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Telomerase enzymatic component hTERT shortens long telomeres in human cells

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Telomere lengths are tightly regulated within a narrow range in normal human cells. Previous studies have extensively focused on how short telomeres are extended and have demonstrated that telomerase plays a central role in elongating short telomeres. However, much about the molecular mechanisms of regulating excessively long telomeres is unknown. In this report, we demonstrated that the telomerase enzymatic component, hTERT, plays a dual role in the regulation of telomere length. It shortens excessively long telomeres and elongates short telomeres simultaneously in one cell, maintaining the optimal telomere length at each chromosomal end for efficient protection. This novel hTERT-mediated telomere-shortening mechanism not only exists in cancer cells, but also in primary human cells. The hTERT-mediated telomere shortening requires hTERT's enzymatic activity, but the telomerase RNA component, hTR, is not involved in that process. We found that expression of hTERT increases telomeric circular DNA formation, suggesting that telomere homologous recombination is involved in the telomere-shortening process. We further demonstrated that shelterin protein TPP1 interacts with hTERT and recruits hTERT onto the telomeres, suggesting that TPP1 might be involved in regulation of telomere shortening. This study reveals a novel function of hTERT in telomere length regulation and adds a new element to the current molecular model of telomere length maintenance.

Introduction

Maintaining genome integrity is essential to normal cell function. For organisms with linear chromosomes, like humans, cells need to differentiate chromosome ends from double-stranded DNA breaks. This is achieved by the specialized nucleoprotein structures at the end of eukaryotic chromosomes, the telomeres.[1] Functional telomeres require the appropriate number of telomeric DNA repeats, as well as the proper binding and amount of telomere-associated proteins (shelterin proteins). Telomere length is ultimately determined by the balance between lengthening and shortening processes, indicating that complex regulatory pathways exist and serve to keep telomere lengths from becoming too long or too short. The best-known mechanism to lengthen short telomeres is the enzyme telomerase, a reverse transcriptase complex that contains hTERT (the reverse transcriptase) and hTR (the RNA template). Telomerase elongates telomeres by adding TTAGGG repeats to the ends of chromosomes.[2-4] The level of telomerase in normal human somatic tissues is insufficient to prevent telomere shortening. Telomeres can be lengthened through increasing telomerase activity by exogenous expression of hTERT or hTR. Cancer cells are known to acquire indefinite replicative capacity to escape from the normal proliferative limitations through maintaining their telomeres, either by upregulation of telomerase[5] or by an alternative lengthening of telomeres (ALT) mechanism.[6-8]

In contrast, very little is known about how the upper telomere length limit is maintained and how excessively long telomeres are shortened in human cells. Several mechanisms were known to shorten telomeres: gradual telomere attrition, which occurs as a result of end-replication limitations and nucleolytic degradation,[1,9] and rapid loss of over-lengthened telomeres by t-loop resolution, referred to as "telomere rapid deletion"[10] or "telomere trimming".[11,12] Telomere rapid deletion in yeast cells is a known RAD52-dependent mechanism that reduces over-elongated telomeres to the wild-type length. Telomere trimming was first reported in human cancer cells with artificially elongated telomeres by overexpression of hTR and was associated with elevated levels of extra-chromosomal telomere circles (t-circles).[11,12] T-circles have also been detected in ALT+ (telomerase-negative) cells[9] and in cells with abnormal telomere rapid deletion.[13] T-circle generation has been shown to involve homologous recombination proteins. However, key factors that contribute to the recognition and initiation of the removal of excessively long telomeres in human cells remain to be identified.

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In the present study, we revealed that hTERT plays a key role in shortening excessively long telomeres. We analyzed single telomere alteration at each chromosomal end and demonstrated that telomerase enzymatic component hTERT shortens excessively long telomeres and elongates short telomeres simultaneously in one cell. We found that this hTERT-mediated telomere-shortening process commonly exists in different types of human cells including normal primary human cells and cancer cell lines. The hTERT-mediated telomere shortening removes large segments of telomere DNA rapidly without inducing telomere dysfunctional foci or affecting cell proliferation; thus, it is mechanistically distinct from telomere rapid deletion.

Results

hTERT decreases the length of long telomeres and increases the length of short telomeres in ALT+ cancer cell lines

ALT+ cancer cells are characterized by having excessively long and heterogeneous telomere lengths across all chromosomal ends without telomerase activity[6-8]. To examine the effects of hTERT in cells with excessively long telomeres, a haemagglutinin (HA)-tagged hTERT was ectopically expressed in U2OS, an osteosarcoma cell line with high ALT activity (**Table 1**; **Fig. S1**). Telomere length (TL) at each of the chromosomal ends was measured by telomere fluorescent in situ hybridization (T-FISH) as previously described[14] and compared between hTERT-expressing cells and vector control cells. Dramatic decrease in the average telomere length (Avg_TL) per telomere was observed in U2OS–hTERT cells compared with that in U2OS–vector control cells (6237 vs. 2314, $P < 0.001$; **Table 1**; **Fig. 1A and B**; **Fig. S2A**). Significant decreases in the telomere length variation (TLV), defined as coefficient of variation (CV%) of all measured telomeres, were observed (76.9 vs. 61.1, $P < 0.001$; **Table 1**). The frequency of excessively long telomeres, defined as TL > 3× average TL for a given cell type, were also seen (average 34 per cell in vector control cells vs. 0.4 per cell in hTERT-expressing cells, $P < 0.001$). Correlation analysis revealed a strong inverse correlation between the lengths of TL at each chromosomal end in vector control cells and the percentage TL change at the corresponding chromosomal end of U2OS–hTERT cells ($r = -0.91$, $P < 0.001$; **Fig. S3A**). The most striking observation is the significant TL shortening at most of chromosomal ends in U2OS-hTERT cells compared with those in U2OS–vector control cells (**Fig. 1A and B**). No significant increases in TL were observed for any of the chromosomal ends. In addition, telomere restricted fragment (TRF) analysis verified these results, as there was a significant decrease in telomere length upon hTERT expression in U2OS cells (**Fig. S3B**). These data suggested that the dominant effect of hTERT expression in U2OS cells was shortening the excessively long telomeres.

We further examined specific chromosome ends possessing long or short TL. In U2OS cells, there is one normal X chromosome. Its long arm (Xq) has the shortest telomere length, while its short arm (Xp) has an excessively long telomere (**Fig. 1B**). We found that expression of hTERT significantly decreased the average TL on Xp (9825 vs. 3141, $P < 0.001$), while it had no significant effect on the TL of Xq (1480 vs. 1747, $P = 0.49$; **Table 1**; **Fig. 1A**

Table 1. Effect of hTERT overexpressing on telomere length

	Telomerase	Avg_TL		Avg_TL	
Cell line, passage #	Activity*	Overall	TLV	Chromosome specific	
				Xp	*Xq*
U2OS-vector, p3	1	6237 ± 968	76.9 ± 6.0	9825 ± 3931	1480 ± 1853
U2OS-hTERT, p3	6.5	2314 ± 710	61.1 ± 10.4	3141 ± 1408	1747 ± 1021
P value		***<0.001***	***<0.001***	***<0.001***	*0.49*
				11p	*11q*
SAOS2-vector, p4	1	1535 ± 305	130.5 ± 34.2	1404 ± 1257	20207 ± 7358
SAOS2-hTERT, p4	8.1	3236 ± 454	75.4 ± 6.9	2957 ± 2317	5168 ± 3121
P value		***<0.001***	***<0.001***	***<0.001***	***<0.001***
				10q	*19p*
IMR90- vector, p3	1	2067 ± 219	83.4 ± 9.3	2878 ± 2093	849 ± 611
IMR90- hTERT, p3	8	2586 ± 341	76.6 ± 6.9	2372 ± 1937	1440 ± 1490
P value		***<0.001***	***<0.001***	***0.030***	***<0.001***
				Xp	*21q*
WI38- vector, p3	1	1682 ± 295	99.7 ± 11.0	2392 ± 2396	898 ± 982
WI38-hTERT, p3	7.2	1781 ± 364	88.6 ± 10.5	1364 ± 1387	1196 ± 1274
P value		*0.26*	***<0.001***	***0.005***	***0.029***

[#]Passage after hTERT or empty vector transfection. *Telomerase activity measured by TRAP assay and expressed as fold change relative to vector control. Avg_TL is the average telomere length per telomere, expressed as fluorescent intensity unites. TLV, telomere length variation (CV%); mean ± SD were reported values in the table. *P* values were based on 2-sided Student t test.

