A translocation-defective telomerase with low levels of activity and processivity stabilizes short telomeres and confers immortalization

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ABSTRACT **Short, repetitive, G-rich telomeric sequences are synthesized by telomerase, a ribonucleoprotein consisting of telomerase reverse transcriptase (TERT) and an integrally associated RNA. Human TERT (hTERT) can repetitively reverse transcribe its RNA template, acting processively to add multiple telomeric repeats onto the same substrate. We investigated whether certain threshold levels of telomerase activity and processivity are required to maintain telomere function and immortalize human cells with limited lifespan. We assessed hTERT variants with mutations in motifs implicated in processivity and interaction with DNA, namely the insertion in fingers domain (V791Y), and the E primer grip motif (W930F). hTERT-W930F and hTERT-V791Y reconstitute reduced levels of DNA synthesis and processivity compared with wild-type telomerase. Of interest, hTERT-W930F is more defective in translocation than hTERT-V791Y. Nonetheless, hTERT-W930F, but not hTERT-V791Y, immortalizes limited-lifespan human cells. Both hTERT-W930F– and hTERT-V791Y–expressing cells harbor short telomeres, measured as signal free ends (SFEs), yet SFEs persist only in hTERT-V791Y cells, which undergo apoptosis, likely as a consequence of a defect in recruitment of hTERT-V791Y to telomeres. Our study is the first to demonstrate that low levels of DNA synthesis—on the order of 20% of wild-type telomerase levels—and extension of as few as three telomeric repeats are sufficient to maintain functional telomeres and immortalize limited-lifespan human cells.**

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INTRODUCTION

Mammalian telomeres are composed of repeating T_2AG_3 DNA sequences (Moyzis *et al.*, 1988) associated with a complex of six proteins collectively referred to as shelterin (de Lange, 2005). The repetitive sequences terminate in a single-stranded G-rich overhang at the 3′ end of the chromosome (Wright *et al.*, 1997; Blackburn, 2001). The 3′ overhang is sequestered within the duplex region of the telomeric DNA, forming a protective structure known as the telomere loop (t-loop). t-loop formation prevents aberrant recombination, end-to-end fusion, and degradation of the chromosomal end (Griffith *et al.*, 1999; Cesare and Griffith, 2004; de Lange, 2005). In the majority of human cells, the DNA replication machinery is unable to fully replicate the ends of chromosomes (Watson, 1972; Olovnikov, 1973). Consequently telomeres progressively shorten at a rate of ~60–120 base pairs/cell division (Harley *et al.*, 1990; Baird *et al.*, 2003).

Telomere length is maintained in germline cells, embryonic stem cells, immortal cell lines, and ~90% of human malignancies (Kim *et al.*, 1994; Wright *et al.*, 1996; Kolquist *et al.*, 1998) by a specialized reverse transcriptase (RT) known as telomerase. Telomerase catalyzes the de novo addition of telomeric DNA repeats to the 3′ ends of chromosomes. It is minimally composed of two subunits: the telomerase reverse transcriptase (TERT) and the telomerase RNA (TR). In most species, the TERT protein is composed of four

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Abbreviations used: FISH, fluorescence in situ hybridization; hTERT, human telomerase reverse transcriptase; hTR, human telomerase RNA; PD, population doubling; RAP, repeat addition processivity; RRL, rabbit reticulocyte lysate; RT, reverse transcriptase; SFE, signal-free end; TRAP, telomere repeat amplification protocol.

structural domains: the telomerase-essential N-terminal (TEN) domain, the telomerase RNA-binding domain (TRBD), the RT domain, and the C-terminus. The central RT domain contains seven motifs (1, 2, A, B′, C, D, and E) that constitute the catalytic site and are evolutionarily conserved among all known RTs (Counter *et al.*, 1997; Harrington *et al.*, 1997; Lingner *et al.*, 1997; Meyerson *et al.*, 1997; Nakamura *et al.*, 1997). These motifs interact with the DNA substrate or primer and incoming nucleotides. The C motif is considered the catalytic center, and the E motif is commonly referred to as the primer grip region. Alteration of conserved sequences in the RT motifs leads to the inactivation of telomerase and reduced telomere length (Lundblad and Szostak, 1989; Counter *et al.*, 1997; Harrington *et al.*, 1997; Lingner *et al.*, 1997; Nakamura *et al.*, 1997). A recent crystal structure of the *Tribolium castaneum* TERT protein reveals that the TRBD, RT, and C-terminal domains fold into a ring structure with a central cavity to accommodate the RNA/DNA hybrid (Gillis *et al.*, 2008; Mitchell *et al.*, 2010).

A unique feature of telomerase, which distinguishes it from other RTs, is its ability to repetitively reverse transcribe its relatively short RNA template after a single primer-binding event, a process known as repeat addition processivity (RAP; Greider and Blackburn, 1987; Greider, 1991). Once the 5′ boundary of the template is encountered after one round of reverse transcription, the enzyme can dissociate from or stay associated to the primer for repetitive addition. Translocation must ensue for another round of extension to occur on the same primer, by which the RNA–DNA hybrid is dissociated from the active site and disrupted, the template and the new 3′ end of the DNA substrate realign, and the new RNA–DNA hybrid is repositioned in the active site for another round of elongation (Greider and Blackburn, 1987; Greider, 1991; Lue, 2004; Autexier and Lue, 2006; Brault *et al.*, 2008; Berman *et al.*, 2011; Qi *et al.*, 2011). Translocation is the rate-limiting step in a telomerase reaction, as indicated by the strong pause after each repeat, giving rise to the characteristic six-nucleotide (nt) banding pattern of telomere products (Greider, 1991).

Several structural elements in TERT not shared by conventional RTs appear to modulate RAP. TERT contains a sizable insertion between A and B′ composed of moderately conserved residues referred to as the insertion in fingers domain (IFD; Lingner *et al.*, 1997; Nakamura *et al.*, 1997; Lue *et al.*, 2003). Est2p (yeast TERT) and human TERT (hTERT) variants containing mutations in the IFD lack processivity (Lue *et al.*, 2003; Xie *et al.*, 2010). The recently characterized motif 3, found between motifs 2 and A and conserved specifically in vertebrate and ciliate TERTs, has been shown to be involved not only in processivity, but also in repeat addition rate (Xie *et al.*, 2010). RAP may also be partly regulated by sequences at the TERT terminal domains. Telomerase–DNA interactions proposed to aid in translocation may occur at a postulated anchor site found within the TEN domain (Wallweber *et al.*, 2003; Jacobs *et al.*, 2006; Romi *et al.*, 2007; Xie *et al.*, 2010; Jurczyluk *et al.*, 2011). Several hTERT C-terminal mutants also exhibit RAP defects, which may result from an inability of TERT to multimerize, leading to improper alignment of the substrate with the template, defects in primer or dNTP binding, or defects in translocation (Huard *et al.*, 2003). It appears that all of the aforementioned domains function in concert to facilitate binding of the RNA/DNA hybrid during template translocation (Xie *et al.*, 2010).

