Nucleolar localization of TERT is unrelated to telomerase function in human cells

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Accepted 18 February 2008
Journal of Cell Science 121, 2169-2176 Published by The Company of Biologists 2008
doi:10.1242/jcs.024091

Summary

Telomerase maintains telomere length and has been implicated in both aging and carcinogenesis of human cells. This enzyme is a specialized ribonucleoprotein (RNP) complex, minimally consisting of two essential components: the protein catalytic subunit TERT (telomerase reverse transcriptase) and the integral RNA moiety TR (telomerase RNA, TERC). Both TERT and TR have been found to localize to nucleoli within the nucleus, leading to the suggestion of nucleoli as the site for telomerase RNP biogenesis in human cells. However, whether this statement is true or not has not yet been convincingly demonstrated. Here, we identify that residues 965-981 of the human TERT polypeptide constitute an active nucleolar-targeting signal (NTS) essential for mediating human TERT nucleolar localization. Mutational inactivation of this NTS completely disrupted TERT nucleolar translocation in both normal and malignant human cells. Most interestingly, such a TERT mutant still retained the capacity to activate telomerase activity, maintain telomere length and extend the life-span of cellular proliferation, as does wild-type TERT, in BJ cells (normal fibroblasts). Therefore, our data suggest that TERT nucleolar localization is unrelated to telomerase function in human cells.

Key words: Nucleolus, TERT, NTS, Localization, Telomerase

Introduction

Telomerase is a specialized ribonucleoprotein (RNP) complex used by most eukaryotes for directing the synthesis of telomeric DNA sequence at chromosomal ends, thereby ensuring replication of the complete genome during cell divisions. Telomerase activity is suppressed in most human somatic cells, and these normal cells suffer from a cell-cycle-dependent telomere-length shortening and eventually enter into senescence when telomeres shorten to a crucial point (Hayflick, 1973). By contrast, telomerase activity is highly upregulated in over 85% of human cancer cells and endows immortalization to cancer cells by maintaining telomere length (Kim et al., 1994; Hahn et al., 1999; Stewart and Weinberg, 2000). These observations have proposed telomerase a plausible anti-cancer target (Parkinson, 2005).

Human telomerase RNP minimally consists of a catalytic subunit protein and an integral single-stranded RNA moiety, called TERT (telomerase reverse transcriptase; also known as hTERT) and TR (telomerase RNA, TERC), respectively (Meyerson et al., 1997; Feng et al., 1995). Early studies have helped to establish TERT as the rate-limiting component for human cellular telomerase regulation (Meyerson et al., 1997), and expression of the *TERT* gene is suppressed by multiple tumor-suppressing pathways in normal somatic cells (Lin and Elledge, 2003). Besides the transcriptional control, TERT protein is still subjected to a highly programmed subcellular transportation that also has important implications in its functional regulations (Aisner et al., 2002; Hathcock et al., 2005).

Recent studies revealed that TERT undergoes a dynamic subnuclear shuttling between the nucleoli and nucleoplasm that is dependent on the cell cycle, DNA damage or cellular transformation (Wong et al., 2002; Zhu et al., 2004; Tomlinson et al., 2006). However, the biological significances of TERT nucleolar localization are still unclear at present. It has been speculated that TERT nucleolar transportation might be a prerequisite step in the process of telomerase RNP biogenesis because its integral binding-partner TR has been shown to be processed and matured within nucleoli (Mitchell et al., 1999; Pogacic et al., 2000; Dez et al., 2001). But whether this assumption is true or not has not yet been convincingly demonstrated. Also, the mechanism underlying the TERT nucleolar translocation is still largely unknown at present.

In this study, we identified a novel nucleolar-targeting signal (NTS) within the C-terminal sequence (aa 965-981) of human TERT. Mutational disruption of this NTS activity abrogates TERT nucleolar localization in both normal and cancerous human cells. Interestingly, ectopic expression of such a TERT mutant in normal human fibroblasts (BJ cells) was still able to activate cellular telomerase, block telomere-length shortening and extend the cellular proliferation life-span in the same way as its wild-type counterpart. Therefore, we provide direct evidence that TERT nucleolar localization is unrelated to telomerase RNP biogenesis and telomerase-mediated telomere maintenance in human cells.

