characterize the effects of the IPF-associated hTERT mutations on telomerase catalytic activity. The CTA measures direct nucleotide addition onto telomeric oligonucleotides that enables us to determine both total DNA synthesis (Fig. 1C) and RAP (Fig. 2). Similar to the results from the TRAP assay, the V144M mutant did not exhibit any defect in activity when tested with an 18-nt telomeric ssDNA primer (bio-TELO18). In contrast, our CTA results showed that the R865C and R865H mutants had a significant decrease in total DNA synthesis (~80%) compared with the WT enzyme. In addition to the decrease in DNA synthesis, we also noticed that the two R865 mutants seemed to be unable to efficiently synthesize multiple telomeric repeats on to the bio-TELO18 primer (Fig. 1C). To further investigate the possibility that the hTERT mutants may affect telomerase RAP, we completed a series of pulse-chase CTA experiments (Fig. 2). hTERT WT and V144M continued to elongate the pulse primer after the addition of chase DNA, thus indicating that the WT and V144M enzymes were able to catalyze processive DNA synthesis. Quantification of three independent pulse-chase experiments revealed that the V144M mutant was not statistically different from the WT enzyme, suggesting that hTERT V144 is not required for DNA synthesis or RAP. Conversely, hTERT R865C was only 12% as processive as the WT enzyme, while the processivity of the R865H mutant was essentially absent of processivity. This is similar to a previous observation where the R865H hTERT mutant protein displayed reduced processivity *in vitro* (Robart & Collins, 2010). This strongly implies that the R865 residue is required for efficient DNA synthesis and RAP, consistent with this residue residing in motif C of the highly conserved RT domain.

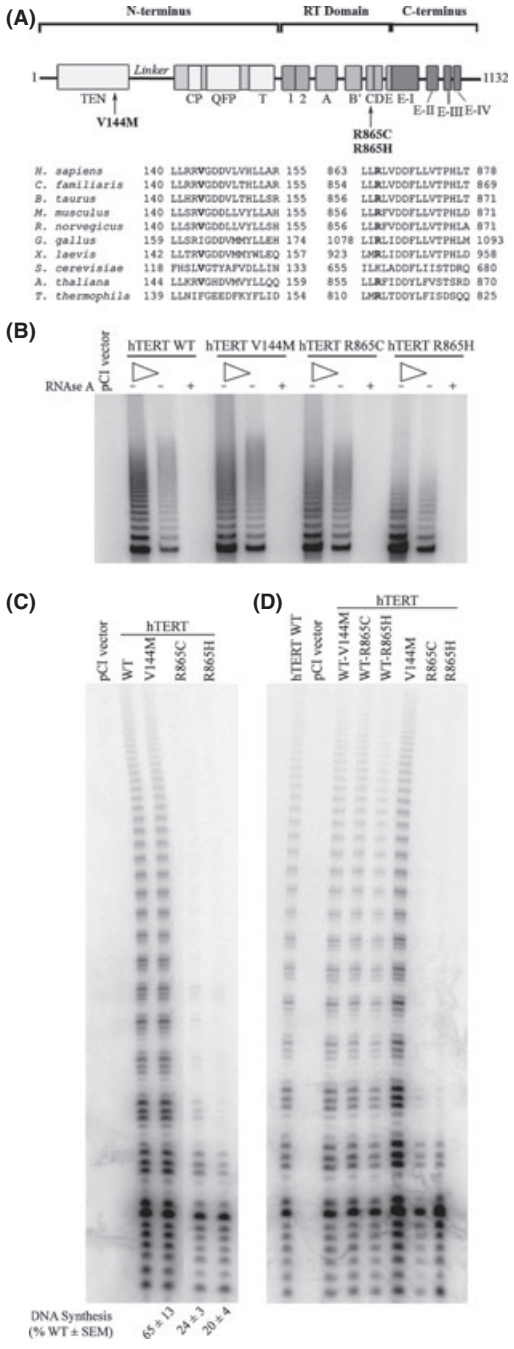

Fig. 1 *In vitro* telomerase activities of three IPF-associated hTERT mutants.
(A) Linear representation of the hTERT protein subunit of telomerase, in which both hTERT V144 and R865 are well conserved among different species.
(B) Reconstituted rabbit reticulocyte lysate (RRL) reactions containing hTERT wild-type (WT), V144M, R865C, or R865H all exhibited *in vitro* telomerase activity using the telomere repeat amplification protocol (TRAP) assay in the absence (–) of RNAse A. Addition (+) of RNAse A abolished telomerase activity. The triangles represent fivefold dilutions of each reaction to ensure that the amplification assays were in the linear range. RRL containing empty pCI vector was the negative control. As the TRAP is a PCR-based assay, it cannot be used to quantify total DNA synthesis or repeat addition processivity; therefore, a more specific conventional telomerase assay (CTA) assay was also performed (C). The CTA showed that hTERT V144M had similar activity levels (represented as DNA synthesis) as hTERT WT; however, hTERT R865C and R865H exhibited a severe defect in DNA synthesis. (D) Co-expression of hTERT WT and hTERT V144M, R865C, or R865H exhibited similar activity levels as hTERT WT alone. IPF, idiopathic pulmonary fibrosis.