Mutational analysis of Est2p indicates that telomerase processivity may control telomere length maintenance. Est2p variants containing mutations in the IFD display defects in RAP and fail to maintain telomere length (Lue *et al.*, 2003). Furthermore, a double-substitution mutation in Est2p converting the yeast E motif to one that more closely resembles that of hTERT and HIV-1 RT confers an increase in processivity and telomere length in cells (Peng *et al.*, 2001; Ji *et al.*, 2005). Despite evidence implicating telomerase processivity as a regulator of telomere length in yeast, several differences exist between human and yeast telomerase in vitro. Telomerases from most vertebrates are highly processive. Human telomerase can add hundreds of nucleotides to a starting primer (Morin, 1989). In contrast, telomerase from yeasts display low processivity (Cohn and Blackburn, 1995; Prescott and Blackburn, 1997; Lue and Peng, 1998). Nonetheless, a few studies implicate human telomerase processivity as a crucial factor in the regulation of telomere length homeostasis and cellular immortalization. Interference with the processivity of telomerase using the pharmacological nonnucleosidic compound BIBR1532, which may promote enzyme dissociation or affect translocation, leads to a reduction in telomere length and growth arrest in cancer cells (Damm *et al.*, 2001; Pascolo *et al.*, 2002). In addition, it was recently shown that an hTERT mutant with increased processivity, L866Y, may initially induce an overlengthening of telomeres in an embryonic kidney cell line with limited lifespan (D'Souza *et al.*, 2013). Based on studies in which telomere length was experimentally altered, it was proposed that, similar to yeast, mammalian telomerase preferentially extends the shortest telomeres (Ouellette *et al.*, 2000; Hemann *et al.*, 2001; Samper *et al.*, 2001; Liu *et al.*, 2002; Britt-Compton *et al.*, 2009). Moreover, under artificial conditions of telomerase inhibition followed by release, several telomerase molecules elongate every telomeric 3′ end by multiple telomeric repeats in a distributive manner (Zhao *et al.*, 2011). In contrast, under telomere length maintenance conditions, such as in cancer cells, every telomere is elongated by a single telomerase molecule that processively adds ~60 nt to the telomeric 3′ end during every cell cycle (Zhao *et al.*, 2009, 2011).

Despite the data described, details of the regulation of telomere length maintenance and immortalization by telomerase processivity in human cells are incomplete. To determine whether certain threshold levels of telomerase activity and processivity are required to maintain telomere function and immortalize human cells with limited lifespan, we created hTERT variants with mutations in two motifs implicated in processivity and interaction with the DNA substrate—hTERT-V791Y in the IFD and hTERT-W930F in the E motif—expressed them in limited-lifespan cells, and analyzed their effect on telomere maintenance and cellular immortalization.

RESULTS

In vitro–expressed hTERT-W930F and hTERT-V791Y reconstitute lower levels of telomerase activity and processivity than does wild-type hTERT

Amino acid alignments of multiple TERT IFD and E motifs from diverse organisms indicates that the valine at position 791 and tryptophan at position 930 in human TERT (boxed in Figure 1A) are well-conserved hydrophobic amino acids (Peng *et al.*, 2001; Lue *et al.*, 2003). In an effort to reduce human telomerase processivity, we mutated the valine and tryptophan to the residues found at similar positions in yeast Est2p, known for exhibiting lower levels of in vitro processivity than wild-type Est2p. To confirm protein stability, we expressed the resulting mutants, hTERT-V791Y and hTERT-W930F, in in vitro rabbit reticulocyte lysate (RRL) in the presence of [^{35}S]methionine. A major band of 130 kDa was observed for both mutants and wild-type (WT) hTERT (Figure 1B, bottom). To determine the telomerase activities of hTERT-W930F and hTERT-V791Y, we expressed the mutants in RRL in the presence of in vitro–transcribed human telomerase RNA (hTR). Activity was assayed over a wide range of protein dilutions using the PCR-based telomere repeat

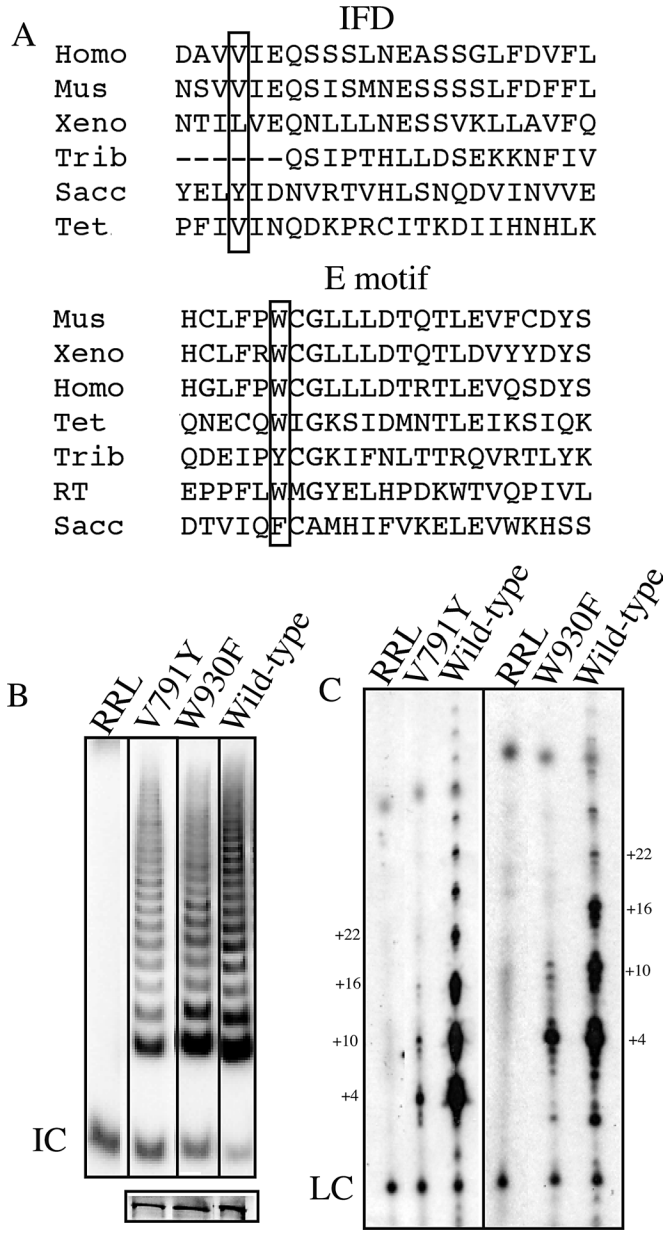

FIGURE 1: Location of IFD and E motif mutations and activity and processivity of in vitro–reconstituted mutant and wild-type telomerase enzymes. (A) Alignment of TERTs from *Homo sapiens* (Homo), *Mus musculus* (Mus), *Xenopus laevis* (Xeno), *Saccharomyces cerevisiae* (Sacc), *Tetrahymena thermophila* (Tet), and *Triboleum castaneum* (Trib), and reverse transcriptase from HIV-1 (RT) was performed using Clustal W. The boxes indicate the valine at position 791 in the IFD and the tryptophan at position 930 in the E motif in *H. sapiens* TERT and the corresponding residues at similar positions within the other TERTs and HIV-1 RT (for E motif). (B) Telomerase mutant and WT complexes were reconstituted in rabbit reticulocyte lysate, and activity was assayed using TRAP. A 1:80 dilution of lysate that reconstitutes telomerase activity in the linear range was quantified. IC, internal control. Activity level corresponding to each complex was quantified from $n = 2$. RRL, rabbit reticulocyte lysate not expressing any protein. Boxes indicate removal of intervening lanes from the original gel. In vitro–synthesized, [^{35}S]methionine-labeled hTERT-V791Y, hTERT-W930F, and WT hTERT were analyzed by SDS–PAGE (bottom). (C) Mutant hTERT-V791Y, hTERT-W930F, and WT telomerases were reconstituted in rabbit reticulocyte lysate, and processivity was assessed by the direct primer extension assay using a biotinylated telomeric $[T_2AG_3]_3$ primer. RAP was quantified from three repeats for hTERT-W930F and from four to six repeats for hTERT-V791Y, $n = 3$. RRL, rabbit reticulocyte lysate not expressing any protein. LC, loading control. +4, +10, +16, and +22 represent the number of nucleotides added to the $[T_2AG_3]_3$ substrate upon reaching the 5′ end of the template. In each T_2AG_3 repeat, these positions represent the first G added. Boxes indicate the presence of data from two different gels.