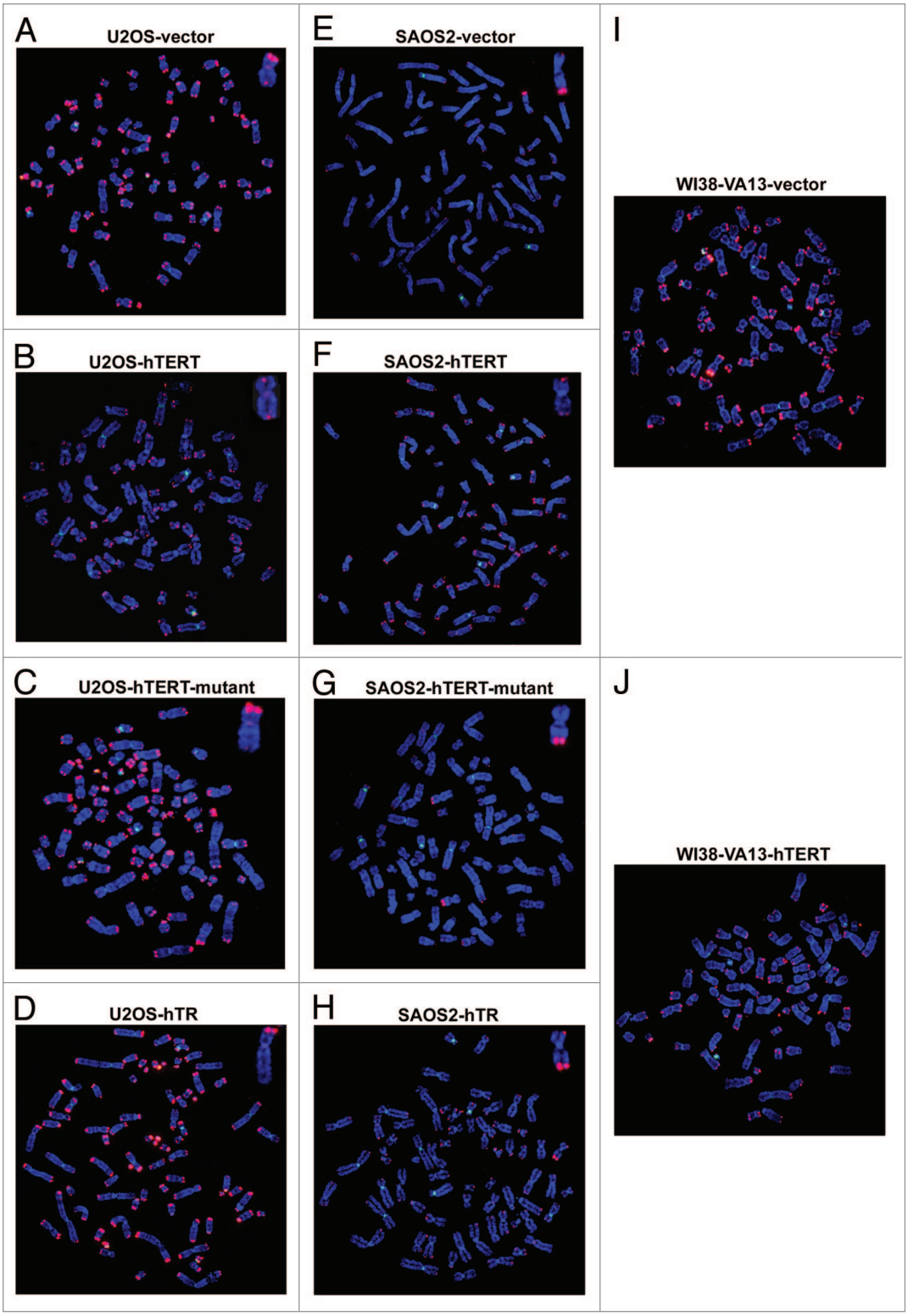

Figure 1. Expression of hTERT shortens long telomeres and elongates short telomeres in ALT+ cancer cells. Empty vector, WT or mutant hTERT, or hTR was expressed in ALT+ U2OS cells (**A–D**), SAOS2 cells (**E–H**), or a hTR-negative cell line WI38-VA13 (**I and J**). Metaphase spreads were prepared and hybridized with a Cy3 (red) telomere PNA probe and FITC (green) label chromosome-specific centromere probes. Thirty cells were analyzed per cell line, and representative metaphase images were displayed. (**A**) U2OS-vector, (**B**) U2OS-hTERT, (**C**) U2OS-mutant-hTERT, (**D**) U2OS-hTR; Amplified chromosome X was showed in the upper-right corner. (**E**) SAOS2-vector, (**F**) SAOS2-hTERT, (**G**) SAOS2-mutant-hTERT, (**H**) SAOS2-hTR; amplified chromosome 11 is shown in the upper right corner. (**I**) WI-38-VA13 vector, and (**J**) WI-38-VA13-hTERT.

and B), indicating that hTERT selectively shortens long telomeres in U2OS cells.

hTERT-mediated shortening of excessively long telomeres was confirmed in another ALT+ cancer cell line, SAOS2. This cell line has low ALT activity and is characterized by having short TL at the most chromosomal ends and one excessively long telomere on the long arm (q) of one of chromosomes 11 (**Fig. 1E**). Expressing hTERT in SAOS2 induced a significant decrease in TLV and increase in the average TL per telomere (**Table 1**; **Fig. S2B**). Correlation analysis revealed a strong inverse correlation between the lengths of TL at each chromosomal end in vector control cells and the percentage TL change at the corresponding chromosomal end of SAOS2–hTERT cells ($r = -0.84$, $P < 0.001$; **Fig. S3C**). Most importantly, we found that expression of hTERT significantly shortened TL on 11q (–56%) and elongated TL on 11p (+248%; **Table 1**; **Fig. 1E and F**), confirming that hTERT selectively shortens long telomeres and elongates short telomeres in ALT+ cancer cells. Moreover, indirect immunofluorescence and telomere FISH displayed the localization of hTERT at those telomeres with high signal intensity, suggesting that hTERT can be recruited to long telomeres (**Fig. S4**).

Together, these data indicate that hTERT plays dual roles in telomere length regulation by

shortening excessively long telomeres and elongating short telomeres, thus maintaining optimal telomere length at each of the chromosomal ends for efficient protection. The data also indicate that the switch between the 2 opposite hTERT functions is dictated by the length of the telomere at a specific chromosomal end.

Expression of hTERT in human primary fibroblasts shortens long telomeres and elongates short telomeres

Wild-type hTERT was overexpressed in IMR90 (primary human fibroblasts) at population doubling (PD) 10, and increased telomerase activity and hTERT expression were confirmed by the qRT-PCR, western blot analysis and the TRAP assay (**Table 1**; **Fig. S1**). Cells were harvested at passage 3 after hTERT transfection, following a standard cytogenetic protocol for chromosome preparation. The average TL per telomere was significantly increased in hTERT-expressing cells compared with that in vector control cells (**Table 1**; **Fig. 2A–C**). We also noticed a significant decrease in TLV across all chromosomal ends (**Table 1**) and a significant decrease in the frequency of excessively long telomeres (average 2.6 per cell in vector controls vs. 1.6 per cell in hTERT-expressing cells, $P = 0.005$). This observation prompted us to examine if changes in TL at each chromosomal end in hTERT-expressing cells were correlated with the TL of the corresponding chromosomal end in vector control cells. Surprisingly, we found a strong inverse correlation between the TL in vector control cells and the percentage TL change in hTERT-expressing cells (Pearson correlation coefficient [r] = -0.80, $P < 0.001$; **Fig. 2A**). More importantly, the data indicated that telomere length at the chromosomal end possessing the longest telomere (10 q) was significantly shortened in hTERT-expressing cells compared with that in control cells (-18%, $P = 0.03$; **Table 1**), while telomere length at the chromosomal end possessing the shortest telomere (19p) showed significant elongation in hTERT-expressing cells compared with that in controls (+70%, $P < 0.001$; **Table 1**). In IMR90 cells, 6 out of 46 (13%) chromosomal ends showed shortened average TL in IMR90-hTERT cells (**Fig. 2A**).

Similar results were obtained with another primary human fibroblast cells (WI38), showing significant decrease in TLV across all chromosomal ends (**Table 1**; **Fig. 2D–F**) in hTERT-expressing cells compared with control cells and strong inverse correlation between the lengths of TL at each chromosomal end in vector control cells and percentage TL change at the corresponding chromosomal end of hTERT-expressing cells (r = -0.66, $P < 0.001$; **Fig. 2D**). A significant decrease (–43%) in average TL at Xp (longest telomere in WI38) and an increase (+51%) in average TL at 21q (the shortest telomere in WI38) were also observed (**Table 1**) upon expressing hTERT. Fifteen out of 46 (33%) chromosomal ends showed shortened average telomere lengths in WI38-hTERT cells (**Fig. 2D**). These data suggested that hTERT plays a dual role in the regulation of telomere length by shortening the long telomeres and extending the short telomeres in normal human fibroblasts.