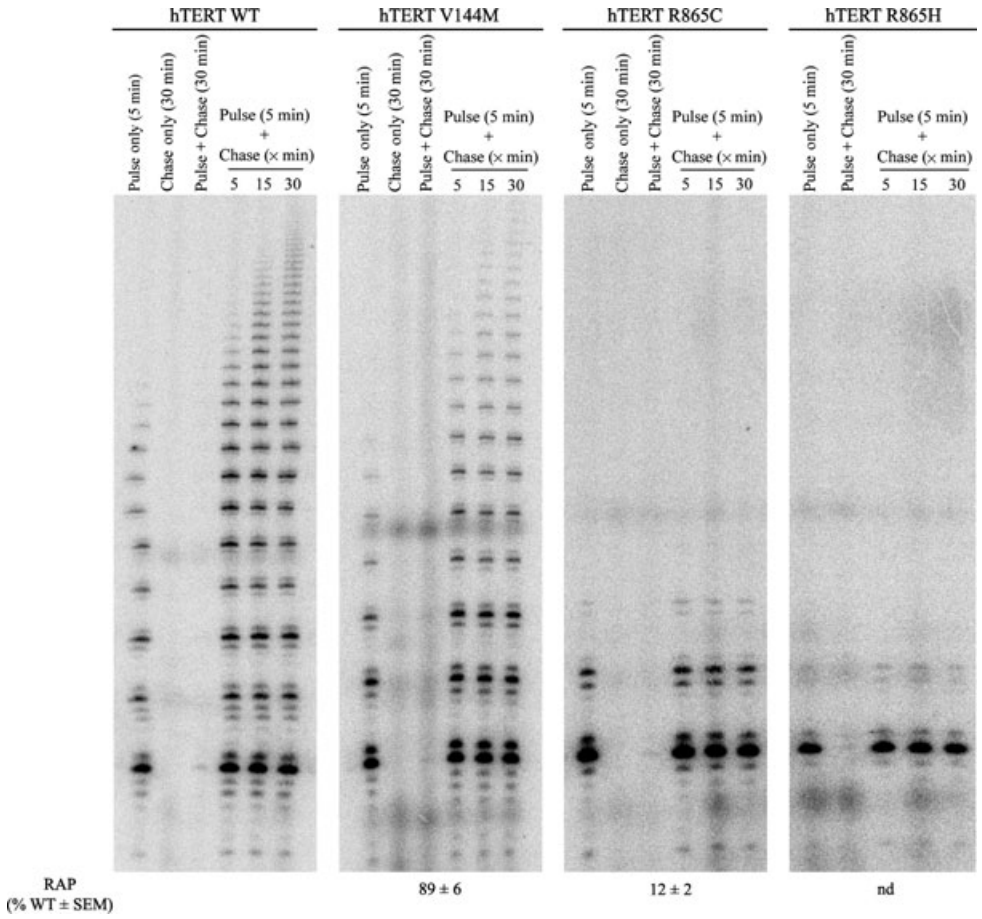

Fig. 2 Repeat addition processivity (RAP) of IPF-associated hTERT mutants. Pulse-chase CTA was used to determine the RAP of hTERT wild-type (WT), V144M, R865C, and R865H. Both reconstituted hTERT WT and V144M continued to elongate the pulse primer (bio-TELO18) even after the addition of the chase primer (TELO18) at various time points (5, 10, 15 min), indicating that both enzymes catalyzed processive DNA synthesis. Conversely, hTERT R865C was only 12% as processive as the WT enzyme, while the processivity of hTERT R865H could not be determined (nd). Control reactions for each panel included: 5-min pulse reaction alone (lane 1) to show that each telomerase enzyme can elongate the primers; 30-min chase reaction alone (lane 2) to ensure that nonbiotinylated products were not being isolated by streptavidin beads; and pulse + chase reaction, in which pulse and chase primers were added simultaneously to show the chase primer was predominantly elongated. CTA, conventional telomerase assay; IPF, idiopathic pulmonary fibrosis.

It has previously been demonstrated that hTERT can multimerize *in vitro* (Beattie *et al.*, 2001; Moriarty *et al.*, 2002). To test whether the presence of IPF-associated hTERT mutants affected the catalytic activity of WT hTERT *in vitro*, we co-expressed both WT and mutant hTERT with hTR in the same rabbit reticulocyte lysate (RRL) reaction, then assayed for telomerase activity using the CTA (Fig. 1D). The mixture of WT and mutant telomerase retained similar levels of DNA synthesis and processivity as the WT enzyme alone, suggesting that the mutant telomerase enzymes do not act as a dominant negative over WT.

To further explore the underlying cause for the activity defects seen with the hTERT R865 mutants, we tested whether the R865 residue (and hTERT V144) helped modulate hTERT stability and ssDNA interactions *in vitro*. Using the RRL system, hTERT WT, V144M, R865C, and R865H were labeled with [^{35}S]cysteine to demonstrate that each of the proteins was stably expressed *in vitro* (Fig. 3A) and to subsequently quantify and normalize the amount of hTERT required for the primer-binding assays. Limited proteolysis experiments with chymotrypsin digestion did not reveal any changes in protein conformation for any of the three mutants when compared with the WT protein (data not shown). One explanation for the observed R865 activity defects could be a compromised ability to bind to ssDNA. To test this, we used a primer-binding assay (Wyatt *et al.*, 2007) to determine whether any of the three hTERT mutations resulted in decreased binding efficiencies to telomeric primers *in vitro* (Fig. 3B). As the hTERT V144M mutant showed no defect in enzyme activity or processivity, it was not surprising to find that this mutant was able to bind various lengths of telomeric ssDNA as efficiently as the WT protein. However, when compared with WT, both R865 mutants showed a significant decrease in binding efficiency to the bio-TELO18 primer. This primer is used for the CTA and pulse-chase CTA assays described earlier; therefore, this decrease in ssDNA binding could explain why we observed a defect in activity and RAP when telomerase was reconstituted with either hTERT R865C or R865H. Although the CTA assay showed addition of the first two telomeric DNA repeats on the bio-TELO18 primer, this could be due to a strong but transient interaction between the mutant R865 hTERT proteins and the telomeric primer, which may be sufficient for addition of the first telomeric repeat. In contrast, a more steady-state, stable interaction, which is being measured by the primer-binding assay, would be needed for efficient RAP, as the enzyme is required to remain stably bound to the telomeric oligonucleotide. Furthermore, the three hTERT mutants retained sequence-specific ssDNA-binding activity. All three

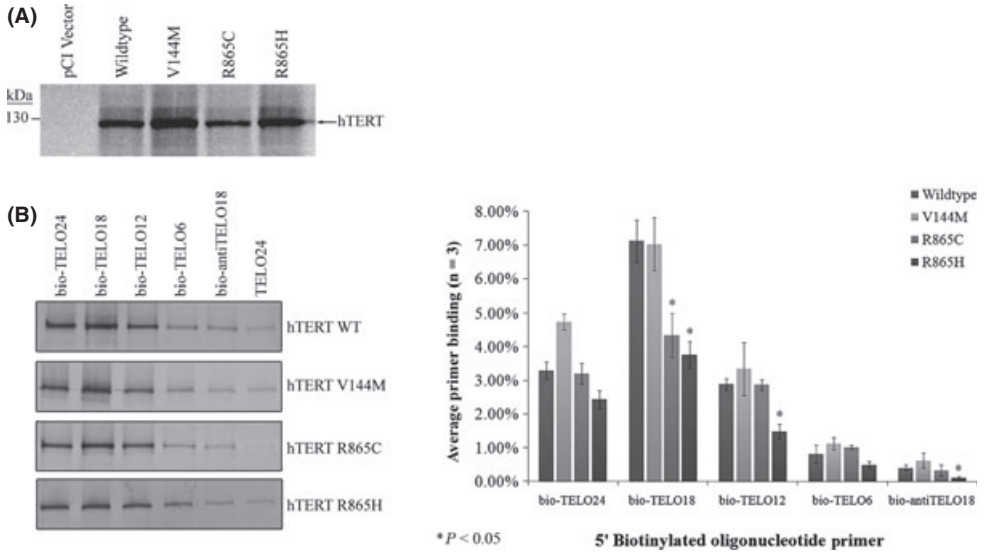

Fig. 3 hTERT mutants interact with telomeric ssDNA. (A) [^{35}S]-cysteine-radiolabeled hTERT wild-type (WT), V144M, R865C, and R865H were all stably expressed *in vitro* [rabbit reticulocyte lysate (RRL)]. This gel was quantified to determine the amounts of RRL to be added to the primer-binding assay. (B) Primer-binding assays were used to investigate the interactions between hTERT WT, V144M, R865C and R865H and 5′-biotinylated ssDNA human telomeric primers of various lengths. hTERT V144M bound all DNA primers as efficiently as hTERT WT. Both hTERT R865 mutants exhibited significantly decreased binding to bio-TELO18 compared with the WT protein. The asterisk (*) denotes statistical significance compared with the interaction between hTERT WT and the corresponding primer; $P < 0.05$.