amplification protocol (TRAP) assay to determine which concentration of protein generates telomerase activity in the linear range (data not shown). Quantification was performed for the 1:80 dilution, which generates telomerase activity within the linear range for both variants and WT-hTERT (Figure 1B). The RRL-reconstituted mutant enzymes both displayed lower levels of telomerase activity compared with WT telomerase. hTERT-W930F and hTERT-V791Y displayed 64.16% (±2.73 SD) and 29.00% (±4.11 SD) of WT levels of overall DNA synthesis, respectively ($n = 2$). Next the RRL-reconstituted human telomerase variants were incubated with biotinylated $[T_2AG_3]_3$ substrate primer, and the extension products were analyzed on sequencing gels (Figure 1C). The mutant enzymes displayed lower levels of processivity than did WT enzyme, as shown by the absence of long extension products. hTERT-W930F consistently synthesized three repeats (+16) and hTERT-V791Y, minimally, four repeats (+22).

Quantification of RAP for the first three repeats for hTERT-W930F and for the first four to six repeats for hTERT-V791Y revealed that hTERT-W930F and hTERT-V791Y, respectively, possessed RAP levels of 75.03% (±3.25 SD) and 87.09% (±0.04 SD) compared with wild-type enzyme ($n = 3$). The non–PCR-based direct primer extension assay was previously reported to be more representative than the TRAP technique for detecting defects in levels of reconstituted telomerase activity or DNA synthesis, largely due to the amplification of low levels of elongation products by the TRAP assay (Huard *et al.*, 2003; Moriarty *et al.*, 2004, 2005a). The variants reconstituted low levels of telomerase activity (DNA synthesis) compared with WT-hTERT. However, hTERT-W930F consistently displayed a higher level of DNA synthesis than hTERT-V791Y, as indicated by the darker band at position +4 (the first G in the first telomeric repeat to be synthesized; Figure 1C), also suggestive of a defect in RAP and translocation.

hTERT-W930F and hTERT-V791Y expressed in cells reconstitute lower levels of telomerase activity and processivity compared with wild-type telomerase

In vitro RRL-reconstituted telomerase containing W930F and V791Y mutations display lower levels of TRAP activity compared with WT telomerase. To confirm these results, we also assayed telomerase activity over a wide range of protein dilutions using extracts from mutant and WT telomerase-expressing HA5 cells. HA5 cells comprise a human embryonic kidney cell line with limited lifespan that expresses SV40 large T and small t antigens and lacks hTERT expression (Stewart and Bacchetti, 1991). HA5-hTERT-V791Y clone D and HA5-hTERT-W930F clone F2 displayed lower levels of activity compared with WT (Figure 2A). Of importance, levels of activity for HA5-hTERT-W930F were never higher than for WT, regardless of the population doubling (PD). Similar results were obtained with HA5-hTERT-V791Y clone E and W930F clone F1 (data not shown). To confirm the processivity defects of the in vitro–reconstituted hTERT-W930F and hTERT-V791Y enzymes, we used cell extracts from 293 cells coexpressing hTR and the various hTERTs in a direct primer

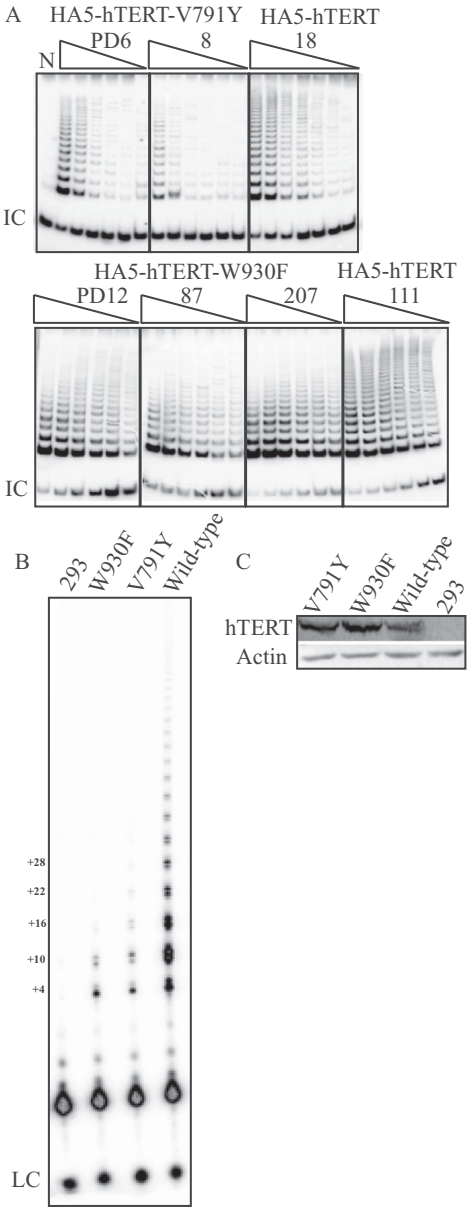

FIGURE 2: Mutant hTERTs reconstituted in HA5 cells and 293 cells display lower levels of activity and processivity compared with wild-type telomerase. (A) Telomerase activity was assayed for clones D and F2 of hTERT-V791Y– and hTERT-W930F–expressing HA5 cells, respectively. Telomerase activity from HA5 cells expressing WT telomerase assayed at the same time as each mutant is shown for comparison. In the final reaction 1, 0.5, 0.25, and 0.125 µg and 62.5 and 31.25 ng of total protein were assayed. N, no cell lysate; IC, internal control. Numbers above indicate population doublings. Boxes indicate the presence of data from two different gels and the removal of intervening lanes from the original gels. (B) Mutant hTERT-W930F and hTERT-V791Y and WT telomerase complexes were reconstituted in 293 cells, and processivity was analyzed using the direct primer extension assay and a biotinylated telomeric $[T_2AG_3]_3$ primer. DNA synthesis was quantified from $n = 3$. RAP was quantified from three repeats for hTERT-W930F and four to six repeats for hTERT-V791Y, $n = 3$. 293, 293 cells not overexpressing exogenous hTERT or hTR. LC, loading control. (C) Mutant hTERT-W930F, hTERT-V791Y, and WT telomerase complexes were reconstituted in 293 cells, and levels of hTERT expression were assessed by Western analysis and compared with levels of actin expression.

extension assay with $[T_2AG_3]_3$ as the substrate. Overexpression of both WT components (sometimes referred to as supertelomerase) generates a sufficient level of telomerase activity required for the direct assay (Cristofari and Lingner, 2006; Xie *et al.*, 2010; D'Souza *et al.*, 2013). Similar to RRL-synthesized and reconstituted mutant hTERT enzymes, mutant telomerases expressed in 293 cells displayed drastically impaired DNA synthesis (hTERT-W930F, 18.36% [±5.68 SD]; hTERT-V791Y, 26.20% [±10.69 SD]; $n = 3$) and processivity in contrast to WT enzyme expressed in 293 cells (Figure 2B). Only two to three or four to five repeats were reproducibly visible for hTERT-W930F and hTERT–V791Y, respectively. Western analysis confirmed that expression levels of mutant hTERTs were not significantly different from that of wild-type hTERT (Figure 2C). Quantification of RAP for the first three repeats for hTERT-W930F and for the first four to six repeats for hTERT-V791Y revealed that hTERT-W930F and hTERT-V791Y, respectively, possessed RAP levels of 25.37% (±12.94 SD) and 70.42% (±11.52 SD) compared with wild-type enzyme ($n = 3$).