Upregulation of endogenous hTERT maintains small TLV in primary epithelia cells

Fibroblast feeder cells, in combination with a Rho kinase inhibitor, have been shown to immortalize human primary epithelia cells that retained a normal karyotype and remained

Table 2. Effect of endogenous hTERT on telomere length in primary and cancer cells

Cell-PD	hTERT mRNA fold change*	Avg_TL	TLV	% of telomeres per cell	
		overall		short	long
Prostate epithelial cells					
GUMC001-PD6	9.4	1879 ± 255	84.9 ± 9.5	10.2 ± 4.1	3.0 ± 2.1
GUMC001-PD24	9.0	2130 ± 298^	77.2 ± 10.1^	7.0 ± 3.6^	2.2 ± 1.9
GUMC001-PD48	9.0	1887 ± 283	86.0 ± 7.5	8.0 ± 4.0^	3.3 ± 2.4
Fibroblast cells					
IMR90-PD10	1.0	2067 ± 219	83.4 ± 9.3	7.17 ± 3.2	2.6 ± 1.3
IMR90-PD20	1.0	1580 ± 255^	100.4 ± 10.9^	14.3 ± 4.9^	4.9 ± 3.1^
IMR90-PD42	0.8	1320 ± 262^	112.0 ± 12.4^	21.6 ± 6.1^	6.2 ± 3.2^
IMR90-PD60†	0.9	1203 ± 242^	124.8 ± 23.2^	19.8 ± 6.3^	7.8 ± 4.8^
Telomerase + cancer cell lines					
MDA231-vector	10	1088 ± 201	79.9 ± 8.0	9.4 ± 3.7	2.1 ± 1.4
MDA231+sh-hTERT	3	813 ± 263	88.2 ± 10.7	12.7 ± 5.3	3.9 ± 4.6
P value		*<0.001*	*0.002*	*0.01*	*0.05*
HT1080-vector	10	688 ± 164	93.6 ± 10.5	16.5 ± 6.9	4.4 ± 3.9
HT1080+sh-hTERT	2	525 ± 146	113.6 ± 9.1	20.2 ± 5.6	7.2 ± 4.6
P value		*<0.001*	*<0.001*	*0.03*	*0.02*

*Human primary karatinocytes was used as a reference to calculate hTERT mRNA fold change. Avg_TL is the average telomere length in fluorescence intensity units. TLV, telomere length variation (CV%); mean ± SD were reported values in the table. ^Significantly different from earliest passage cells ($P < 0.05$). †Pre-senescent cell, only 14 metaphase cells were available for analysis. Short, very short telomeres, TL < 10% of the average TL. Long, very long telomeres, TL > 3 times of the average TL.

non-tumorigenic.[15] This culture condition dramatically upregulates endogenous hTERT expression level (8- to 14-fold increase) and telomerase activity.[15] Under the feeder layer co-culture condition, GUMC001 cells (human primary prostate epithelia cells) maintained relatively small and constant TLV and no significant change in average telomere length per telomere from PD6 to PD48, whereas the number of excessively long telomeres remained stably low, and the number of very short telomeres decreased from PD6 to PD48 (**Table 2**). The observed small TLV and low number of excessively long and very short telomeres in GUMC001 cells are comparable with what was observed in hTERT-expressing IMR90 cells (**Table 1**). In contrast, IMR90

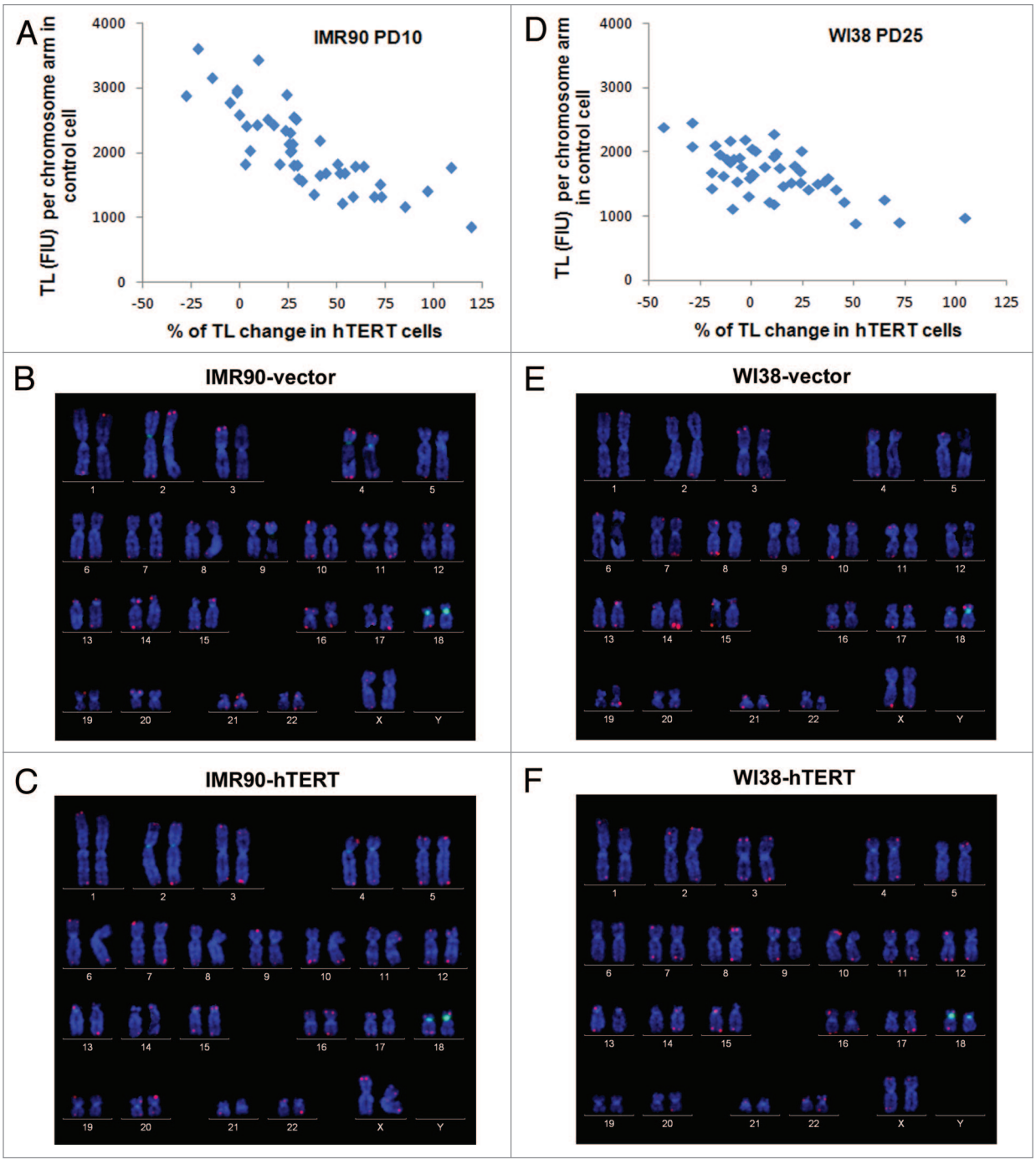

Figure 2. Expression of hTERT shortens long telomeres and elongates short telomeres in human primary fibroblasts. Expression of hTERT in human primary fibroblasts IMR90 and WI38 shortened long telomeres and elongated short telomeres. Metaphase spreads were prepared and hybridized with a Cy3 (red) telomere PNA probe and FITC (green) label chromosome specific centromere probes. **Figure 1A and D** show correlations between the average TL at specific chromosomal ends in vector control cells and percents of TL change at the corresponding chromosomal ends in hTERT-overexpressing cells. Each dot represents a chromosomal end. Thirty cells were analyzed per cell line. Representative metaphase images were displayed. (**B**) IMR90-vector, (**C**) IMR90-hTERT, (**E**) WI38-vector, (**F**) WI38-hTERT.