Results

The short unique C-terminal sequence of TERT encodes nucleolar-targeting information

TERT possesses a short unique C-terminal polypeptide that shows little homology in both amino acid sequences and biological function with its yeast counterpart. Previously, we showed that ectopic overexpression of a TERT C-terminal polypeptide fragment specifically inhibits telomerase-positive cancer-cell growth without affecting cellular telomerase activity and overall telomere length (Huang et al., 2002). In those studies, we observed that, in a

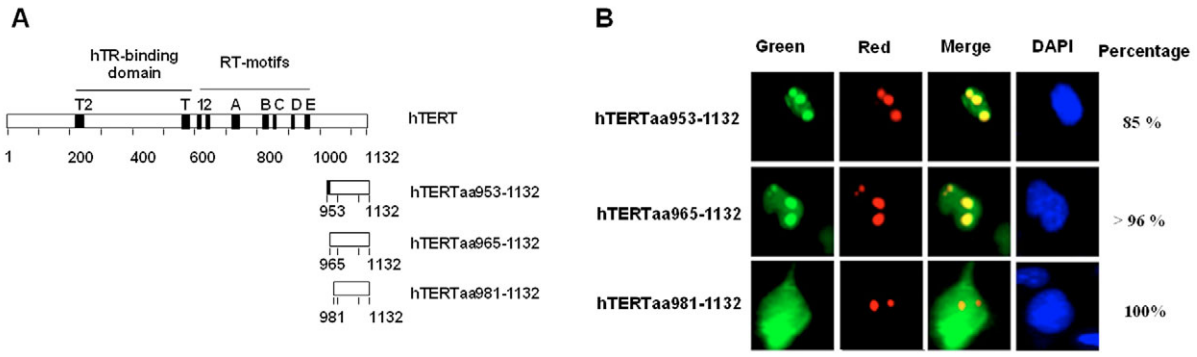

Fig. 1. Subcellular distributions of TERT C-terminal polypeptide sequences expressed in HeLa cells. (A) Schematic presentation of full-length TERT and its indicated C-terminal polypeptide fragments used in this study. (B) An example of the subcellular distribution patterns of different TERT C-terminal fragments in transiently transfected HeLa cells (refer to text for detailed description). 400× magnification. Green, signal for ectopically expressed GFP-tagged fragments; red, signal for the co-expressed RFP-B23; blue, DAPI nuclear staining. The merge shows the overlay images of GFP-TERT fragments and the co-expressed RFP-B23 nucleolar protein within nuclei. The percentages of cells with the represented GFP staining patterns in the corresponding transfected cell populations are shown to the right (>400 expressing cells counted for each group).

substantial fraction of transfected cells, the expressed TERT C-terminal fragment displayed specific intranuclear-foci localization corresponding to the nucleolar structures identified under optic-microscope observation. To determine whether the TERT C-terminal sequence contains information for nucleolar localization, the three TERT C-terminal peptides with different N-terminal truncations (Fig. 1A) were fused with GFP and were transiently co-expressed with the RFP-tagged nucleolar protein B23 (nucleophosmin) (Ye, 2005) in HeLa cells (a human cervical cancer cell line). Fluorescent-microscope observations indicated that the two longer fragments (GFP-$TERT_{aa953-1132}$ and GFP-$TERT_{aa965-1132}$) co-stained exactly with RFP-B23 within nucleoli in almost all expressing HeLa cells, whereas the fragment GFP-$TERT_{aa981-1132}$ was homogeneously distributed in the whole cell without colocalization with RFP-B23 (Fig. 1B). These results clearly indicated that the TERT C-terminal residues contain nucleolar-targeting information and residues 965-981 might be a functional motif for this activity.