Despite reduced levels of DNA synthesis and processivity reconstituted by hTERT-W930F and hTERT-V791Y enzymes, hTERT-W930F, but not hTERT-V791Y, expression can immortalize HA5 cells

To determine the in vivo effects of decreased DNA synthesis and processivity resulting from mutated IFD and E motifs, we expressed the variants and WT hTERTs in HA5 cells. The ability of WT hTERT to maintain telomeres and immortalize HA5 cells has been demonstrated (Counter *et al.*, 1998; Armbruster *et al.*, 2001; Moriarty *et al.*, 2005b). Several dozen colonies were obtained after retroviral infection of HA5 cells with hTERT-W930F or WT hTERT. Similar to WT hTERT, the expression of hTERT-W930F was able to immortalize HA5 cells. Two clones of each were selected and passaged for ~150–200 PDs (Figure 3A). In contrast to hTERT-W930F, only five colonies of HA5 cells expressing hTERT-V791Y and seven colonies of HA5 cells infected with empty vector were obtained. Colonies of each variant were expanded to monitor growth. hTERT-V791Y–expressing cells behaved similarly to empty vector–containing cells in culture. They both failed to immortalize HA5 cells and died between PDs 6 and 10 (Figure 3B). It was also important to confirm the expression of the WT and mutant proteins to assess whether protein levels fluctuated during culture or differed between mutant hTERT- and WT hTERT-expressing cells. hTERT protein levels remained relatively unchanged during growth (Figure 3C). The levels of hTERT-W930F expressed by the HA5 cells were never higher than the levels of hTERT-V791Y for the different clones. hTERT protein was not detected in empty vector–containing HA5 cells.

HA5 cells expressing hTERT-V791Y display signal-free ends, which persist with increasing population doubling

Typically, telomeres of transformed cells such as HA5 cells shorten with successive cell division, eventually leading to unprotected telomeres, genomic instability, and cell death associated with crisis (Counter *et al.*, 1992, 1994; Shay *et al.*, 1993; Klingelhutz *et al.*, 1994). To determine whether HA5 cells expressing hTERT-V791Y or empty vector were undergoing apoptosis, we performed fluorescence-activated cell sorting (FACS) analysis (Figure 4A). hTERT-V791Y–expressing and empty vector–containing cells experienced high levels of apoptosis compared with hTERT-W930F and WT telomerase-expressing cells. In addition, the levels of apoptosis increased with increasing PD, as shown by empty vector clone H and all of the V791Y-telomerase–expressing clones. We predicted that hTERT-V791Y–expressing cells, which experienced high levels of

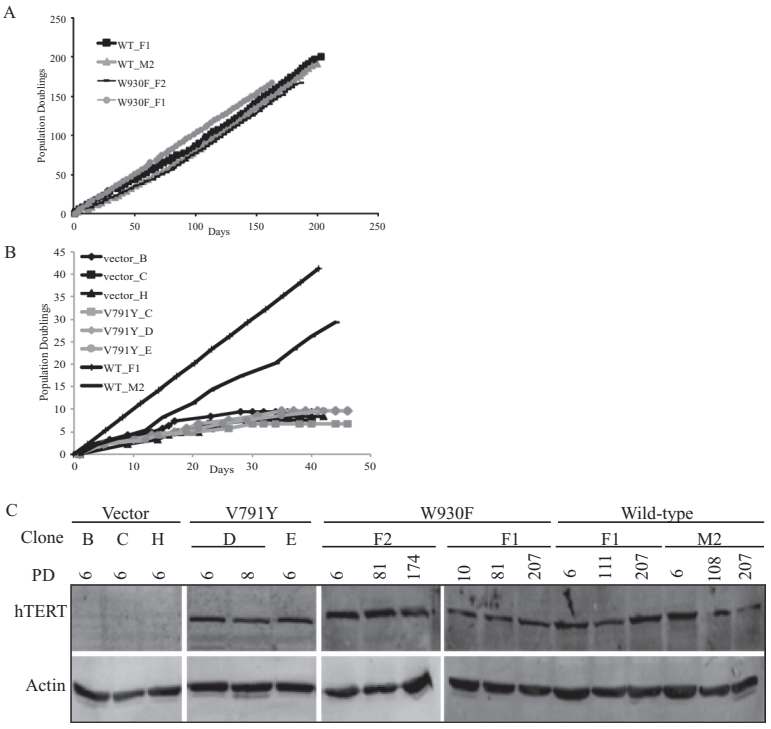

FIGURE 3: hTERT-W930F, but not hTERT-V791Y, expression can immortalize HA5 cells. (A, B) Growth curves of HA5 cells infected with retroviruses stably expressing hTERT-W930F or WT hTERT (A) or expressing hTERT-V791Y or containing empty vector (B). Growth curves of WT-hTERT shown in A are also shown in B for comparison purposes. Three clones each of HA5 cells containing empty vector or expressing hTERT-V791Y and two clones each of HA5 cells expressing hTERT-W930F or WT hTERT were selected for passage. (C) HA5 cells containing empty vector or expressing hTERT-V791Y, hTERT-W930F, and WT 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. Clone and PD are indicated above each lane. Boxes indicate the presence of data from different gels and the removal of intervening lanes.

apoptosis, might display an increased frequency of chromosomes with short telomeres or signal-free ends (SFEs) compared with hTERT-W930F–expressing cells. Similar to empty vector–containing cells, HA5 cells expressing hTERT-V791Y exhibited a high frequency of chromosomes with SFEs (Figure 4B). HA5 cells expressing hTERT-V791Y-clone D displayed a frequency of 10% SFEs at PD6. The frequency did not change significantly at higher PD. HA5 cells expressing WT telomerase displayed very low levels of SFEs. Surprisingly, both clones of hTERT-W930F expressing HA5 cells also displayed SFEs, evident by early and/or mid passage. However, the majority of these SFEs disappeared by late passage (Figure 4B). The appearance of SFEs could be a consequence of reduced levels of DNA synthesis and processivity exhibited by the hTERT-W930F enzyme. Furthermore, the absence of SFEs at late passage may explain why hTERT-W930F–expressing HA5 cells are able to survive in culture. However, residue 791 within the hTERT IFD appears to be critical for telomere length maintenance and cell immortalization.