(human primary fibroblast cells) cultured in conventional condition resulted in rapid loss of telomere length and significantly increased TLV from PD10 to PD60. The number of excessively long telomeres also showed a significant trend of increase (*P* for trend < 0.001, **Table 2**). IMR90 cells grown in conventional cell culture condition typically have very low levels of hTERT expression and telomerase activity (**Table 2**). These data suggest that upregulation of endogenous hTERT in prostate epithelial cells may have shortened excessively long telomeres and elongated short telomeres to maintain a small TLV, which is consistent with the results obtained by ectopically overexpressing hTERT in human primary fibroblast cells. Because GUMC001 does not grow in conventional cell culture conditions without feeder layer, we could not obtain chromosome preparations without hTERT upregulation to show a direct comparison.

hTERT knockdown in telomerase-positive cancer cells decreases the telomere length

To investigate the effects of downregulation of hTERT on telomere length in telomerase-positive cancer cells, we used siRNA to knockdown hTERT in MDA231, a breast cancer cell line. hTERT knockdown efficiency was confirmed by TRAP assay and qRT-PCR. Downregulation of hTERT significantly decreased average telomere length per telomere and increased TLV in MDA231 cells. The increase in TLV is accompanied by increases in the frequency of very short telomeres and excessively long telomeres (**Table 2**). Similar results were obtained for another telomerase-positive fibrosarcoma cell line, HT1080 (**Table 2**). Our data indicates that knockdown hTERT in telomerase-positive cancer cell lines affects telomere elongation and results in shorter telomeres across all chromosomal ends.

The enzymatic domain of hTERT is required for hTERT-mediated telomere shortening

To investigate the role of telomerase activity in hTERT-mediated telomere shortening, we used a haemagglutinin (HA)-tagged hTERT mutant construct in which the aspartic acid and valine residues at positions 710 and 711 in the third reverse transcriptase motif of hTERT were substituted with alanine and isoleucine, respectively.[16] This mutant construct lacks telomerase activity (enzymatically dead) and has a dominate-negative effect on telomere elongation. Consistent with previous reports,[16,17] no telomerase activity was detected in HA immunoprecipitates after the mutant construct was expressed (**Fig. S1**). Expressing this mutant hTERT in U2OS cells did not affect average TL per telomere and TLV (**Table 3**; **Fig. 1C**). We observed no significant change in TL on both Xp (long telomere) and Xq (short telomere; **Table 3**; **Fig. 1C**). These data suggest that the catalytic domain of hTERT is required for shortening the excessively long telomeres.

Expressing the mutant hTERT in SAOS2 cells induced a significant decrease in average TL per telomere and increase in TLV. The increase in TLV in cells expressing the mutant hTERT was primarily driven by significant increases in the frequency of very short telomeres (average 10.6 per cell in vector control cells

Table 3. Effect of expressing hTERT-mutant, hTR and hTERT on telomere length

Cell line, passage #	Telomerase activity*	RNA fold change*	Avg_TL overall	TLV	Avg_TL chromosome specific	
					Xp	Xq
U2OS-vector, p4	1	1	7779 ± 1348	73.0 ± 7.3	11379 ± 5128	3573 ± 4859
U2OS-hTERT-Mutant, p4	1	8	7459 ± 1529	72.1 ± 7.2	12140 ± 7725	3199 ± 4292
P value			*0.45*	*0.52*	*0.58*	*0.76*
					11p	11q
SAOS2-vector, p4	1	1	1175 ± 224	111.0 ± 25.0	1122 ± 1191	11051 ± 5585
SAOS2-hTERT-Mutant, p4	1	9	820 ± 196	179.8 ± 60.0	533 ± 478	14296 ± 7476
P value			***<0.001***	***<0.001***	***0.016***	*0.06*
					Xp	Xq
U2OS-vector, p4	1	1	5313 ± 751	83.5 ± 6.0	6872 ± 3812	553 ± 675
U2OS-hTR, p4	4	8	6454 ± 383	79.5 ± 10.5	8006 ± 5521	2967 ± 3720
P value			***<0.001***	***0.08***	0.38	***0.002***
					11p	11q
SAOS2-vector, p4	1	1	1531 ± 250	102.6 ± 19.6	1719 ± 1385	12809 ± 5405
SAOS2-hTR, p4	3	7	1356 ± 298	109.3 ± 23.0	1785 ± 2248	11138 ± 5664
P value			***0.02***	*0.24*	*0.89*	*0.25*
WI-38 VA13-vector, p4	1	1	5841 ± 1955	90.1 ± 11.2	ND	ND
WI-38 VA13-hTERT, p4	6	7	3407 ± 1169	96.2 ± 7.5	ND	ND
P value			***<0.001***	***0.017***		

[#]Passage after hTERT-mutant or hTR or hTERT or empty vector transfection. *Telomerase activity and hTR mRNA fold change relative to vector control. Avg_TL is the average telomere length in fluorescence intensity units. TLV, telomere length variation (CV%). Mean ± SD were reported values in the table.

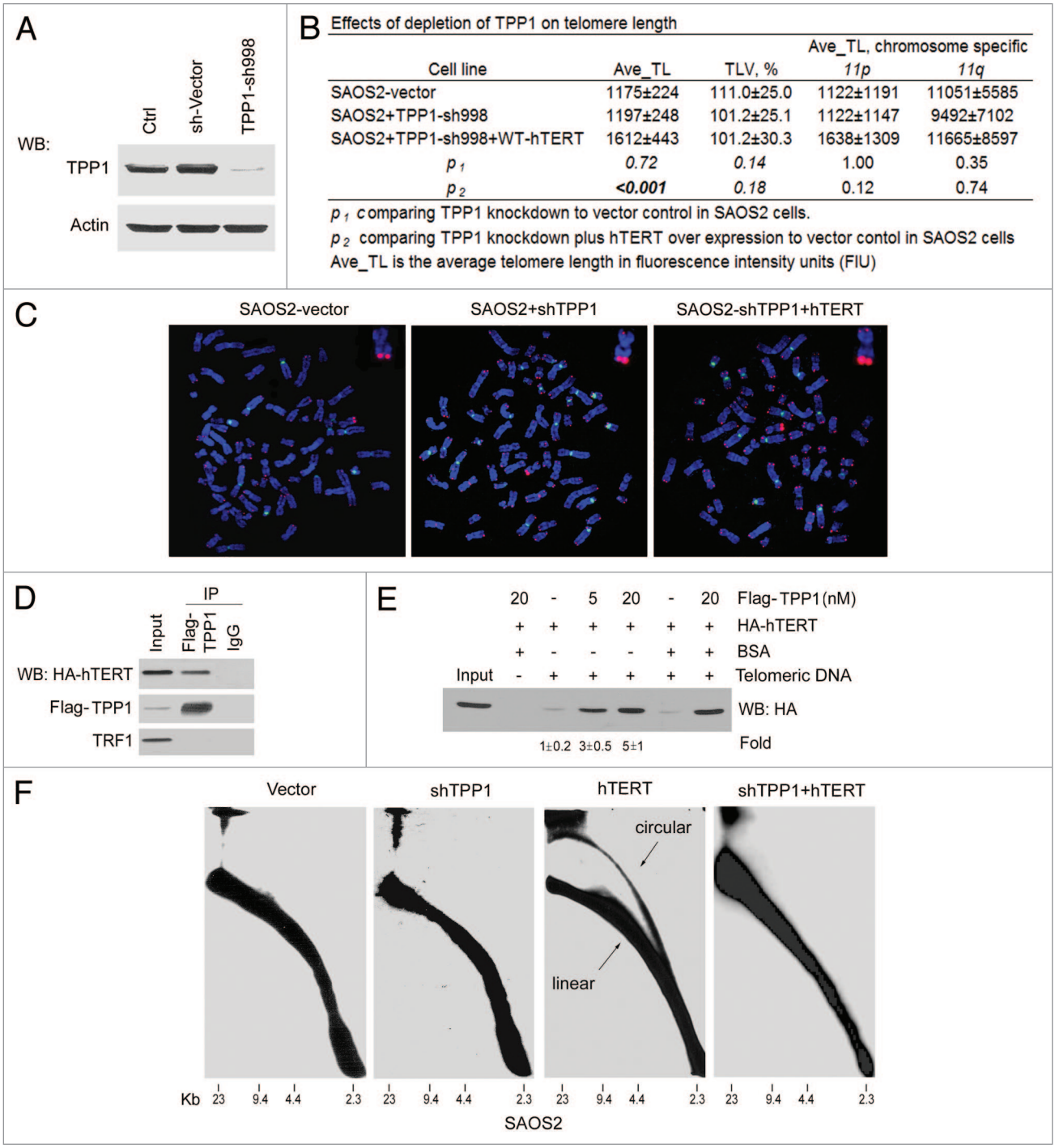

Figure 3. TPP1 physically and functionally interacts with hTERT to regulate telomere length. (**A**) Depletion of TPP1 by shRNA. Lentiviral empty vector or TPP1 shRNAs were expressed in SAOS2 cells. Protein expression was analyzed by western blotting with indicated antibodies. (**B**) Effects of depletion of TPP1 on telomere length. hTERT was expressed in ALT+ SAOS2 cells with or without expression of TPP1 shRNA. Thirty cells were analyzed per cell line. (**C**) Representative metaphase images of SAOS2 cell with or without shTPP1 and/or hTERT expression. Amplified chromosome 11 was shown in the upper right corner. (**D**) TPP1 binds to hTERT in SAOS2 cells by coimmunoprecipitation. Nuclear extracts (0.5 mg) were treated with DNase I and then subjected to immunoprecipitation with the anti-Flag (TPP1) antibody, followed by western blotting with indicated antibodies. IgG was used as a control. Fifty micrograms of nuclear extract was loaded as input. (**E**) Recombinant TPP1 recruits hTERT binding to the telomere DNA substrate. The biotin-D-loop DNA was incubated with in vitro-translated HA-hTERT in the absence or presence of various amounts of recombinant TPP1 (5 and 20 nmol). The biotinylated DNA substrate and its bound proteins on the beads were analyzed by SDS-PAGE gel. Quantitative data is from 3 independent experiments (mean ± S.D.). Bovine serum albumin did not affect TPP1-enhanced hTERT binding to the D-loop substrate. HA-hTERT was detected by WB with the anti-HA antibody. Input, 50% of the total HA-hTERT used in each reaction. (**F**) hTERT generates t-circle formation, and TPP1 is required for hTERT-mediated t-circles. Empty vector and hTERT were expressed in ALT+ SAOS2 cells with or without expression of shTPP1, and 2D assay was performed to detect t-circle formation.