Residues 965-981 of the TERT polypeptide constitute an active NTS with functional conservation in both human and mouse cells

Alignment analysis showed that the peptide sequence aa965-981 of human TERT is highly conserved in its counterparts from both mice and *Xenopus*, and that it is characterized by the enrichment of positively charged amino acid residues (Fig. 2A), which is a marker of the NTS sequences found in other nucleolar proteins (Hatanaka, 1990). To clarify whether residues 965-981 of TERT constitute an active NTS, the three conserved positively charged residues (indicated in Fig. 2) were substituted with alanines on the fragment GFP-$TERT_{aa965-1132}$. The resultant mutant, named GFP–$TERT_{aa965-1132}$-3A, was transiently transfected into human HeLa cells and mouse NIH3T3 fibroblasts. As expected, all expressed GFP–$TERT_{aa965-1132}$-3A showed a clear nucleolar-exclusion pattern within the nucleus of both the human and mouse transfected cells (Fig. 2B). We also fused the peptide sequence aa965-981 of TERT to GFP and found that this peptide sequence alone was able to deliver GFP into nucleoli both in transfected HeLa and NIH3T3 cells (Fig. 2C). We thus conclude that aa965-981 of TERT is an active NTS with functional conservation in both human and mouse cells.

Mutational inactivation of the C-terminal NTS completely abolishes TERT nucleolar localization in both normal and cancerous human cells

To test the role of the C-terminal NTS identified here in the process of TERT nucleolar translocation, the aforementioned substitution mutations were introduced into full-length TERT. The wild-type GFP-TERT and the mutated GFP–TERT-3A were first introduced into the normal diploid BJ fibroblasts for stable expression by recombinant lentiviral infection. Consistent with other previous reports (Wong et al., 2002; Masutomi et al., 2003), we found that nearly all (98% in 500 cells counted) GFP-TERT-infected BJ cells displayed the nuclear-expressing pattern with an obvious concentrated nucleolar staining (Fig. 3A). However, all (~100%) GFP–TERT-3A-infected BJ cells exhibited a nucleolar-exclusion staining within the expressing nuclei (Fig. 3A), indicating that mutational inactivation of the C-terminal NTS completely destroys TERT nucleolar localization in normal cells. We also introduced GFP-TERT and GFP–TERT-3A into three different human cancer cell lines – HeLa, H1299 (a human small lung cancer cell line with p53 genetically deletion) and U2OS (a human sarcoma cell line without TERT/telomerase expression) – by the same method to analyze their subcellular localization in these malignant cells. It has been reported that the phenotype of TERT nucleolar localization in normal cells is widely affected in human cancer cells by transformation events (Wong et al., 2002). In agreement, in all tested GFP-TERT-infected cancer-cell populations, only a few cells (~5%, 4% and 4% for HeLa, U2OS and H1299, respectively; >400 cells counted in each group) displayed a discernable nucleolar-accumulation staining (data not shown). However, unlike the report that ectopically expressed GFP-TERT showed a dominant nucleolar-exclusion distribution in transfected cancer-cell populations (Wong et al., 2002), we found that only a small fraction of infected cancer cells (around 20% on average in 400 cells counted for each group) exhibited the distinct GFP-TERT nucleolar-exclusion staining pattern. Instead, we found that most GFP-TERT-infected cancer cells (~74% on average, >400 cells counted) showed a predominant nuclear diffuse distribution pattern without impressive nucleolar concentration in all tested malignant cells (Fig. 3B). Similar observations of the subcellular localizations of GFP-TERT in different transfected cancer-cell populations were also reported by others (Yang et al., 2002; Etheridge et al., 2002). Actually, a recent