To examine telomere length maintenance in cells expressing hTERT-W930F, we extracted genomic DNA from cells at different population doublings. The average telomere lengths of HA5 clones expressing hTERT-W930F were measured by TRF analysis (Figure 4C). Telomere length decreased from 2.5 kb at PD6 to ~1.7 kb at PD54 and subsequently increased to reach an average telomere length of 1.9 kb at PD165 in clone F2, whereas telomere length decreased from ~3 kb at early PD to 1.3 kb at PD123 in clone F1. The decrease in average telomere length followed by an increase in clone F2 paralleled the increased SFEs observed at middle passage followed by a decreased number of SFEs at late passage (Figure 4B). Such a correlation was not observed for clone F1, which, despite initially longer average telomere lengths compared with clone F2, also harbored very short telomeres at early and middle passages (Figure 4, B and C). Of importance, at late passage, the average telomere lengths of both HA5 clones hTERT-W930F-F2 (1.9 kb) and hTERT-W930F-F1 (1.3 kb) were consistently less than the average telomere lengths of two HA5 clones expressing hTERT-WT (clone F1, 2.2 kb; clone M2, 3.2 kb; D'Souza *et al.*, 2013).

hTERT-W930F is more defective in translocation than hTERT-V791Y

Next we investigated the translocation efficiency of the hTERT mutant enzymes to determine whether hTERT-V791Y's inability to elongate short telomeres and immortalize HA5 cells was due to a defect in translocation. During extension of a primer by telomerase, translocation must occur once the 5′ boundary of the template is encountered in order for another round of extension to proceed. The efficiency of translocation was measured using a recently developed assay (Latrick and Cech, 2010) that allows only a single template translocation event to occur. Extension of the primer 26GTT (TTATTATTAGGGTTAGGGTTAGGGTT) results in two products, a +2nt product and a +4nt product. The +2nt product is generated by the addition of one dATP and one dGTP. After their addition, translocation occurs. During the next round of extension two additional dGTPs are added to generate the +4nt product. Further nucleotide addition cannot occur due to the absence of dTTP in the reaction. Telomerase enzymes were reconstituted in 293 cells coexpressing hTR and incubated with 26GTT and a 10-fold excess of an unextendable 3′-phosphorylated competitive primer (A5P: TTAGGGTTAGCGTTAGGGp) to minimize the frequency of reinitiation on the same 26GTT primer. Western analysis confirmed that expression levels of mutant hTERTs were not significantly different from that of wild-type hTERT (Figure 2C). The expected +2nt and +4nt products can be seen at most of the time points for the telomerase mutant and WT enzymes (Figure 5A). The +3nt product is unrelated to template translocation (Qi *et al.*, 2011). The efficiency of translocation corresponds to the ratio of the +4nt signal compared with the total signal at the +2 and +4 positions at the last time point and reflects the fraction of telomerase that has translocated. The translocation efficiency for WT telomerase was 68.14% [(±0.83 SD; $n = 2$), which is in agreement with the previously reported value (Latrick and Cech, 2010). Both hTERT-W930F and hTERT-V791Y were impaired in translocation compared with WT hTERT, which may contribute to the decrease in processivity displayed by both RRL- and 293 cell–reconstituted mutant telomerase enzymes. Surprisingly, an increased number of stalled or dissociated telomerase complexes was seen for hTERT-W930F (translocation efficiency, 36.00% [±5.06 SD], $n = 2$)

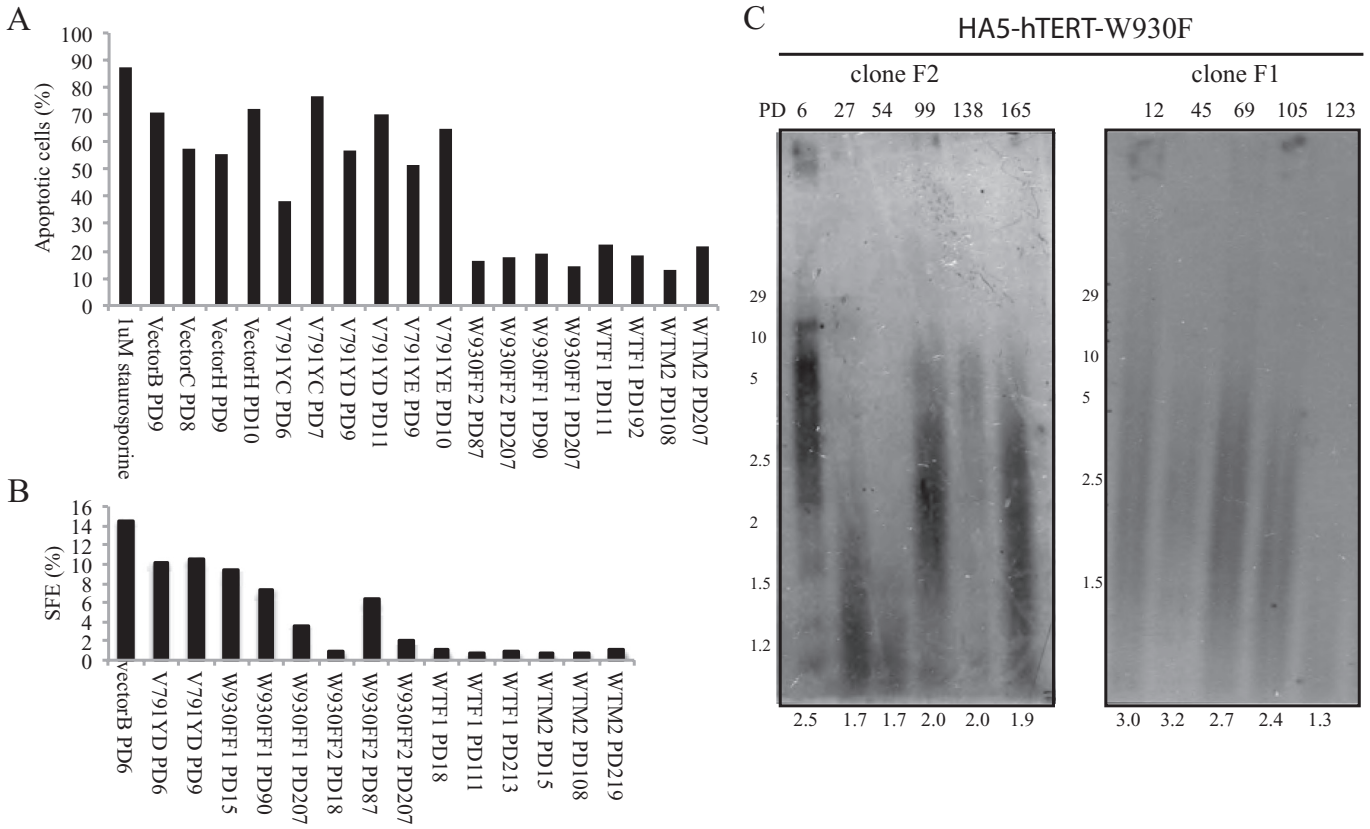

FIGURE 4: hTERT-V791Y–expressing HA5 cells undergo apoptosis and display persistent signal-free ends. (A) FACS analysis was performed, and percentage apoptosis is shown for HA5 cells treated with staurosporine, containing empty vector (clones B, C, and H) or expressing hTERT-V791Y (clones C, D, and E), hTERT-W930F (clones F1 and F2), or WT-hTERT (clones F1 and M2). PDs are also indicated. (B) Percentage of SFEs for HA5 cells containing empty vector (clone B) or expressing hTERT-V791Y (clone D), hTERT-W930F (clones F1 and F2), or WT-hTERT (clone F1 and M2) at different indicated PDs. Approximately 3000 ends were scored for hTERT-V791Y–expressing or empty vector–containing cells. Approximately 1000 ends were scored for HA5 cells expressing hTERT-W930F or WT-hTERT. (C) TRF analysis of genomic DNA digested with *Hin*fI and *Rsa*I from HA5 clones F2 and F1 expressing hTERT-W930F at increasing PD. Selected marker sizes (kb) are shown to the left of each TRF. Average telomere length at each PD is shown below each TRF.

compared with hTERT-V791Y (translocation efficiency, 58.55% [±0.54 SD], $n = 2$; Figure 5B), despite hTERT-W930F's ability to immortalize HA5 cells.