vs. 16.3 per cell in hTERT-mutant expressing cells, $P < 0.001$) (**Table 3**). While short telomeres (i.e., 11p) had significantly decreased TL (–52%, $P = 0.02$), in contrast, excessively long telomere on 11q had slightly increased TL (+29%, **Table 3**; **Fig. 1G**), confirming that this mutant hTERT failed to shorten excessively long telomeres. Together, our data indicated that the enzymatic domain is required for hTERT-mediated telomere shortening.

hTR is not required for hTERT-mediated telomere shortening

Previous reports showed that overexpression of hTR increased telomerase activity and elongated telomere length.[11] To examine the effect of hTR on telomere shortening, we expressed hTR in U2OS and SAOS2 cells. The function of hTR construct was first tested by expressing it in HT1080, and hTR expression resulted in 8- and 6-fold increase in hTR mRNA level and telomerase activity, respectively, consistent with previous reports.[11,12]

Expressing hTR in U2OS cells significantly increased average TL per telomere and borderline significantly decreased TLV, due to a decrease in the frequency of very short telomeres. Although hTR expression significantly increased telomere length on short telomeres (Xq, +436%), it had no significant effect on the length of the long telomeres on Xp (**Table 3**; **Fig. 1D**). Expressing hTR in SAOS2 cells did not shorten the telomere length on chromosome 11q (**Table 3**; **Fig. 1H**), although hTR mRNA levels increased 6–8-fold (**Fig. S1**). These data indicate that overexpression of hTR alone does not shorten long telomeric DNA.

To further confirm whether hTR is required for hTERT-mediated telomere shortening, we expressed wide-type hTERT in a hTR-negative cancer cell line, WI38 VA13. T-FISH analysis revealed that expression hTERT dramatically shortened average telomere length per telomere and decreased frequency of excessively long telomeres from an average of 20 per cell in vector controls cells to 6 per cell in hTERT expression cells ($P < 0.001$, **Table 3**, **Fig. 1I and J**), indicating that hTR is not required in hTERT-mediated telomere shortening.

TPP1 is required for hTERT-mediated telomere shortening

TPP1 is a shelterin protein and interacts with telomerase for telomere elongation.[18-23] We examined its role on hTERT-mediated telomere shortening. TPP1 knockdown did not affect average TL in SAOS2 ($P = 0.72$) and had no significant effect on the length of very long telomeres on 11q (**Fig. 3A–C**). In TPP1-depleted SAOS2 cells, overexpressing hTERT significantly increased average TL per telomere ($P < 0.001$) and had no significant effect on TLV ($P = 0.14$). Analysis of chromosome-specific TL revealed that expression of hTERT did not shorten long telomeres on 11q in TPP1-depleted SAOS2 cells (**Fig. 3B and C**), suggesting that hTERT-mediated telomere shortening requires TPP1.

Using immunoprecipitation assays, we found that Flag-TPP1 co-precipitated with HA-hTERT (**Fig. 3D**). Treatment with DNase-I did not prevent the TPP1–hTERT interaction, excluding bridging effects of nucleic acids. We then tested whether recombinant TPP1 regulates the binding of HA-hTERT to telomere DNA. Immunoblot analysis with anti-HA antibody showed that, in the absence of TPP1, HA-hTERT was associated with biotinylated-telomeric DNA substrates that were retrieved by streptavidin beads (**Fig. 3E**). The addition of TPP1 significantly increased the binding of HA-hTERT to the telomeric DNA substrates in a dose-dependent manner (**Fig. 3E**). Bovine serum albumin did not affect the telomere DNA binding activity of HA-hTERT. These data indicate that TPP1 dramatically enhances hTERT binding to the telomeric DNA substrates.

hTERT-mediated telomere shortening increases t-circle formation

T-circle formation has been shown to involve the telomere recombination mechanism,[13,24-26] and the increase in t-circles in ALT+ cells is considered a by-product of removed long telomeres.[11] Expression of hTERT in SAOS2 cells increased t-circles as measured by the 2D gel assay (**Fig. 3F**). The increase in hTERT-induced t-circles was also confirmed in primary human fibroblasts (IMR90) by overexpression of hTERT (data not shown). Further, we found that TPP1 depletion prevented hTERT-induced t-circle formation in SOAS2 cells (**Fig. 3F**), consistent with our T-FISH findings that hTERT overexpression failed to shorten long telomeres on 11q in TPP1-depleted SAOS2 cells (**Fig. 3B and C**). Together, these data suggest that telomere recombination is likely involved in hTERT-mediated telomere shortening, and TPP1 might regulate the hTERT loading on telomeres for telomere recombination during the hTERT-mediated telomere-shortening process.

hTERT-mediated telomere shortening does not induce telomere signal-free ends and telomere dysfunction

We found that overexpression of hTERT did not increase the number of telomere signal-free chromosomal ends by T-FISH analysis, nor did it induce telomere dysfunctional foci formation (**Fig. S5**), which is consistent with previous reports.[24,25] In fact, expression of hTERT reduced the number of telomere signal-free ends in all the cell lines tested. Our observations are in agreement with previous reports that overexpression of hTERT in human primary cells leads to continued cell growth and extended life span in the absence of a DNA damage response.[1,27-29] These data suggest that hTERT-mediated telomere shortening does not produce telomere-free chromosomal ends and is different from aberrant rapid telomere deletion, which triggers a DNA damage response and ultimately initiates cell cycle arrest.

Discussion

In the present study, we demonstrated that telomerase enzymatic component hTERT plays an important role in maintaining the upper limit of telomere length by shortening excessively long telomeres in human primary and cancer cells. Our results revealed a novel mechanism, which involves hTERT, possibly TPP1, and telomere homologous recombination (HR) to shorten the excessively long telomeres. hTERT-mediated telomere shortening induces t-circle formation that has been show to involve telomere HR process. Telomere HR has been demonstrated to takes place during the G_2/M phase of the cell cycle, after DNA synthesis.[9] Thus hTERT-mediated telomere shortening is likely to occur during G_2/M phase of cell cycle (post-telomere synthesis). Additionally, hTERT-mediated telomere shortening does not require the telomerase RNA component, hTR, indicating

that the role of hTERT in telomere shortening is mechanistically distinct from its involvement in telomere elongation. We further demonstrated that telomere shelterin protein, TPP1, is required for hTERT-mediated t-circle formation and telomere shortening, supporting the TPP1-hTERT-telomere HR-based model to shorten long telomeres in human cells.