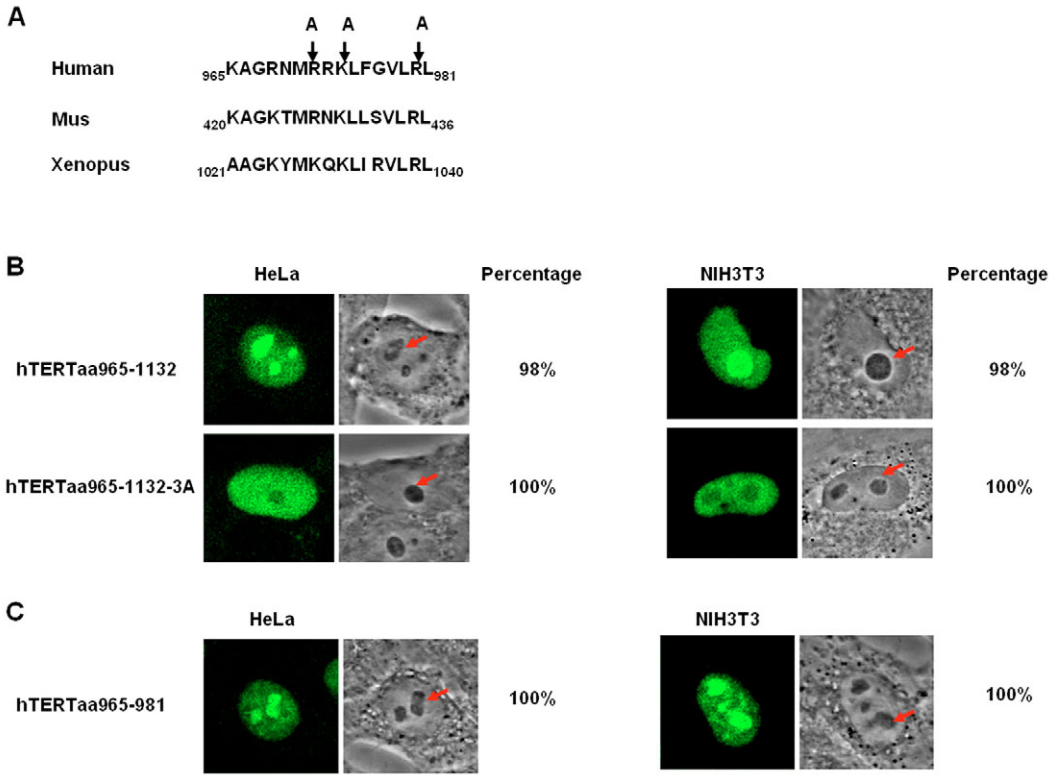

Fig. 2. Residues 965-981 of the TERT polypeptide are an active NTS sequence. (A) Alignment analysis shows that the sequence aa965-981 of TERT is highly conserved in its counterparts from mouse and *Xenopus*, and that it is enriched with positively charged residues. (B) Substitution of the three conserved positively charged residues indicated in A with alanines abolished nucleolar localization of the fragment GFP-TERT$_{aa965-1132}$ in both human (HeLa) and mouse (NIH3T3) cells. Arrows indicate nucleoli. (C) Fusion of the aa965-981 sequence of TERT to GFP leads to nucleolar accumulation of the expressed fusion protein in both HeLa and NIH3T3 cells. The fluorescence signal shows the localization patterns of the GFP fusion proteins in their corresponding transfected cells. Arrows indicate nucleoli that were visualized by using an optic microscope. 400× magnification. The percentage of cells with the represented GFP staining patterns in the corresponding transfected cell populations are shown to the right (>400 expressing cells counted for each group).

study has demonstrated that endogenous TERT is transported to nucleoli during the S-phase of the cell cycle of cancer cells (Tomlinson et al., 2006). We thus prefer to propose that TERT localizes to nucleoli in both normal and malignant human cells but that the dynamic behavior of TERT nucleolar localization between normal and cancerous cells is clearly different. Interestingly, the expressed GFP–TERT-3A mutant protein displayed the distinct nucleolar-exclusion pattern, without any obvious nucleolar staining, in all infected malignant cells (Fig. 3B). Taken together, these results unambiguously demonstrate that mutational inactivation of the C-terminal NTS completely destroys TERT nucleolar localization in both normal and cancerous human cells.

Mutational inactivation of C-terminal NTS abrogates the response of TERT to DNA-damage-stimulated nucleolar translocation