hTERT mutants were also observed to bind hTR as efficiently as WT (data not shown).

hTERT residues V144 and R865 are critical for telomerase function in human cells

In an extension of our *in vitro* studies, we examined how the IPF-associated hTERT mutations affect the biological functions of telomerase in human cells. We stably expressed the three hTERT mutants (hTERT V144M, R865C, and R865H), a WT positive control and an empty vector negative control in both immortalized telomerase-positive cells (SV40 large T-antigen-transformed embryonic kidney cells; 293T) and telomerase-negative primary cells (human fibroblasts; BJ). The three hTERT mutants were expressed in 293T cells to assess the function of these mutations in the presence of WT telomerase and to determine whether the mutant proteins could behave as dominant negatives in cells. Figure 4A shows that immunoprecipitated hTERT V144M, R865C, and R865H all exhibited similar levels of TRAP activity compared with the hTERT WT control. Interestingly, hTERT R865H does not exhibit the longer PCR extension products seen with the WT enzyme. We were unable to extract sufficient amounts of telomerase activity from the overexpressing 293T cell lysates to perform a CTA or a pulse-chase assay to directly examine possible defects in RAP. To address activity by an alternative method, telomere length was used as a readout for functional telomerase activity in 293T cells. As previously reported (Bodnar *et al.*, 1998; Zhang *et al.*, 1999), overexpression of hTERT WT resulted in telomere elongation (Fig. 4B). However, the expression of hTERT V144M and the two R865 mutants did not exhibit the same phenotype, indicating that these mutants are unable to catalyze telomere synthesis in cells despite retaining catalytic activity. This inability to elongate telomeres was not because of the exclusion of the mutant hTERT proteins from the nucleus (data not shown). Furthermore, none of the key features of previously characterized dominant negative telomerase enzymes, such as proliferation defects or cell apoptosis (Zhang *et al.*, 1999), were seen in the 293T cells expressing any of the hTERT mutants.

In addition to 293T cells, we also studied the effects of the IPF-associated hTERT mutations on telomerase function in telomerase-negative normal diploid (BJ) cells. As BJ cells have a finite lifespan, these cells enabled us to determine whether any of the three hTERT mutants were able to elongate telomeres and bypass the replicative senescence checkpoint. As shown in Fig. 5A, antiflag immunoprecipitation of hTERT WT, V144M, R865C, and R865H exhibited similar levels of TRAP activity; however, hTERT R865H did not exhibit the longer PCR extension products similar to what was seen in the 293T cells. Upon examination of telomere length in the different cell strains, BJ cells overexpressing hTERT WT demonstrated telomere elongation over successive mean population doublings (mpd) (Fig. 5B) and the ability to undergo three times more mpd compared with the negative control (Fig. 5C). In addition, the WT-expressing cells did not stain positive for β-galactosidase activity (Fig. 5D), which is consistent with these cells bypassing replicative senescence. In contrast, cells expressing hTERT V144M or R865H exhibited telomere shortening similar to that of the negative control (Fig. 5B). However, despite continuous telomere shortening, these mutants paradoxically extended BJ cell lifespan. Furthermore, hTERT V144M-expressing cells did not exhibit β-galactosidase activity until they completed approximately two times more mpd compared with the negative control, while hTERT R865H-expressing cells extended cellular lifespan by approximately 10 mpd. Finally, BJ cells stably expressing hTERT R865C exhibited telomere shortening in the early passages, but the telomeres seemed to be maintained in later passages (Fig. 5B). Even though this mutant did not elongate telomeres in the BJ cells, the growth curve for these cells was similar to the hTERT WT control, in which the R865C-expressing cells underwent three times more mpd than the negative control and did not stain positive for β-galactosidase activity. Together, the 293T and BJ cell studies revealed that the IPF-associated hTERT mutations were unable to maintain robust telomere synthesis in human cells despite retaining some *in vitro* biochemical telomerase activity and that R865C can immortalize cells in the absence of altering telomere length.

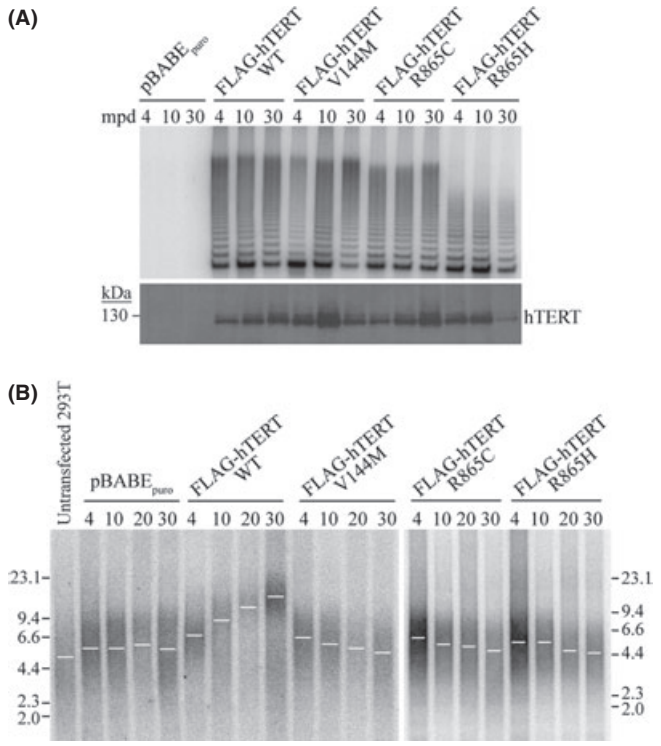

Fig. 4 hTERT V144 and R865 are required for robust telomere synthesis in transformed human cells. 293T cells were stably transfected to express $pBABE_{puro}$ or $pBABE_{puro}$-FLAG-hTERT wild-type (WT), V144M, R865C, or R865H. (A) Immunoprecipitated FLAG-hTERT was assayed for telomerase activity via the TRAP assay (top panel). All hTERT mutants exhibited similar levels of telomerase activity in 293T cells. However, hTERT R865H shows some defect in RAP compared with WT, which was not a result of decreased protein expression (bottom panel). (B) Mean telomere length (represented by white bars) was determined by the terminal restriction fragment analysis. Overexpression of hTERT WT resulted in telomere elongation; however, expression of the three mutants did not exhibit the same phenotype. RAP, repeat addition processivity; TRAP, telomere repeat amplification protocol.