hTERT-V791Y is impaired in recruitment to telomeres

Finally, we tested whether the presence of SFEs in HA5-hTERT-V791Y cells and the inability of hTERT-V791Y to immortalize HA5 cells were due to a lack of proper localization of mutant telomerase to telomeres. Sensitive and specific fluorescence in situ hybridization (FISH) procedures using overexpressed telomerase components have been used, enabling visualization of the localization of the telomerase RNA component to the telomere (Tomlinson *et al.*, 2006, 2008; Cristofari *et al.*, 2007; Abreu *et al.*, 2010; Stern *et al.*, 2012; Zhong *et al.*, 2012). The localization of hTR to the telomere is dependent on the presence of hTERT (Tomlinson *et al.*, 2008). These studies revealed that the telomere-lengthening activity of telomerase is controlled in part by regulated trafficking of telomerase components. hTR and mutant or WT hTERT were coexpressed in HeLa cells, and similar expression levels of W930F-, V791Y- and WT-hTERT were confirmed by Western analysis (Figure 6C). FISH was performed using three hTR-specific Cy3-conjugated probes (magenta) and one Oregon Green–conjugated, telomere-specific probe (green; Figure 6A). Cy3 foci were not visible in HeLa cells in the absence of overexpressed hTERT and hTR. Colocalization of telomeres and hTR was evident in HeLa cells coexpressing hTR and WT hTERT or hTERT-W930F (average of 7.14 [±0.74 SD] vs. 6.13 [± 0.57 SD] colocalizations/cell, respectively). hTR was observed at five or more telomeres in 90.75% of HeLa cells coexpressing WT telomerase and in 77.26% of hTERT-W930F–expressing cells. In contrast, an average of only 1.86 colocalizations (±0.18 SD) was seen per HeLa cell coexpressing hTERT-V791Y and hTR. The percentage of cells containing hTR associations with five or more telomeres decreased to 0.70% in cells coexpressing hTERT-V791Y and hTR. These results indicate that this mutant telomerase enzyme may be defective in recruitment to telomeres.

DISCUSSION

Telomerase is a processive enzyme that reverse transcribes its relatively short RNA template onto the 3′ end of chromosomal DNA. The synthesis of a telomeric repeat requires the 3′ end of the DNA primer to base pair with the RNA template to form an RNA/DNA hybrid positioned within the active site. DNA polymerization then proceeds by reverse transcription of the RNA template

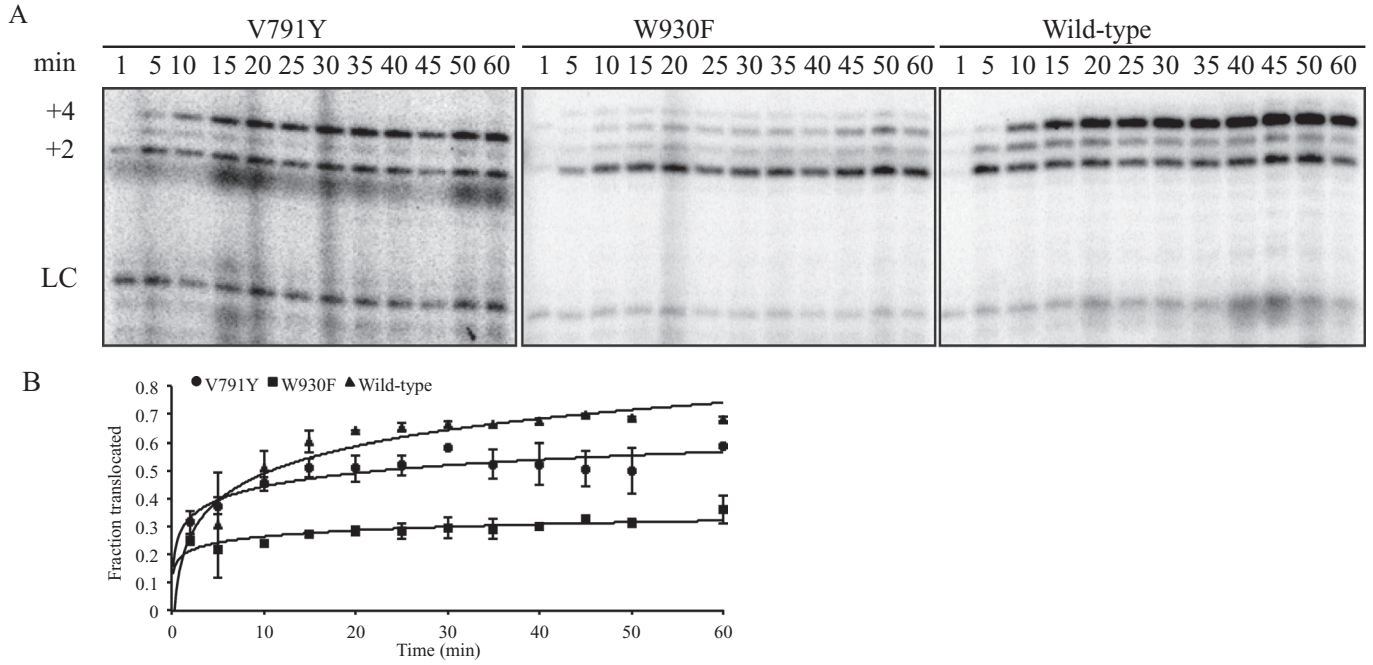

FIGURE 5: hTERT-V791Y and hTERT-W930F display reduced levels of translocation efficiency compared with wild-type enzyme. (A) Translocation time-course assay of hTERT-V791Y, hTERT-W930F, or WT telomerases reconstituted in 293 cells. +2 and +4 bands are indicated. LC, loading control. Boxes indicate the presence of data from experiments performed at the same time but from different gels. (B) The fraction of hTERT-V791Y, hTERT-W930F, or WT telomerase translocated at each time point was quantified and plotted. $n = 2$. Quantification is always performed using a wild-type enzyme assessed at the same time as the mutant enzymes and from reactions run concurrently.

onto the 3′ end of the DNA primer. A unique biochemical attribute of vertebrate and ciliate telomerase is RAP, by which hundreds of DNA repeats can be synthesized onto a given DNA primer without complete dissociation from the enzyme (Lue, 2004). Telomerase processivity relies on a "template translocation" mechanism to regenerate access to the RNA template after each repeat is synthesized. The processivity of the reaction is determined by the efficiency of RNA/DNA realignment over complete product release during translocation (Qi *et al.*, 2011). We identified two residues in hTERT—V791 within the IFD and W930 within the E motif—that both regulate processivity and DNA synthesis but influence telomere function and immortalization differently. Despite decreased levels of DNA synthesis and processivity reconstituted by both hTERT variants, only hTERT-W930F was able to elongate short telomeres and immortalize HA5 cells. HA5 cells expressing hTERT-V791Y underwent apoptosis after ~10 PDs. Of interest, despite hTERT-W930F's ability to maintain telomere function, it was also shown to possess lower RAP and translocation efficiency than hTERT-V791Y.

According to the high-resolution structural model of *T. castaneum* TERT, the N- and C-termini are postulated to come together to form a ring-like structure (Mitchell *et al.*, 2010). The IFD is located at the periphery of the N-terminus and RT domain, implying that this region plays an important role in the structural organization of TERT (Gillis *et al.*, 2008). The IFD is unique to telomerase and is not found in other RTs. Telomerase has evolved a more elaborate fingers domain than HIV-1 RT to optimize substrate interaction and mediate synthesis of multiple telomeric repeats (Lue *et al.*, 2003). Mutation of residues within Est2p's IFD indicates that this region is required for normal telomere maintenance in vivo and maximal telomerase activity in vitro. The quadruple Est2p LYID589AAAA mutant and corresponding hTERT VVIE790AAAA mutant manifest primer-specific defects, being selectively impaired in extending primers that form short hybrids with telomerase RNA (Lue *et al.*, 2003; Qi *et al.*, 2011). Furthermore, translocation efficiency of these mutant TERTs is severely affected, confirming that this region is also required for processivity (Lue *et al.*, 2003; Xie *et al.*, 2010; Qi *et al.*, 2011).