It has been reported that DNA damage can triggers a reaction of TERT re-accumulation from the nucleoplasm to the nucleolus in cancer cells (Wong et al., 2002). To determine whether the C-terminal NTS of TERT is essential for mediating this response, three different human cancer cells, including HeLa, H1299 and U2OS, with either GFP-TERT or GFP–TERT-3A lentiviral infection were treated with 50 μM of etoposide, an anti-cancer reagent inducing double-strand DNA breaks via the inhibition of topoisomerase II activity. After 6 hours of treatment, the subcellular distributions of GFP-TERT and GFP–TERT-3A expressed in these cells were analyzed by confocal fluorescent microscope observation. As indicated in Fig. 4A, we found a remarkable change in the subnuclear distribution of GFP-TERT from the predominant nuclear diffuse localization before etoposide treatment to the apparently nucleolar accumulation pattern after the treatment in all these tested cancer cell lines. These results further confirmed that induction of TERT nucleolar concentration constitutes an acute phase of DNA-damage response in human cancer cells. Because the H1299 cancer cell line has a genetic deletion of the *p53* tumor-suppressor gene (Wang et al., 1999), our data also suggest that the cellular response of DNA-damage-induced TERT nucleolar localization is via a p53-independent signaling transduction pathway. Interestingly, we found that etoposide stimulation did not affect the subnuclear localization of the GFP–TERT-3A mutant in all tested cells. All (~100%) GFP–TERT-3A-expressing cells still retained the nucleolar exclusion staining within the nuclei before and after etoposide treatment (Fig. 4B). We therefore conclude that the C-terminal NTS is also essential for mediating the response of TERT to DNA-damage-induced nucleolar accumulation.

In addition to the C-terminal NTS, two independent NTSs were also previously identified within the N-terminal section of the TERT polypeptide and were shown to have effects on regulating TERT nucleolar localization (Yang et al., 2002; Etheridge et al., 2002). We thus constructed two such TERT mutants either by deletion of the extreme N-terminal 15-amino-acid sequence from full-length TERT or by substitution of the six-amino-acid sequence $_{386}$YWGMRP of