Discussion

Recently, mutations in the genes encoding two critical components of telomerase, *hTERT* and *hTR*, have been identified in a subset of patients with adult-onset IPF. Patients harboring these mutations had shorter telomeres compared with age-matched family members without these mutations, suggesting that telomerase deficiency could be the underlying cause of this phenotype. It is postulated that hTERT or hTR mutations will lead to improper telomerase function and, as a result, contribute to the development and progression of IPF. Although these telomerase mutations have been identified, little is known about how these mutations affect telomerase structure and function and how they manifest as shortened telomeres in affected patients. Our study identified two critical hTERT residues that play crucial roles in telomere length maintenance.

hTERT V144M is important for telomere maintenance but not telomerase activity

The V144 residue is located in the TEN domain of the hTERT subunit of telomerase, a region that has been implicated in TERT multimerization, ssDNA binding, enzyme activity, processivity, and localization (Jacobs *et al.*, 2006). Sequence analysis revealed that this residue is well conserved among all TERT proteins (Jacobs *et al.*, 2006), suggesting that this residue plays an important role in telomerase function. Our study revealed that although the V144M mutation did not affect telomerase catalytic activity or RAP, this mutation caused a severe defect in the ability for the enzyme to catalyze telomere synthesis in human cells. This may be due to a variety of potential factors, such as improper localization of the enzyme to the 3′-end of the DNA substrate or the disruption of critical protein–protein interactions that are required for proper telomere synthesis. Interestingly, we found that despite a defect in telomere synthesis, the overexpression of hTERT V144M in BJ cells extended cellular lifespan. This implies that hTERT may have functions independent of telomere length maintenance, which is consistent with growing evidence that suggests telomerase has additional nontelomeric roles in cells (see below).

hTERT R865 is required for telomerase processivity and function

Tsakiri *et al.* also identified two missense mutations, which correspond to the R865C and R865H amino acid mutations. Our studies have demonstrated that the R865 residue plays a crucial role in both robust DNA synthesis and RAP. This defect in telomerase function may be due to the fact that this arginine (Arg) residue resides in motif C of the highly conserved RT domain. Specifically, the residue forms part of the RhhDD motif that is shared by almost all TERT proteins (Drosopoulos & Prasad, 2007). The invariant aspartic acid (Asp) residues form part of a conserved Asp triad that is involved in nucleotide addition and is coordinated by a two metal ion mechanism. Previous studies have shown that mutating these Asp residues in HIV-1 RT and in TERT proteins resulted in inactive enzymes (Lowe *et al.*, 1991; Lingner *et al.*, 1997). In addition, the determination of the TERT structure in *Tribolium castaneum* revealed that the valine residue (which is conserved in humans) directly preceding the Asp doublet forms part of the TERT nucleotide-binding pocket (Gillis *et al.*, 2008). The positive nature of this highly conserved Arg residue makes it ideal for binding negatively charged groups, such as nucleotides; thus, this residue may also reside in the nucleotide-binding pocket. Impaired RAP may be a common mechanism by which telomerase mutations manifest as shortened telomeres in patients with IPF. It has recently been reported that another IPF associated hTERT mutation, V867M, also displayed defects in RAP (Alder et al., 2011).

Expression of hTERT R865C and R865H in 293T and BJ cells did not result in telomere elongation as observed with the WT enzyme. However, both mutants were able to extend the cellular lifespan of the BJ fibroblasts compared with the negative control. While the telomeres of the hTERT R865H-expressing cells shortened similarly to the negative control, the cells did proliferate for an additional 10 mpd. Most interestingly, the BJ cells expressing the hTERT R865C mutant not only exhibited delayed telomere shortening compared with the vector-only cells, but the mean telomere lengths in these cells became more homogenous and were maintained over successive mpd. This observation is consistent with previous studies that had determined that the shortest telomeres are preferentially elongated (Chang *et al.*, 2007). The ability for the R865C mutant enzyme to maintain telomeres in the BJ cells may have enabled these cells to proliferate like the positive control and seemingly bypass replicative senescence. It is unlikely that simply overexpressing hTERT R865C can overcome its diminished biochemical activities and bypass senescence in cells, because the R865H mutant, which exhibits similar biochemical properties both *in vitro* and in cells, was unable to elongate telomeres or immortalize the BJ fibroblasts. Therefore, despite the defect in total DNA