Telomere length in normal human cells is tightly regulated within a narrow range, and its regulatory system is complex. The best-known mechanism to counteract telomere attrition is the enzyme telomerase, a reverse transcriptase complex that contains hTERT and hTR. Telomerase elongates telomeres by adding TTAGGG repeats to the ends of chromosomes.[2-4] In contrast, very little is known about how cells maintain the upper limit of telomere length. Our data indicate that hTERT plays a central role in maintaining the upper limit of telomere length by shortening the excessively long telomeres in human cells. hTERT regulates telomere length homeostasis by shortening long telomeres and elongating short telomeres, and these 2 opposing but biologically interactive functions appear to be regulated by local factors, such as telomere length at a specific chromosomal end, and are likely to involve telomere length counting as illustrated in budding yeast, in which Rif1 and Rif2 were proposed molecules to be counted for length determination, and Rap1, a telomere binding protein, recruits Rif proteins to the telomeric track.[30,31] The mechanism underlying the telomere length counting in human cells is largely unknown. Our data indicated that TPP1, a member of shelterin protein complex, is required in the hTERT-mediated telomere-shortening process. It is possible that shelterin protein binding initiates telomere length "counting" in human cells. TPP1 recruits hTERT onto telomeres to recognize the length set-point for shortening, or the TPP1–hTERT complex may act as effectors to recruit HR proteins, which carry out the reaction to remove excessive telomeric DNA at the long telomeres, and this process generates t-circles. Our observation that expressing hTERT in human fibroblasts and ALT+ cancer cells significantly increased amount of t-circles suggests that HR is involved in hTERT-mediated telomere shortening. HR-mediated resolution of t-loops can result in loss of telomeric DNA equivalent to the length of the t-loop and can generate extrachromosomal t-circles.[13] This process depends on the HR proteins XRCC3 and MRN complex, which have been implicated in resolvase activity. ATM plays a critical role in maintenance of telomeres and may also be involved in this telomere metabolism process.[32-35] T-circles are prevalent in cells utilizing the ALT mechanism and are also found in telomerase-positive cancer cell lines following progressive telomere lengthening by exogenous telomerase activity.[11]

hTERT-mediated telomere shortening might require endonucleases. A recent paper has shown that SLX4 assembles with a telomere maintenance toolkit by bridging multiple endonucleases with telomeres.[36] SLX4 interacts with endonucleases SLX1, XPF, and MUS81 and also interacts with telomeric protein TRF2. It negatively regulates telomere length through SLX1-catalyzed nucleolytic resolution of telomeric DNA structures. However, whether SLX4 protein complex is involved in hTERT-mediated telomere shortening remains to be illustrated. Another candidate endonuclease that could play a role in hTERT-mediated telomere shortening is the TERRA complex. TERRA promotes telomere shortening through exonuclease 1-meidated resection of chromosome ends.[37] Other human telomerase-associated nucleases might also associate with hTERT-mediated telomere shortening.[24,25,38] Future studies will determine whether these factors are required for hTERT-mediated telomere-shortening process.

Our data indicated that the enzymatic domain of hTERT is needed for hTERT-mediated telomere shortening. But depletion of hTR did not affect hTERT-mediated telomere shortening, suggesting that direct hTERT reverse transcriptase function may not be required for the telomere-shortening process. Recruitment of hTERT onto telomeres depends on its binding partners and telomeric DNA status.[34,39,40] It is possible that the enzymatic domain of hTERT is required for protein–protein interactions involved in one or multiple steps in hTERT-mediated telomere shortening, such as hTERT recruitment onto telomeric DNA, an initiated sensor for telomere length "counting" and/or regulation of endonucleases. The mutation in the enzymatic domain of hTERT may have altered its protein surface structure to lose the binding ability. A recent paper reported that a translocation-defective telomerase with low levels of activity and processivity stabilizes short telomeres and confers immortalization.[41] These data support the notion that the different hTERT functional domains could associate with hTERT-mediated telomere length shortening.

Gradual telomere attrition and telomere trimming have been implicated in telomere length shortening.[2-4,10-12] The hTERT-mediated telomere-shortening process is distinct from telomere attrition. It is well known that telomere attrition is the result of the end-replication problem.[42-44] Telomere attrition, induced by successive cell division, occurs on all telomeres, including very short telomeres and leads to loss of telomere protection that triggers a DNA damage response and ultimately induces cell cycle arrest.[10] In contrast, hTERT-mediated telomere shortening only shortens excessively long telomeres and does not trigger a DNA damage response. It removes excessive telomeric DNA to maintain the telomere length equilibrium (near the average telomere length) at the processed chromosomal ends. It is likely that hTERT-mediated telomere shortening is part of the routine telomere post-synthesis processing and critical to maintain upper telomere length limit in human cells, since it exists in several human primary cells and cancer cell lines.

Telomere trimming induced by overexpression of hTR is the HR-mediated resolution of the terminal t-loop structure.[11,12] hTERT-mediated telomere shortening may share the same processing factors to generate t-circles as telomere trimming. It is currently unknown whether hTERT participates in the initiation of "telomere trimming", since the entire process of "telomere trimming" is not well defined, and the factor(s) that triggers the "telomere trimming" remains to be identified. Our data indicates that hTR is not required for hTERT-mediated telomere shortening, suggesting that the mechanism may be different from "telomere trimming". In addition, hTERT-mediated telomere shortening significantly decreases telomere length variation across all chromosomal ends, while telomere trimming induces long and heterogeneous telomere lengths in the cells.[11,12]

Most human somatic cells have little or no telomerase to prevent telomere loss; in such a situation, successive cell divisions lead to progressive telomere attrition due to the end-replication problem.[42-44] Critically short telomeres trigger cellular growth arrest that drives progressive atrophy and functional decline in high-turnover tissues.[45,46] Thus, progressive telomere loss was proposed to be the basis of cellular senescence and aging.[47] On the other hand, telomere-associated replicative senescence is regarded as an important tumor-suppressive mechanism.[48] While the detrimental effects of short telomeres on human health have been intensively studied, very little is known about the health consequences of excessively long telomeres. If only short telomeres are deleterious to cells, why aren't telomere lengths in human cells longer in order to buffer the telomere attrition, and why do telomerase-positive cancer cells predominantly maintain substantially shorter telomeres?[49-51] Perhaps excessively long telomeres are simply more costly to maintain, leading to reduced fitness in cell production to replenish high-turnover tissues and affecting human longevity. Excessively long telomeres may cause disruption of proper telomere-capping functions, including t-loop formation and associated telomere-binding proteins, leading to harmful consequences such as chromosome instability. Excessively long telomeres are vulnerable to replication slippage, stalled replication forks, or the formation of secondary structures that disturb normal cell cycle progression. In these regards, shortening excessively long telomeres is likely to be equally important as elongating very short telomeres for proper cell proliferation and efficient cell production.

Given the importance of telomere biology in cancer and other aging-associated diseases,[52-55] targeted manipulation of telomere maintenance mechanisms not only provides a promising strategy for treating cancer and aging-associated diseases, but, in certain circumstances, may also be useful for the prevention of aging-related health conditions. Unveiling the dual regulatory roles of hTERT in maintaining telomere length homeostasis adds a new element to the current molecular model of telomere length maintenance. Telomerase is regarded as a promising target for cancer therapy, and the treatment efficacy of telomerase inhibition has been shown to be related to telomere length in the tumor cells.[56] Our discovery reminds us of the ever complex role of telomerase in the regulation of telomere length and other critical cellular functions and adds a new domain to be considered when designing drugs that target telomerase for cancer therapy and disease prevention.

Materials and Methods

Cell culture, western blot analyses, and immunoprecipitation assays

ALT cell lines U2OS, SAOS-2, WI-35-VA13, and non-ALT cell lines MCF7, HT1080, IMR90, and WI38 cells were cultured in respective mediums (Clontech). Western blotting analyses and immunoprecipitation were essentially as described previously.[57]

Ectopical expression of hTERT, lentiviral shRNA constructs, and antibodies

The lentiviral HA-tagged hTERT construct was transfected into 293T cells. After 48 h, supernatant media with virus were collected. When an equal titer of supernatant from hTERT construct or a vector control was transduced into different cell lines, almost all the cells became puromycin resistant, showing high transduction efficiency in the experimental system. After 48 h of infection with hTERT vector, hTERT mRNA and protein expressions were examined.

hTERT and TPP1 shRNAs were designed and cloned to the lentiviral vector (Sigma) (shRNA sequences are available upon request). The following antibodies were used: anti-mouse HA (abCAM) and anti-rabbit Flag (Sigma).

Measurement of chromosome-specific telomere length by fluorescent in situ hybridization

Chromosome arm-specific telomere length was measured by telomere quantitative fluorescent in situ hybridization (TQ-FISH) as previously described.[14] Briefly, chromosome preparations were dropped onto clean microscopic slides and hybridized with 15 μl of hybridization mixture consisting of 0.3 μg/ml Cy3-labeled telomere-specific peptide nucleic acid (PNA) probe, 1 μl of cocktails of FITC-labeled centromeric PNA probes specific for chromosomes 2, 4, 8, 9, 13, 15, 18, 20, and 21, and 20 μg/ml of Cy3-labeled centromeric PNA probes specific for chromosome X (Biomarkers), in hybridization buffer containing 50% formamide, 10 mM TRIS-HCl, pH 7.5, and 5% blocking reagent. Slides were denatured and then hybridized at 30 °C for 3 h. After hybridization, the slides were sequentially washed 10 min each at 42 °C: once in 1 × SSC, once in 0.5 × SSC, and once in 0.1 × SSC. The slides were then mounted in anti-fade mounting medium containing 300 ng/ml DAPI.