A number of inherited human diseases, including idiopathic pulmonary fibrosis, are linked to telomerase gene mutations, which result in telomere shortening–mediated stem cell defects (Alder *et al.*, 2011; Armanios and Blackburn, 2012). An ancestral mutation in hTERT, V867M-V791I, leading to decreased RAP, was previously shown to correlate with the development of idiopathic pulmonary fibrosis in members of two families (Alder *et al.*, 2011). Mutation carriers were shown to have telomere lengths below the 10th percentile of a normal distribution compared with age-matched controls. Defects in RAP were not evident for the single-substitution mutant hTERT-V791I in vitro. On the other hand, hTERT-V867M displayed processivity defects under conditions of low nucleotide concentrations, indicating that the development of shorter telomeres in double-mutation carriers was mainly due to the effects of V867M. Our results indicate that mutation at the same position, V791, but to a tyrosine results in decreased processivity. It is possible that hTERT-V791Y could not maintain telomeres or immortalize late-passage HA5 cells because these cells presumably have shorter telomeres, in accordance with the role of this region in telomerase function when the RNA–DNA hybrid is short (Xie *et al.*, 2010). However, the levels of processivity and DNA synthesis displayed by hTERT-V791Y were never lower than that displayed by hTERT-W930F, a mutant able to immortalize late-passage HA5 cells, indicating that RAP defects are not sufficient to explain apoptosis of HA5 cells expressing hTERT-V791Y. It is likely that this mutant suffers from defects in assembly or recruitment. A primary mechanism involved in the regulation of telomerase activity and processivity is through intracellular assembly and trafficking of the enzyme's components during S phase of the

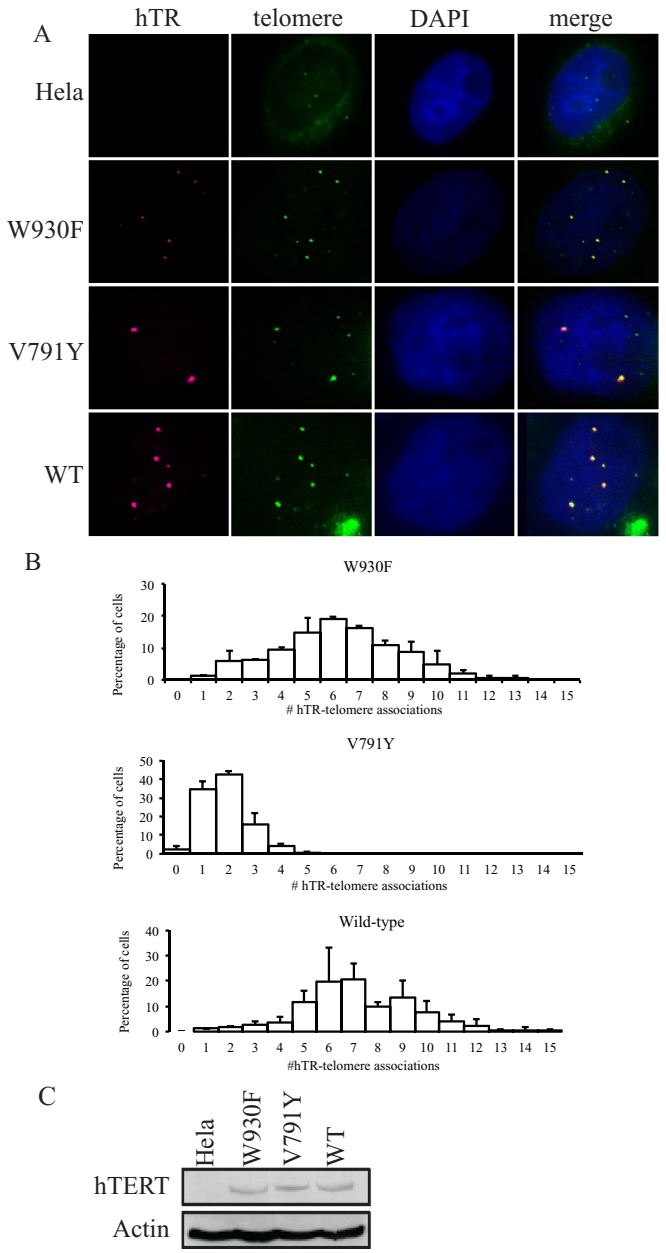

FIGURE 6: hTERT-V791Y is defective in recruitment to telomeres. (A) hTR (magenta foci) and telomere (green foci) FISH was performed on untransfected HeLa cells (top), coexpressing hTR and hTERT-W930F (second from top), hTERT-V791Y (third from top), or WT-hTERT (bottom). hTR colocalizations with telomeres are represented by yellow foci in the last column (merge). (B) Frequency of colocalization of hTR with telomeres. The number of cells in which the indicated number of colocalizations occurs is expressed as a percentage of the total cells counted, $n = 2$. Colocalizations were counted in ~200 cells per n. (C) Mutant hTERT-W930F and hTERT-V791Y and WT telomerase complexes were reconstituted in HeLa cells, and levels of hTERT expression were assessed by Western analysis and compared with levels of actin expression.

cell cycle (Jady *et al.*, 2006; Tomlinson *et al.*, 2006, 2008; Cristofari *et al.*, 2007; Stern *et al.*, 2012; Zhong *et al.*, 2012). When this mutant enzyme is expressed in HA5 cells, it is not able to extend telomeres, likely due to an inability to reach the chromosome ends. We speculate that telomeres in HA5 cells expressing hTERT-V791Y become fusogenic due to the presence of SFEs, eventually leading to apoptosis. These results also suggest that recruitment defects can sometimes be masked by activity or processivity defects, underscoring the importance of examining telomere recruitment in telomerase insufficiency diseases.

The E motif, commonly referred to as the primer grip region, is a universally conserved processivity determinant (Peng *et al.*, 2001). The structure of *T. castaneum* TERT also indicates that the E motif positions the 3′-end hydroxyl of the DNA primer at the active site of the enzyme for nucleotide addition and is in close proximity to the incoming nucleotides (Mitchell *et al.*, 2010). The four amino acids located at the C-terminal part of this motif in hTERT are W-C-G-L, whereas the corresponding sequence in Est2p is F-C-A-M (Figure 1A). A double-residue substitution that converts the yeast motif to one that imitates HIV-1 RT and hTERT's E motif exhibits increased processivity, longer telomeres, and normal growth (Peng *et al.*, 2001; Ji *et al.*, 2005). In contrast, a five-alanine substitution mutation in the hTERT primer grip sequence leads to a complete loss of telomerase activity and defects in binding short telomeric primers in vitro (Wyatt *et al.*, 2007), consistent with a role for this region in interacting with the DNA substrate. Mutation of the F to an A at position 720 in Est2p leads to shortened telomeres and has a detrimental effect on cell growth (Peng *et al.*, 2001). Like Est2p F720A, the hTERT-W930F enzyme displays decreased processivity and translocation efficiency, in addition to the presence of SFEs. However, growth of HA5 cells expressing hTERT-W930F was not significantly affected compared with cells expressing WT-hTERT.