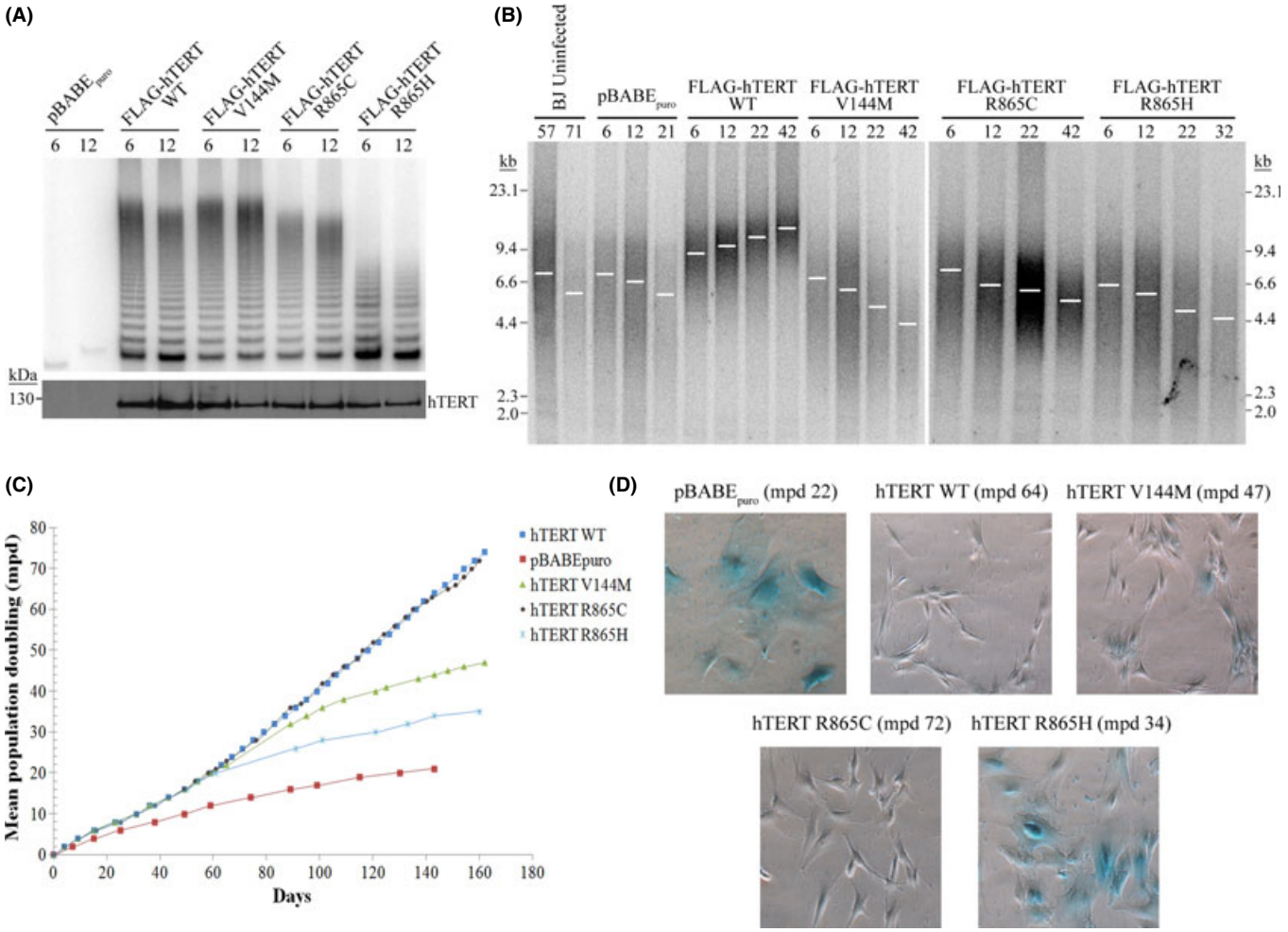

Fig. 5 hTERT V144M and R865H are required for telomere elongation and fibroblast immortalization. BJ cells were stably infected to express pBABE$_{puro}$, or pBABE$_{puro}$-FLAG-hTERT wild-type (WT), V144M, R865C, or R865H. (A) Immunoprecipitated FLAG-hTERT was assayed for telomerase activity via the TRAP assay (top panel), in which hTERT V144M, R865C, and R865H exhibited similar activity levels as hTERT WT. The R865H mutant showed a slight defect in RAP and was not because of decreased protein expression (bottom panel). (B) Mean telomere length (represented by white bars) was determined by the terminal restriction fragment analysis. Overexpression of hTERT WT led to telomere elongation, while cells expressing hTERT V144M and R865H exhibited telomere shortening similar to the negative control. Telomeres in the hTERT R865C cells showed some telomere shortening in early passages, but the telomeres became more homogenous in later mpd. Uninfected BJ cells at mpd 57 and 71 correspond to stably infected BJ cells at mpd 6 and 21, respectively. (C) Growth curves of the different BJ cell lines showed that hTERT R865H cells ceased proliferation shortly after the negative control, while hTERT V144M extended BJ cell lifespan by an additional 20 mpd. hTERT R865C had a similar growth curve as hTERT WT. (D) β-galactosidase activity at pH 6.0 (blue staining) was used as a marker of replicative senescence and showed that the negative control, hTERT V144M, and R865H cells eventually underwent senescence, while cells expressing hTERT WT and R865C bypassed senescence. RAP, repeat addition processivity; TRAP, telomere repeat amplification protocol.

synthesis and RAP, the R865C telomerase mutant was still able to add a sufficient number of telomeric repeats to partly circumvent normal telomere shortening. Alternatively, it may also be involved in one of the many recently reported noncanonical roles that telomerase plays in cells (see below). Therefore, the hTERT R865C mutant may be less detrimental than the R865H mutation, and this difference may account for the observation that the R865C mutation was only identified in one sporadic case of IPF, while the R865H mutant was found in a subset of patients with familial IPF (Tsakiri *et al.*, 2007).

Given that patients harboring these mutations retain one WT *hTERT* allele, we postulate that only half the amount of processive, fully functional telomerase is recruited to the telomere for proper telomerase function. Together, these mechanisms could result in improper telomere elongation and thereby manifest as shortened telomeres in patients carrying these *hTERT* mutations.

Expression of mutant hTERT proteins confers a growth advantage in human cells

We found that expression of hTERT V144M, R865C, or R865H in BJ cells conferred a growth advantage compared with the negative control and was independent of telomere elongation. As a result, we have uncoupled the role of telomerase in telomere elongation and in lifespan extension. The extended lifespan of the BJ cells upon expression of the mutant hTERT proteins could be due to a variety of different factors. Increasing evidence suggests that in addition to its function in telomere length maintenance, telomerase plays critical roles in pathways that do not involve chromosome ends. In these noncanonical roles, telomerase may function in the promotion of tumourigenesis (Stewart *et al.*, 2002) and cell survival (Lee *et al.*, 2008), the prevention of apoptosis (Santos *et al.*, 2006), or as a transcriptional regulator for several genes involved in cell proliferation

(Sarin *et al.*, 2005; Choi *et al.*, 2008; Park *et al.*, 2009) and in the DNA damage response (Sharma *et al.*, 2003). However, prolonged cell survival is likely associated with telomerase activity because overexpression of dominant negative and other inactive telomerase mutants do not exhibit the same phenotype (Counter *et al.*, 1998; Wyatt *et al.*, 2009). It remains possible that cells expressing these hTERT mutants have a diminished DNA damage response or reduced ability to detect DNA damage, and therefore, the shortened telomeres in these cells are unable to elicit the appropriate cellular response. Additional studies will be required to further elucidate the mechanism behind prolonged cell survival.

Potential consequences of hTERT mutations in the development of familial IPF

IPF, which is characterized by alveolar scarring, is thought to develop as a result of improper repair and renewal of the lung tissue (Dempsey, 2006). Different progenitor cell populations in different regions of the lung contribute to tissue repair and maintenance (Thannickal & Loyd, 2008). Alveolar damage is believed to be repaired and renewed by progenitor cells called alveolar epithelial type II cells or AE2 cells (Brody & Williams, 1992). Telomerase activity has been suggested to play a crucial role in lung tissue renewal. Although not completely representative of human IPF, lung epithelial cell injury in rodent models of pulmonary fibrosis led to increased telomerase expression (Fridlender *et al.*, 2007; Liu *et al.*, 2007). This implies that telomerase may have a protective role in the disease process and enzyme deficiency may lead to tissue fibrosis. In our studies, we have shown that three different IPF-associated hTERT mutations: V144M, R865C, and R865H, all led to a defect in telomerase function, in which these mutant enzymes were unable to catalyze robust telomere synthesis in human cells. As patients carrying these *hTERT* mutations are heterozygous, they express both WT and mutant hTERT and therefore might only express half the amount of normal functional telomerase. Through a mechanism of haploinsufficiency, we speculate that AE2 cells harboring these *hTERT* mutations would exhibit accelerated telomere shortening and decreased replicative potential compared with normal AE2 cells. Insufficient levels of functional telomerase may result in increased cell apoptosis, fibrosis, and/or cellular senescence, which may ultimately lead to a loss of AE2 cell populations and therefore impair their ability to respond to and repair injured or damaged alveoli.