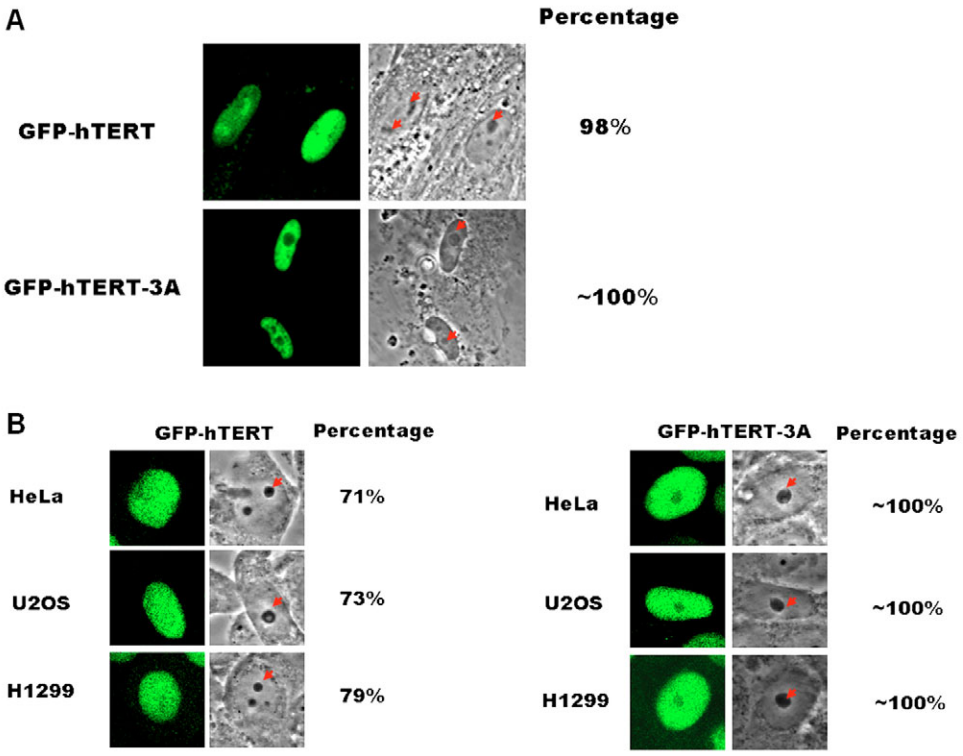

Fig. 3. Mutational inactivation of the C-terminal NTS of TERT completely disrupts TERT nucleolar localization in both normal and malignant human cells. Wild-type GFP-TERT and the mutant GFP–TERT-3A, which contains the substitution mutations indicated in Fig. 2A, were individually introduced, through recombinant lentiviral infection, into BJ cells (normal human fibroblasts) or into HeLa, H1299 or U2OS cancerous cells for stable expression. (A) A representative example showing that ectopically overexpressed GFP-TERT (green, upper panel) concentrates within nuclear foci identical to the nucleolar structures observed under optic microscope observation. However, overexpressed GFP–TERT-3A (green, lower panel) distributed diffusely in the nucleoplasm, outside nucleoli, in stable BJ cells. 100× magnification. (B) Images showing that overexpressed GFP-TERT exhibited a predominant nuclear diffusion pattern in all indicated transfected cancer cell lines and that GFP–TERT-3A displayed a predominant nucleolar-exclusion pattern in these cell lines. 400× magnification. Arrows indicate nucleolar regions identified under optic microscope observation. The percentages of cells with each representative image in the corresponding cell populations are presented to the right.

TERT with the sequence NAAIRS, according to the following references (Yang et al., 2002; Etheridge et al., 2002). The resultant mutants (GFP–TERT-N1 and GFP–TERT-N2, respectively) were individually introduced into the HeLa cell line through lentiviral infection, and their subcellular localization patterns in infected cell populations were compared with wild-type GFP-TERT and mutant GFP–TERT-3A. As summarized in Table 1, under normal culture conditions, the percentage of cells with a nucleolar-exclusion staining pattern in both GFP–TERT-N1- and GFP–TERT-N2-infected cells (65% and 58%, respectively) was substantially increased as compared with GFP-TERT-infected cells (24%), but were not as remarkably increased as the GFP–TERT-3A-infected cell population, in which all GFP–TERT-3A-expressing cells (100%) exhibited the nucleolar-exclusion pattern. When these cells were treated with 50 μM etoposide for 6 hours, we found that the incidence of DNA-damage-induced TERT nucleolar accumulation observed in GFP-TERT-infected cells (68%) was also greatly reduced in both GFP–TERT-N1- and GFP–TERT-N2-infected cells (21% and 27%, respectively). However, all GFP–TERT-3A-infected cells (100%) still retained the nucleolar-exclusion staining pattern under the same treatment. These results thus indicate that the C-terminal NTS identified here plays an essential role in regulating TERT nucleolar

Table 1. Subnuclear distributions of GFP-TERT, GFP–TERT-N1, GFP–TERT-N2 and GFP–TERT-3A in HeLa cells under normal and DNA-damage stress growth conditions

	TERT		TERT-N1		TERT-N2		TERT-3A	
Staining pattern*	N	DDS	N	DDS	N	DDS	N	DDS
Nuclear diffusion (%)	71	29	34	38	40	42	0	0
Nucleolar concentration (%)	5	68	1	21	2	27	0	0
Nucleolar exclusion (%)	24	3	65	41	58	31	100	100

The GFP-tagged TERT constructs were introduced into HeLa cells through lentiviral infection. The subcellular distributions of the indicated fusion proteins in the corresponding infected cell populations under normal (N) and etoposide-induced DNA-damage stress conditions (50 μM; DDS) were observed by fluorescence microscopY. A total of 400 expressing cells were counted for the analysis in each group. *See Fig. 4 for the representative images of the described subnuclear staining patterns.