Telomerase preferentially extends the shortest telomeres when telomerase is expressed in telomerase-negative cells (Ouellette *et al.*, 2000; Hemann *et al.*, 2001; Samper *et al.*, 2001; Britt-Compton *et al.*, 2009). We speculate that low levels of DNA synthesis and processivity and reduced translocation efficiency reconstituted by hTERT-W930F may lead to problems in telomere elongation that result in the generation of SFEs that we observe in early- and middle-passage HA5 cells expressing hTERT-W930F. Subsequently, hTERT-W930F is likely recruited to the shortest telomeres, observed as an absence of SFEs by late passage. The ability of hTERT-W930F to elongate short telomeres is probably sufficient to prevent telomere uncapping, genomic instability, and cell death associated with crisis, thus leading to the immortalization of these cells despite decreased levels of processivity and translocation efficiency. hTERT-V791Y is also deficient in translocation; however, its inability to be recruited to telomeres likely results in defective telomere extension, telomere uncapping, genomic instability, and apoptosis of HA5 cells.

Of importance, our results highlight that low levels of DNA synthesis and processivity displayed by the W930F enzyme are sufficient to maintain telomere function and impart immortalization to limited-lifespan cells. It has been speculated that a threshold level of enzyme activity, and perhaps processivity, is necessary to prevent telomere shortening, senescence, or crisis and that levels of telomerase activity and telomere length might correlate in human cancer cells, although such a correlation in cancer cells has been difficult to demonstrate (Autexier and Lue, 2006). Low levels of telomerase are detected in normal human somatic cells (Broccoli *et al.*, 1995; Masutomi *et al.*, 2003) but are insufficient to prevent telomere shortening. Bone marrow cells and peripheral blood leukocytes contain 0.2–40% of the specific TRAP activity of a HeLa cell reference extract (Broccoli *et al.*, 1995). By using TRAP assays to quantify telomerase activity, Hamad *et al.* (2002) showed that levels of telomerase activity >5% and <50% of WT enzyme were required for immortalization of limited-lifespan cells. BJ normal human diploid foreskin fibroblasts expressing hTERT were reported to stabilize their telomere

size at subsenescent average lengths with TRAP telomerase activity levels on the order of 1–5% of a control lung adenocarcinoma reference cell line H1299 (Ouellette *et al.*, 2000). Our study is the first to demonstrate, using a direct primer extension assay, that low levels (20% of WT telomerase) of DNA synthesis and extension of as few as three telomeric repeats are sufficient to maintain functional telomeres and immortalize limited-lifespan cells.

Of interest, the phenotype of W930F is reminiscent of mTERT$^{+/-}$ ES cell lines, which, despite short telomere lengths similar to the telomere lengths of mTERT$^{-/-}$ ES cell lines, do not display end-to-end fusions or genomic instability, suggesting that telomerase levels in heterozygote cells are sufficient to provide a protective advantage from end-to-end fusion and genome instability (Liu *et al.*, 2002). At late PD, HA5 cells expressing W930F could not maintain the average telomere length of 2.5–3.0 kb observed at early PD, and average telomere lengths were consistently below the average telomere length of HA5 cells expressing WT-hTERT (D'Souza *et al.*, 2013). However, the elongation of short telomeres by the W930F enzyme, observed by a decrease in SFEs (Figure 4B) and an increase in average telomere length from middle to late population doublings in clone F2 (Figure 4C), is sufficient to maintain functional telomeres.

We showed that telomerase processivity may affect telomere length maintenance, which is required for growth of cancer cells. A better understanding of processivity may facilitate the design of anticancer therapeutics that target domains regulating telomerase processivity. Genetic factors that regulate telomerase localization to the telomere may also be important determinants of telomere length regulation. Drugs that augment telomerase recruitment to telomeres could provide therapies for individuals diagnosed with telomerase insufficiency diseases.

MATERIALS AND METHODS

Plasmid construction

The pMSCV-puromycin-FLAG-hTERT plasmid was constructed by insertion of *Bgl*II- and *Eco*RI-digested PCR products encoding FLAG-hTERT into pMSCV-puromycin (kind gift from Gerardo Ferbeyre, Université de Montréal, Montréal, Canada). The plasmids pET28b-hTERT (Bachand and Autexier, 1999), pMSCV-puromycin-FLAG-hTERT, and pcDNA6/myc-His C-hTERT (Cristofari and Lingner, 2006; kind gift from Joachim Lingner, Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland) were used as templates to generate pET28b-hTERT-W930F and -V791Y, pMSCV-puromycin-FLAG-hTERT-W930F and -V791Y, and pcDNA6/myc-His C-hTERT-W930F and -V791Y 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

Retroviral and transient transfections were performed as previously described (D'Souza *et al.*, 2013). HA5 cells were a gift from Silvia Bacchetti, Istituto Regina Elena, Rome, Italy.

In vitro transcription and translation, SDS–PAGE, TRAP assay, and direct primer extension assays

Reconstitution of WT and telomerase variants, SDS–PAGE, TRAP assay, and direct primer extension were performed as described (D'Souza *et al.*, 2013).

Quantification of RAP

RAP was quantified as described (Hardy *et al.*, 2001; Moriarty *et al.*, 2004).

Protein analysis

Western blots were performed as described (D'Souza *et al.*, 2013).

Apoptosis analysis by FACS

HA5 cells expressing telomerase variants were grown to confluence in 10-cm plates, trypsinized, washed, and collected. As a positive control, HA5 cells treated with 1 µM staurosporine were also collected. Cells were treated with propidium iodide (Sigma-Aldrich, St. Louis, MO) and Annexin V–fluorescein isothiocyanate (BD Biosciences, San Diego, CA), incubated for 15 min, and passed through a FACSCalibur (BD Biosciences) at the Lady Davis Institute Flow Cytometry Facility. Results were analyzed using CellQuest Pro and compiled with Excel (Microsoft, Redmond, WA).

Telomere restriction fragment analysis

TRF analysis was performed as described (D'Souza *et al.*, 2013).

Fluorescence in situ hybridization

Metaphase spreads and Q-FISH analyses for the detection of SFE were performed as described (D'Souza *et al.*, 2013). Images were captured using an Axio Imager M1 (63×; Carl Zeiss, Jena, Germany). Quantitative analysis of SFE was performed with TFL-Telo, version 2.0, software (kindly provided by Peter Lansdorp, British Columbia Cancer Center, Vancouver, Canada). hTR-telomere FISH was performed using three different Cy3-conjugated hTR probes (Tomlinson *et al.*, 2008) and an Oregon Green–conjugated telomeric probe (Abreu *et al.*, 2010) on HeLa cells coexpressing supertelomerase or supertelomerase variants and hTR (Abreu *et al.*, 2011). Cy3 monoreactive dye was from GE Healthcare (Piscataway, NJ), Oregon Green 488 from Invitrogen, and probes from Operon (Huntsville, AL). Imaging was performed using an Axio Imager M1 (63×).

Translocation assay

Translocation assays and quantification were carried out as previously described, with minor modifications (Latrick and Cech, 2010; Qi *et al.*, 2011). Briefly, 30 µg of supertelomerase whole-cell extract was incubated with the telomerase substrate oligonucleotide 26GTT, an A5P chase primer to prevent telomerase from reinitiating on the same 26GTT primer, dATP, and radiolabeled dGTP (PerkinElmer, Waltham, MA). The assay was conducted at 4°C in the presence of 0.1 mM Mg^{2+} to slow the reaction. Samples were removed at various time points and stopped with 100 µl of 3.6 M ammonium acetate with 20 µg of glycogen (Invitrogen), ethanol precipitated at –80°C overnight, and electrophoresed on a 10% denaturing gel. The percentage of translocated product was calculated by dividing the +4 band by the total count (+2 and +4 band). The +3nt product is unrelated to template translocation (Qi *et al.*, 2011). The fraction of translocated products was then plotted versus time.

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