Genetic factors, such as mutations in *hTERT*, are not the only factors that play a role in the onset and development of IPF. This is supported by the observation that patients with IPF who carry an *hTERT* mutation still had shorter telomeres compared with healthy family members with the same mutations (Tsakiri *et al.*, 2007), indicating that other factors also contribute to the onset and development of IPF. Most notably, environmental factors that lead to oxidative stress may influence cell proliferation, cell injury, and telomere loss (Taskar & Coultas, 2006). For example, cigarette smoking is considered a major risk factor for IPF, and it has been associated with telomere shortening in a dose-dependent manner (Morla *et al.*, 2006). Interestingly, Tsakiri *et al.* found that *hTERT* mutation carriers who smoked died on average 10 years earlier than mutation carriers who did not smoke. This reinforces the idea that other environmental factors, and not only *hTERT* mutations, have roles in the pathogenesis of IPF. Taken together, we can speculate that people who carry mutations in the *hTERT* gene could be at greater risk of developing IPF; however, the clinical manifestation of the disease will also depend on other environmental and genetic factors.

We have characterized the effects of three naturally occurring hTERT mutations on telomerase function. To our knowledge, this is the first report that describes how hTERT V144 and R865 affect telomerase function and telomere elongation. The R865 residue is crucial for telomeric DNA synthesis and RAP as it is located in the highly conserved nucleotide-binding pocket of the RT domain. These mechanisms that cause a defect in telomere elongation, in combination with other genetic and environmental factors, can ultimately lead to rapid telomere shortening seen phenotypically in *hTERT* mutation carriers. Additional investigations will be required to further uncover the role and mechanisms that telomerase mutants may play in the pathogenesis of IPF, and this may eventually result in the identification and development of novel preventative and therapeutic strategies for patients with pulmonary fibrosis.

Experimental procedures

For details please see Supporting Information S1.

Oligonucleotides

All oligonucleotides were synthesized by University Core DNA Services (University of Calgary, Calgary, AB, Canada).

In vitro reconstitution of human telomerase

hTERT constructs were cloned using site-directed mutagenesis as described by Wyatt *et al.* (2007). Full-length human telomerase RNA (nt 1-451) was cloned (Wyatt *et al.*, 2007) and synthesized *in vitro* using MEGAscript® T7 Transcription Kit (Ambion, Inc., Austin, TX, USA). Human telomerase was reconstituted *in vitro* using the RRL TNT® T7-Coupled Transcription/Translation System (Promega, Fisher Scientific Ltd., Ottawa, ON, Canada), according to manufacturer's instructions and the study by Wyatt *et al.* (2007).

Cell line generation and culture conditions

293T cells (Simian virus 40 (SV40)-transformed human embryonic kidney cells) were cultured in GIBCO high-glucose DMEM containing 10% fetal bovine serum (FBS), 50 U mL^{-1} penicillin G, and 50 μg mL^{-1} streptomycin sulfate (Invitrogen, Burlington, VT, Canada). 293T cells were transfected with 1 μg of DNA from the appropriate construct using FuGENE® 6 Transfection Reagent (Roche Applied Sciences, Laval, QC, Canada). Forty-eight hour after transfection, cultures were split and stable polyclonal populations were selected in growth media supplemented with 1 μg mL^{-1} puromycin (Sigma-Aldrich, St. Louis, MO, USA). The first plate that reached confluency under selection was arbitrarily defined as mpd 0. 293T cells were passaged continuously at 1:5.

Primary human foreskin fibroblasts (BJ cells) were grown in Lonza BioWhittaker EMEM with EBSS (Fischer Scientific Ltd., Ottawa, ON, Canada) supplemented with 10% FBS, 1% L-glutamine, 50 U mL^{-1} penicillin G, and 50 μg mL^{-1} streptomycin sulfate (Invitrogen). For BJ cell infection, amphotrophic retroviruses were packaged in 293T cells using 1 μg of helper plasmid pCL-10A1 and the appropriate control or hTERT construct DNA. 293T cells were transiently transfected with the appropriate pBABE$_{puro}$ construct (1 μg) using FuGENE® 6 Transfection Reagent (Roche Applied Sciences). Twenty-four hour after transfection, filtered viral supernatant was combined with polybrene (final concentration 8 μg mL^{-1}) and applied to the BJ cells. Forty-eight hour after retroviral infection, BJ cells were split 1:4 in growth media supplemented with 0.1 μg mL^{-1} puromycin (Sigma-Aldrich). Once the selected cells reached confluency, they were arbitrarily defined as mpd 0. BJ cells were continuously passaged at 1:4 until the cells divided three times more than the vector-only control or until they reached replicative senescence (positive β-galactosidase staining at pH 6.0).

Cell lysis

293T and BJ cell pellets were lysed in NP-40 lysis buffer (10 mM Tris–HCl, pH 7.5; 1 mM EGTA; 1 mM $MgCl_2$; 150 mM NaCl; 1% NP-40; 10% glycerol) containing 5 mM β-mercaptoethanol, 10 U mL^{-1} RNaseOUT™ (Invitrogen), and Protease Inhibitor Cocktail tablet (Roche Applied Sciences) on ice (30 min) followed by rocking at 4 °C (30 min), then centrifuged at 12 100 *g* for 30 min (4 °C). The supernatant was precleared with protein G Sepharose (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) for 30 min (4 °C) with rocking and centrifuged at 800 *g*. The protein concentration of the lysates was determined using the Bio-Rad Protein Detection Assay (Bio-Rad, Mississauga, ON, Canada).

Immunoprecipitations

Using precleared soluble protein lysate (1000 μg), FLAG-hTERT was immunoprecipitated with anti-FLAG M2 affinity resin (Sigma-Aldrich) in IP buffer-150 (10 mM Tris–Cl, pH 7.5; 1 mM EGTA; 1 mM $MgCl_2$; 150 mM NaCl; 1% NP-40; 10% glycerol) supplemented with 10 U mL^{-1} RNaseOUT™ (Invitrogen) and a Proteinase Inhibitor Cocktail tablet (Roche Applied Science) for 2 h (4 °C). The protein-bead complexes were centrifuged at 300 *g* for 30 s at room temperature (RT), washed in IP buffer-150 (washes 1 and 4) and IP buffer-300 (IP buffer containing 300 mM NaCl; washes 2 and 3). FLAG-hTERT was eluted from the affinity resin by resuspending the beads in 30 μL IP buffer-150 containing 2 mg mL^{-1} 3× FLAG peptide (Sigma-Aldrich), rocking at 4 °C (30 min) and centrifuging at 800 *g* for 3 min (4 °C).