After TQ-FISH, cells were analyzed using an epifluorescence microscope equipped with a charge-coupled device (CCD) camera. Metaphase cells were captured with exposure times of 0.15, 0.25, and 0.05 s for Cy3, FITC, and DAPI signals, respectively. Digitized metaphase images were analyzed using the Isis software (MetaSystems Inc), which permits the measurement of 92 telomere signals simultaneously after karyotyping. Telomere fluorescent intensity units (FIU) were recorded as an indirect measurement of telomere length. For each study subject, 30 metaphase cells were analyzed.

Immunofluorescence and in combination with telomeric DNA-FISH assays

Cells were cultured on 4-well chamber slides, and immunofluorescence staining was performed after pre-extract in 0.5% Triton/PBS for 1 min on ice, as described previously.[58] To combine with telomeric DNA-FISH, after immunostaining, slides were fixed, treated with 0.5 μg/ml RNase A in 2 × SSC for 45 min at 37 °C, and then dehydrated in 70%, 80%, and 100% ethanol. After being denatured at 80 °C for 3 min, slides were hybridized for 2 h in hybridization solution contained 0.3 μg/ml Tel-C PNA telomere probe (Applied Biosystems) with 70% formamide, 1% BSA, and 10 mM Tris, pH 7.2. Finally, slides were washed, dehydrated, and covered with anti-fading 4'-6-diamidino-2-phenylindole (DAPI) mounting medium.

2D gel assays and TRF assays

Genomic DNA was isolated following standard procedure[59] and digested with Hinf I. Samples were separated by electrophoresis on 0.5% agarose gel at 1 v/cm for 18–20 h. For the TRF

assay, alkaline transfer was performed. For the 2D gel assay, the separated DNA samples were cut from the gel and put into 1% agarose gel and continued to run for the secondary dimension at 5 v/cm for 2.5 h. Then the alkaline transfer was conducted. Membranes were hybridized with ^{32}P-labeled telomere probe.

TPP1-hTERT in vitro DNA binding assay

Buffers used and binding procedures were described previously.[60] Briefly, telomeric DNA substrates were biotinylated with a kit from BioServer Biotechnologies. In vitro-translated HA-hTERT (2 μL; Promega) and the biotinylated DNA substrates (0.1 pmol) were incubated in the absence or presence of various amounts of recombinant Flag-TPP1 for 30 min. The biotinylated DNA substrates were retrieved with streptavidin beads. After the unbound proteins were washed away with the binding buffer, HA-hTERT bound to the biotinylated DNA was eluted and detected by western blotting using anti-HA antibodies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author contributions

Q.Y. and Y.L.Z. generated the hypotheses, designed experiments and analyzed data. F.Z., B.S., J.Z., Y.W., L.T., K.K., C.K.S., J.Y., and X.L. performed experiments and generated data. Q.Y. and Y.L.Z. wrote the manuscript. R.S. contributed to data interpretation and edited manuscript. All authors contributed to the final revision of the manuscript.

Supplemental Materials

Supplemental materials may be found here:
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Reference List

1. Blackburn EH. Switching and signaling at the telomere. Cell 2001; 106:661-73; PMID:11572773; http://dx.doi.org/10.1016/S0092-8674(01)00492-5
2. Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell 1985; 43:405-13; PMID:3907856; http://dx.doi.org/10.1016/0092-8674(85)90170-9
3. Greider CW, Blackburn EH. A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. Nature 1989; 337:331-7; PMID:2463488; http://dx.doi.org/10.1038/337331a0
4. Greider CW. Telomerase activity, cell proliferation, and cancer. Proc Natl Acad Sci U S A 1998; 95:90-2; PMID:9419332; http://dx.doi.org/10.1073/pnas.95.1.90
5. Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell 1985; 43:405-13; PMID:3907856; http://dx.doi.org/10.1016/0092-8674(85)90170-9
6. Murnane JP, Sabatier L, Marder BA, Morgan WF. Telomere dynamics in an immortal human cell line. EMBO J 1994; 13:4953-62; PMID:7957062
7. Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA, Reddel RR. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. Nat Med 1997; 3:1271-4; PMID:9359704; http://dx.doi.org/10.1038/nm1197-1271
8. Bryan TM, Englezou A, Gupta J, Bacchetti S, Reddel RR. Telomere elongation in immortal human cells without detectable telomerase activity. EMBO J 1995; 14:4240-8; PMID:7556065
9. Cesare AJ, Reddel RR. Alternative lengthening of telomeres: models, mechanisms and implications. Nat Rev Genet 2010; 11:319-30; PMID:20351727; http://dx.doi.org/10.1038/nrg2763
10. Lustig AJ. Clues to catastrophic telomere loss in mammals from yeast telomere rapid deletion. Nat Rev Genet 2003; 4:916-23; PMID:14634639; http://dx.doi.org/10.1038/nrg1207
11. Pickett HA, Cesare AJ, Johnston RL, Neumann AA, Reddel RR. Control of telomere length by a trimming mechanism that involves generation of t-circles. EMBO J 2009; 28:799-809; PMID:19214183; http://dx.doi.org/10.1038/emboj.2009.42
12. Pickett HA, Henson JD, Au AY, Neumann AA, Reddel RR. Normal mammalian cells negatively regulate telomere length by telomere trimming. Hum Mol Genet 2011; 20:4684-92; PMID:21903669; http://dx.doi.org/10.1093/hmg/ddr402
13. Wang RC, Smogorzewska A, de Lange T. Homologous recombination generates T-loop-sized deletions at human telomeres. Cell 2004; 119:355-68; PMID:15507207; http://dx.doi.org/10.1016/j.cell.2004.10.011
14. Zheng YL, Zhou X, Loffredo CA, Shields PG, Sun B. Telomere deficiencies on chromosomes 9p, 15p, 15q and Xp: potential biomarkers for breast cancer risk. Hum Mol Genet 2011; 20:378-86; PMID:20956286; http://dx.doi.org/10.1093/hmg/ddq461
15. Liu X, Ory V, Chapman S, Yuan H, Albanese C, Kallakury B, Timofeeva OA, Nealon C, Dakic A, Simic V, et al. ROCK inhibitor and feeder cells induce the conditional reprogramming of epithelial cells. Am J Pathol 2012; 180:599-607; PMID:22189618; http://dx.doi.org/10.1016/j.ajpath.2011.10.036
16. Hahn WC, Stewart SA, Brooks MW, York SG, Eaton E, Kurachi A, Beijersbergen RL, Knoll JH, Meyerson M, Weinberg RA. Inhibition of telomerase limits the growth of human cancer cells. Nat Med 1999; 5:1164-70; PMID:10502820; http://dx.doi.org/10.1038/13495
17. Masutomi K, Yu EY, Khurts S, Ben-Porath I, Currier JL, Metz GB, Brooks MW, Kaneko S, Murakami S, DeCaprio JA, et al. Telomerase maintains telomere structure in normal human cells. Cell 2003; 114:241-53; PMID:12887925; http://dx.doi.org/10.1016/S0092-8674(03)00550-6
18. Latrick CM, Cech TR. POT1-TPP1 enhances telomerase processivity by slowing primer dissociation and aiding translocation. EMBO J 2010; 29:924-33; PMID:20094033; http://dx.doi.org/10.1038/emboj.2009.409
19. Zaug AJ, Podell ER, Nandakumar J, Cech TR. Functional interaction between telomere protein TPP1 and telomerase. Genes Dev 2010; 24:613-22; PMID:20231318; http://dx.doi.org/10.1101/gad.1881810
20. Nandakumar J, Bell CF, Weidenfeld I, Zaug AJ, Leinwand LA, Cech TR. The TEL patch of telomere protein TPP1 mediates telomerase recruitment and processivity. Nature 2012; 492:285-9; PMID:23103865; http://dx.doi.org/10.1038/nature11648
21. Nandakumar J, Cech TR. Finding the end: recruitment of telomerase to telomeres. Nat Rev Mol Cell Biol 2013; 14:69-82; PMID:23299958; http://dx.doi.org/10.1038/nrm3505
22. Wang F, Podell ER, Zaug AJ, Yang Y, Baciu P, Cech TR, Lei M. The POT1-TPP1 telomere complex is a telomerase processivity factor. Nature 2007; 445:506-10; PMID:17237768; http://dx.doi.org/10.1038/nature05454
23. Zhong FL, Batista LF, Freund A, Pech MF, Venteicher AS, Artandi SE. TPP1 OB-fold domain controls telomere maintenance by recruiting telomerase to chromosome ends. Cell 2012; 150:481-94; PMID:22863003; http://dx.doi.org/10.1016/j.cell.2012.07.012
24. Zeng S, Xiang T, Pandita TK, Gonzalez-Suarez I, Gonzalo S, Harris CC, Yang Q. Telomere recombination requires the MUS81 endonuclease. Nat Cell Biol 2009; 11:616-23; PMID:19363487; http://dx.doi.org/10.1038/ncb1867
25. Zeng S, Yang Q. The MUS81 endonuclease is essential for telomerase negative cell proliferation. Cell Cycle 2009; 8:2157-60; PMID:19617716; http://dx.doi.org/10.4161/cc.8.14.9149
26. Cesare AJ, Griffith JD. Telomeric DNA in ALT cells is characterized by free telomeric circles and heterogeneous t-loops. Mol Cell Biol 2004; 24:9948-57; PMID:15509797; http://dx.doi.org/10.1128/MCB.24.22.9948-9957.2004
27. Yang Q. Cellular senescence, telomere recombination and maintenance. Cytogenet Genome Res 2008; 122:211-8; PMID:19188689; http://dx.doi.org/10.1159/000167806
28. Reddel RR. Alternative lengthening of telomeres, telomerase, and cancer. Cancer Lett 2003; 194:155-62; PMID:12757973; http://dx.doi.org/10.1016/S0304-3835(02)00702-4
29. Blackburn EH. Telomere states and cell fates. Nature 2000; 408:53-6; PMID:11081503; http://dx.doi.org/10.1038/35040500
30. Lue NF, Yu EY, Lei M. A popular engagement at the ends. Nat Struct Mol Biol 2013; 20:10-2; PMID:23288360; http://dx.doi.org/10.1038/nsmb.2483
31. Lue NF. Closing the feedback loop: how cells "count" telomere-bound proteins. Mol Cell 2009; 33:413-4; PMID:19250901; http://dx.doi.org/10.1016/j.molcel.2009.02.001