Western analysis

FLAG-hTERT immunoprecipitates from 293T and BJ cells were detected via Western blotting using a polyclonal anti-hTERT antibody (1:500; Rockland Immunochemical Inc., Gilbertsville, PA, USA) as previously described (Wyatt *et al.*, 2009).

Telomere repeat amplification protocol

A modified version of the TRAP (Kim *et al.*, 1994) was used to detect telomerase activity. RRL reactions (1 μL) or 3X-FLAG peptide eluate (2 μL) were incubated in a 50-μL volume reaction, and reactions were carried out as previously described by Wyatt *et al.* (2009).

Conventional telomerase assay

The CTA assay was used to measure the activity of human telomerase reconstituted in the RRL as described previously (Wyatt *et al.*, 2007). The processivity of telomerase was determined by the pulse-chase CTA assays. In the pulse-chase, RRL containing human telomerase (24.5 μL) was incubated in a 40-μL reaction for 5 min at 30 °C (pulse). The pulse reaction was then incubated with 200 mM nonbiotinylated $(TTAGGG)_3$ at 30 °C for 5, 15 or 30 min (chase) and terminated with stop buffer at 37 °C for 15 min. The elongated biotinylated pulse primers were isolated from the nonbiotinylated primers following the previously described protocols (Wyatt *et al.*, 2007).

Telomerase activity was determined using data collected from the standard CTA assay, while RAP was calculated using data from the pulse-chase CTA assay. The Quantity One® version 4.5.0 software (Bio-Rad, Hercules, CA, USA) was used to quantify the signal intensities of each elongation product, which was normalized to the total number of incorporated [α-^{32}P]dGTP. Telomerase activity was calculated following the methods described by Wyatt *et al.* (2009).

Primer-binding assay

DNA-hTERT-binding efficiency was determined using the previously described DNA-binding assay (Wyatt *et al.*, 2007). Using the TNT T7 Transcription–Translation System (Promega), hTERT was radiolabeled with [^{35}S]cysteine in the absence of hTR. Radiolabeled WT and mutant hTERT proteins were tested for the ability to bind 5′-biotinylated oligonucleotides of various lengths. Phosphorimaging was used to visualize and quantify reaction products, which were subsequently analyzed following the methods described by Wyatt *et al.* (2007).

Terminal restriction fragment analysis

Using an in-gel hybridization technique, telomeres were visualized using a radiolabeled telomeric DNA probe (generously provided by Dr J. Karlseder, Salk Insitute for Biological Studies, La Jolla, CA, USA), $(TTAGGG)_x$, labeled with Klenow and [α-^{32}P]dGTP (3000 Ci $mmol^{-1}$; PerkinElmer) as previously described by Wyatt *et al.* (2009).

β-galactosidase staining

BJ cells grown in 6-cm culture dishes were washed with PBS (pH 7.2) and fixed for 5 min at RT in PBS (pH 7.2) containing 0.5% gluteraldehyde (Sigma-Aldrich). After fixing, cells were washed in PBS (pH 7.2) containing 1 mM $MgCl_2$ and stained overnight with fresh β-galactosidase staining solution (PBS (pH 6.0) with 1 mg mL^{-1} bromo-chloro-indolyl-galactopyranoside, 1 mM $MgCl_2$, 120 μM potassium ferricyanide, and 120 μM potassium ferrocyanide). The next day, stained cells were washed with PBS (pH 7.2) and visualized using bright-field microscopy.

Acknowledgments

The authors thank Dr K. Riabowol for helpful discussions on the manuscript and D. Lobb and members of the Beattie laboratory for technical assistance. This work was funded by operating grants to TLB from Alberta Innovates Health Solutions, the Alberta Cancer Foundation and the Canadian Institutes for Health Research. ART is the recipient of a studentship from the Alberta Cancer Foundation, and TLB is a scholar of the Alberta Cancer Foundation.

Author contributions

ART, HDMW, and NSYT performed the experiments. Data was analyzed by ART, HDMW, and TLB. Paper was written by ART and TLB.

References

Alder JK, Cogan JD, Brown AF, Anderson CJ, Lawson WE, Lansdorp PM, Phillips JA, Loyd JE, Chen JJ, Armanios MY (2011) Ancestral mutation in telomerase causes defects in repeat addition processivity and manifest as familial pulmonary fibrosis. *PLoS Genetics* **7**, e1001352.

Armanios MY, Chen JJ, Cogan JD, Alder JK, Ingersoll RG, Markin C, Lawson WE, Xie M, Vulto I, Phillips JA, Lansdorp PM, Greider CW, Loyd JE (2007) Telomerase mutations in families with idiopathic pulmonary fibrosis. *N. Engl. J. Med.* **356**, 1317–1326.

Autexier C, Lue NF (2006) The structure and function of telomerase reverse transcriptase. *Annu. Rev. Biochem.* **75**, 493–517.

Bailey S, Murnane J (2006) Telomeres, chromosome instability and cancer. *Nucleic Acids Res.* **34**, 2408–2417.

Beattie TL, Zhou W, Robinson MO, Harrington L (2001) Functional multimerization of the human telomerase reverse transcriptase. *Mol. Cell. Biol.* **21**, 6151–6160.

Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE (1998) Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**, 349–352.

Brody JS, Williams MC (1992) Pulmonary alveolar epithelial cell differentiation. *Annu. Rev. Physiol.* **54**, 351–371.

Chang M, Arneric M, Lingner J (2007) Telomerase repeat addition processivity is increased at critically short telomeres in a Tel1-dependent manner in *Saccharomyces cerevisiae*. *Genes Dev.* **21**, 2485–2494.

Choi J, Southworth LK, Sarin KY, Venteicher AS, Ma W, Chang W, Cheung P, Jun S, Artandi MK, Shah N, Kim SK, Artandi SE (2008) TERT promotes epithelial proliferation through transcriptional control of a Myc- and Wnt-related developmental program. *PLoS Genet.* **4**, e10.

Counter CM (1996) The roles of telomeres and telomerase in cell life span. *Mutat. Res.* **366**, 45–63.

Counter CM, Hahn WC, Wei W, Caddle SD, Beijersbergen RL, Lansdorp PM, Sedivy JM, Weinberg RA (1998) Dissociation among in vitro telomerase activity, telomere maintenance, and cellular immortalization. *Proc. Natl Acad. Sci. USA* **95**, 14723–14728.

Dempsey OJ (2006) Clinical review: idiopathic pulmonary fibrosis – past, present and future. *Respir. Med.* **100**, 1871–1885.

Drosopoulos WC, Prasad VR (2007) The active site residue Valine 867 in human telomerase reverse transcriptase influences nucleotide incorporation and fidelity. *Nucleic Acids Res.* **35**, 1155–1168.

Fridlender ZG, Cohen PY, Golan O, Arish N, Wallach-Dayan S, Breuer R (2007) Telomerase activity in bleomycin-induced epithelial cell apoptosis and lung fibrosis. *Eur. Respir. J.* **30**, 205–213.

Garcia CK, Wright WE, Shay JW (2007) Human diseases of telomerase dysfunction: insights into tissue aging. *Nucleic Acids Res.* **35**, 7406–7416.

Gillis AJ, Schuller AP, Skordalakes E (2008) Structure of the *Tribolium castaneum* telomerase catalytic subunit TERT. *Nature* **455**, 633–637.

Greider CW (1991) Telomerase is processive. *Mol. Cell. Biol.* **11**, 4572–4580.

Greider CW, Blackburn EH (1985) Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell* **43**, 405–413.

Greider CW, Blackburn EH (1989) A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. *Nature* **337**, 331–337.

Harley CB, Futcher AB, Greider CW (1990) Telomeres shorten during ageing of human fibroblasts. *Nature* **345**, 458–460.

Jacobs SA, Podell ER, Cech TR (2006) Crystal structure of the essential N-terminal domain of telomerase reverse transcriptase. *Nat. Struct. Mol. Biol.* **13**, 218–225.

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**, 2011–2015.

Lee J, Sung YH, Cheong C, Choi YS, Jeon HK, Sun W, Hahn WC, Ishikawa F, Lee HW (2008) TERT promotes cellular and organismal survival independently of telomerase activity. *Oncogene* **27**, 3754–3760.

Lingner J, Hughes TR, Shevchenko A, Mann M, Lundblad V, Cech TR (1997) Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science* **276**, 561–567.

Liu T, Chung MJ, Ullenbruch M, Yu H, Jin H, Hu B, Choi YY, Ishikawa F, Phan SH (2007) Telomerase activity is required for bleomycin-induced pulmonary fibrosis in mice. *J. Clin. Invest.* **117**, 3800–3809.

Lowe DM, Parmar V, Kemp SD, Larder BA (1991) Mutational analysis of two conserved sequence motifs in HIV-1 reverse transcriptase. *FEBS Lett.* **282**, 231–234.

Marney A, Lane KB, Phillips JA, Riley DJ, Loyd JE (2001) Idiopathic pulmonary fibrosis can be an autosomal dominant trait in some families. *Chest* **120**, 56S.

Moriarty TJ, Huard S, Dupuis S, Autexier C (2002) Functional multimerization of human telomerase requires an RNA interaction domain in the N terminus of the catalytic subunit. *Mol. Cell. Biol.* **22**, 1253–1265.

Morla M, Busquets X, Pons J, Sauleda J, MacNee W, Agusti AGN (2006) Telomere shortening in smokers with and without COPD. *Eur. Respir. J.* **27**, 525–528.

Olovnikov AM (1973) A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. *J. Theor. Biol.* **41**, 181–190.

Palm W, de Lange T (2008) How shelterin protects mammalian telomeres. *Annu. Rev. Genet.* **42**, 301–334.

Park JI, Venteicher AS, Hong JY, Choi J, Jun S, Shkreli M, Chang M, Meng Z, Cheung P, Ji H, McLaughlin M, Veenstra TD, Nusse R, McCrea PD, Artandi SE (2009) Telomerase modulates Wnt signalling by association with target gene chromatin. *Nature* **460**, 66–72.

Robart AR, Collins K (2010) Investigation of human telomerase holoenzyme assembly, activity, and processivity using disease-linked subunit variants. *J. Biol. Chem.* **285**, 4375–4386.

Santos JH, Meyer JN, Van Houten B (2006) Mitochondrial localization of telomerase as a determinant for hydrogen peroxide-induced mitochondrial DNA damage and apoptosis. *Hum. Mol. Genet.* **15**, 1757–1768.

Sarin KY, Cheung P, Gilison D, Lee E, Tennen RI, Wang E, Artandi MK, Oro AE, Artandi SE (2005) Conditional telomerase induction causes proliferation of hair follicle stem cells. *Nature* **436**, 1048–1052.

Sharma GG, Gupta A, Wang H, Scherthan H, Dhar S, Gandhi V, Iliakis G, Shay JW, Young CS, Pandita TK (2003) hTERT associates with human telomeres and enhances genomic stability and DNA repair. *Oncogene* **22**, 131–146.

Shay JW, Wright WE (2007) Hallmarks of telomeres in ageing research. *J. Pathol.* **211**, 114–123.

Stewart SA, Hahn WC, O'Connor BF, Banner EN, Lundberg AS, Modha P, Mizuno H, Brooks MW, Fleming M, Zimonjic DB, Popescu NC, Weinberg RA (2002) Telomerase contributes to tumorigenesis by a telomere length-independent mechanism. *Proc. Natl Acad. Sci. USA* **99**, 12606–12611.

Taskar VS, Coultas DB (2006) Is idiopathic pulmonary fibrosis an environmental disease? *Proc. Am. Throac. Soc.* **3**, 293–298.

Thannickal VJ, Loyd JE (2008) Idiopathic pulmonary fibrosis: a disorder of lung regeneration? *Am. J. Respir. Crit. Care Med.* **178**, 663–665.

Tsakiri KD, Cronkhite JT, Kuan PJ, Xing C, Raghu G, Weissler JC, Rosenblatt RL, Shay JW, Garcia CK (2007) Adult-onset pulmonary fibrosis caused by mutations in telomerase. *Proc. Natl Acad. Sci. USA* **104**, 7552–7557.

Vaziri H, Benchimol S (1998) Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr. Biol.* **8**, 279–282.

Wyatt HDM, Lobb DA, Beattie TL (2007) Characterization of physical and functional anchor site interactions in human telomerase. *Mol. Cell. Biol.* **27**, 3226–3240.

Wyatt HDM, Tsang AR, Lobb DA, Beattie TL (2009) Human telomerase reverse transcriptase (hTERT) Q169 is essential for telomerase function in vitro and in vivo. *PLoS ONE* **4**, e7176.

Zhang X, Mar V, Zhou W, Harrington L, Robinson MO (1999) Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. *Genes Dev.* **13**, 2388–2399.

Supporting Information

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Data S1. Experimental procedures.

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