32. Sharma GG, Gupta A, Wang H, Scherthan H, Dhar S, Gandhi V, Iliakis G, Shay JW, Young CS, Pandita TK. hTERT associates with human telomeres and enhances genomic stability and DNA repair. Oncogene 2003; 22:131-46; PMID:12527915; http://dx.doi.org/10.1038/sj.onc.1206063
33. Sharma GG, Hwang KK, Pandita RK, Gupta A, Dhar S, Parenteau J, Agarwal M, Worman HJ, Wellinger RJ, Pandita TK. Human heterochromatin protein 1 isoforms HP1(Hsalpha) and HP1(Hsbeta) interfere with hTERT-telomere interactions and correlate with changes in cell growth and response to ionizing radiation. Mol Cell Biol 2003; 23:8363-76; PMID:14585993; http://dx.doi.org/10.1128/MCB.23.22.8363-8376.2003
34. Pandita TK. ATM function and telomere stability. Oncogene 2002; 21:611-8; PMID:11850786; http://dx.doi.org/10.1038/sj.onc.1205060
35. Wood LD, Halvorsen TL, Dhar S, Baur JA, Pandita RK, Wright WE, Hande MP, Calaf G, Hei TK, Levine F, et al. Characterization of ataxia telangiectasia fibroblasts with extended life-span through telomerase expression. Oncogene 2001; 20:278-88; PMID:11313956; http://dx.doi.org/10.1038/sj.onc.1204072
36. Wan B, Yin J, Horvath K, Sarkar J, Chen Y, Wu J, Wan K, Lu J, Gu P, Yu EY, et al. SLX4 assembles a telomere maintenance toolkit by bridging multiple endonucleases with telomeres. Cell Rep 2013; 4:861-9; PMID:24012755; http://dx.doi.org/10.1016/j.celrep.2013.08.017
37. Pfeiffer V, Lingner J. TERRA promotes telomere shortening through exonuclease 1-mediated resection of chromosome ends. PLoS Genet 2012; 8:e1002747; PMID:22719262; http://dx.doi.org/10.1371/journal.pgen.1002747
38. Oulton R, Harrington L. A human telomerase-associated nuclease. Mol Biol Cell 2004; 15:3244-56; PMID:15121883; http://dx.doi.org/10.1091/mbc.E04-03-0178
39. Pandita TK, Hunt CR, Sharma GG, Yang Q. Regulation of telomere movement by telomere chromatin structure. Cell Mol Life Sci 2007; 64:131-8; PMID:17219023; http://dx.doi.org/10.1007/s00018-006-6465-0
40. Teixeira MT, Arneric M, Sperisen P, Lingner J. Telomere length homeostasis is achieved via a switch between telomerase- extendible and -nonextendible states. Cell 2004; 117:323-35; PMID:15109493; http://dx.doi.org/10.1016/S0092-8674(04)00334-4
41. D'Souza Y, Chu TW, Autexier C. A translocation-defective telomerase with low levels of activity and processivity stabilizes short telomeres and confers immortalization. Mol Biol Cell 2013; 24:1469-79; PMID:23447707; http://dx.doi.org/10.1091/mbc.E12-12-0889
42. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. Nature 1990; 345:458-60; PMID:2342578; http://dx.doi.org/10.1038/345458a0
43. Olovnikov AM. A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. J Theor Biol 1973; 41:181-90; PMID:4754905; http://dx.doi.org/10.1016/0022-5193(73)90198-7
44. Watson JD. Origin of concatemeric T7 DNA. Nat New Biol 1972; 239:197-201; PMID:4507727; http://dx.doi.org/10.1038/newbio239197a0
45. Krizhanovsky V, Xue W, Zender L, Yon M, Hernando E, Lowe SW. Implications of cellular senescence in tissue damage response, tumor suppression, and stem cell biology. Cold Spring Harb Symp Quant Biol 2008; 73:513-22; PMID:19150958; http://dx.doi.org/10.1101/sqb.2008.73.048
46. Harley CB. Telomere loss: mitotic clock or genetic time bomb? Mutat Res 1991; 256:271-82; PMID:1722017; http://dx.doi.org/10.1016/0921-8734(91)90018-7
47. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. Nature 1990; 345:458-60; PMID:2342578; http://dx.doi.org/10.1038/345458a0
48. de Lange T. Telomeres and senescence: ending the debate. Science 1998; 279:334-5; PMID:9454329; http://dx.doi.org/10.1126/science.279.5349.334
49. Counter CM, Avilion AA, LeFeuvre CE, Stewart NG, Greider CW, Harley CB, Bacchetti S. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. EMBO J 1992; 11:1921-9; PMID:1582420
50. Counter CM, Botelho FM, Wang P, Harley CB, Bacchetti S. Stabilization of short telomeres and telomerase activity accompany immortalization of Epstein-Barr virus-transformed human B lymphocytes. J Virol 1994; 68:3410-4; PMID:8151802
51. Engelhardt M, Drullinsky P, Guillem J, Moore MA. Telomerase and telomere length in the development and progression of premalignant lesions to colorectal cancer. Clin Cancer Res 1997; 3:1931-41; PMID:9815582
52. Calado RT, Young NS. Telomere diseases. N Engl J Med 2009; 361:2353-65; PMID:20007561; http://dx.doi.org/10.1056/NEJMra0903373
53. Prescott J, Wentzensen IM, Savage SA, De V. I. Epidemiologic evidence for a role of telomere dysfunction in cancer etiology. Mutat Res 2012; 730:75-84; PMID:21756922
54. Zhu H, Belcher M, van der Harst P. Healthy aging and disease: role for telomere biology? Clin Sci (Lond) 2011; 120:427-40; PMID:21271986; http://dx.doi.org/10.1042/CS20100385
55. Sanders JL, Fitzpatrick AL, Boudreau RM, et al. Leukocyte Telomere Length Is Associated With Noninvasively Measured Age-Related Disease: The Cardiovascular Health Study. J Gerontol A Biol Sci Med Sci 2012; 67:409-16; PMID:21934123
56. Harley CB. Telomerase and cancer therapeutics. Nat Rev Cancer 2008; 8:167-79; PMID:18256617; http://dx.doi.org/10.1038/nrc2275
57. Yang Q, Zheng YL, Harris CC. POT1 and TRF2 cooperate to maintain telomeric integrity. Mol Cell Biol 2005; 25:1070-80; PMID:15657433; http://dx.doi.org/10.1128/MCB.25.3.1070-1080.2005
58. Jiang WQ, Zhong ZH, Henson JD, Reddel RR. Identification of candidate alternative lengthening of telomeres genes by methionine restriction and RNA interference. Oncogene 2007; 26:4635-47; PMID:17297460; http://dx.doi.org/10.1038/sj.onc.1210260
59. Karlseder J, Smogorzewska A, de Lange T. Senescence induced by altered telomere state, not telomere loss. Science 2002; 295:2446-9; PMID:11923537; http://dx.doi.org/10.1126/science.1069523
60. Zou L, Elledge SJ. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science 2003; 300:1542-8; PMID:12791985; http://dx.doi.org/10.1126/science.1083430