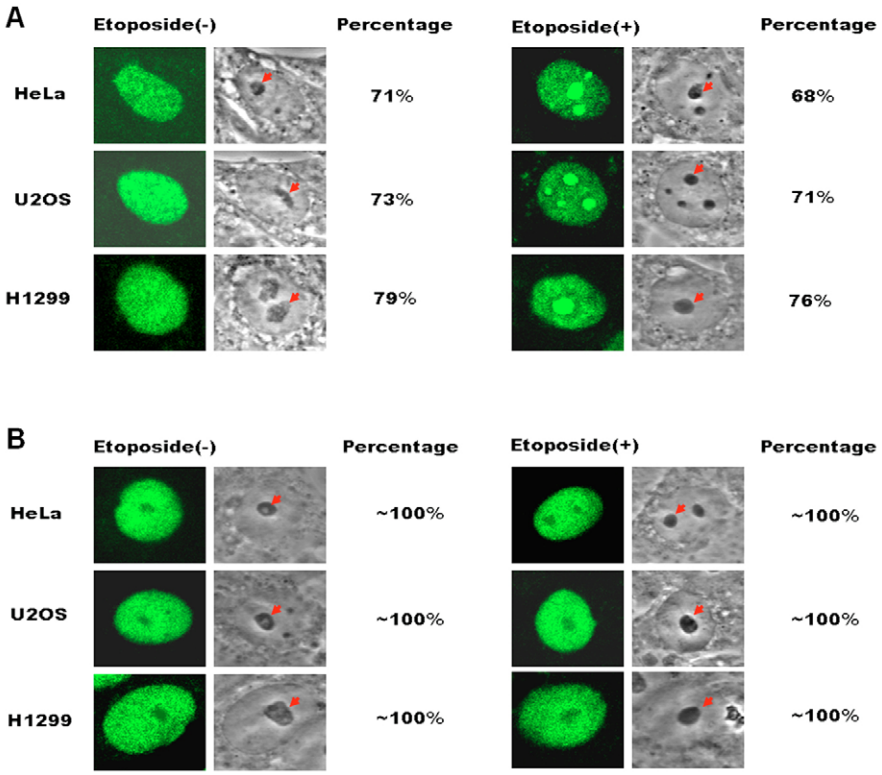

Fig. 4. Mutational inactivation of the C-terminal NTS of TERT abrogates the DNA-damage-induced nucleolar localization of TERT. Wild-type GFP-TERT and the mutant GFP–TERT-3A were stably expressed in the indicated human cancer cell lines through lentiviral infection. (A) Treatment with 50 μM etoposide for 6 hours stimulated the expressed GFP-TERT to translocate from the nucleoplasm to nucleoli in all tested stable cancer cell lines. (B) The same etoposide treatment did not alter the intranuclear distribution of the expressed GFP-TERT mutant in all tested stable cells. Green represents the fluorescence signal for expressed GFP-TERT or GFP–TERT-3A in the corresponding stable cells before and after etoposide treatment. Arrows indicate the nucleolar structures observed under the optic microscope. 400× magnification. The percentage of cells with the represented GFP staining patterns in the corresponding transfected cell populations are shown on the right (>400 expressing cells counted for each group).

localization but also suggest that its activity alone is not sufficient to fully mediate this process in human cells under both normal conditions and DNA-damage stress conditions.

Disrupting TERT nucleolar localization does not affect its in vivo function on telomerase activation

The nucleolus is well-known for its function in directing the biogenesis of ribosomes as well as other RNP complexes in cells. It has also been proposed that nucleoli might be involved in the biogenesis of human telomerase RNP because both TERT and TR localize to this subnuclear compartment in human cells. But such a statement remains controversial. An early study showed that specific point mutations within the extreme N-terminal region of TERT resulted in a declined efficacy of TERT nucleolar localization associated with the loss of its capacity to activate telomerase in vivo (Yang et al., 2002). But, because this TERT mutant still displays the nucleolar-localization phenotype in a substantial fraction of its expressing cells (Yang et al., 2002) (Table 1), its loss of telomerase function might be most likely due to other causes rather than the defect of nucleolar localization per se. The fact that mutational destruction of the C-terminal NTS completely abrogates TERT nucleolar localization provides an opportunity to address whether TERT nucleolar transportation is a prerequisite step for the biogenesis of telomerase RNP in human cells. We first detected cellular-telomerase enzymatic activity in BJ cells that were lentivirally infected with wild-type GFP-TERT or mutant GFP–TERT-3A. Stable expression of GFP-TERT and GFP–TERT-3A in the corresponding transfected cell masses was confirmed to be at approximately the same level by western blotting assay (Fig. 5A), suggesting that such a mutation does not affect the protein expression levels of TERT in BJ cells. TRAP assay indicated that robust telomerase activities were readily detectable in both GFP-TERT- and GFP–TERT-3A-expressing BJ cells, but not in vector-control BJ cells (Fig. 5B). We further examined whether the TERT-3A mutant could reconstitute telomerase activity in the telomerase-negative U2OS cancer cell line. The ectopic expression levels of GFP-TERT and GFP–TERT-3A were also approximately the same in the corresponding U2OS cell masses (Fig. 5C). Again, we found that the same robust telomerase enzymatic activity was detectable in both GFP-TERT- and GFP–TERT-3A-infected U2OS cell masses (Fig. 5D). These data thus indicate that nucleolar localization of TERT is not required for its effect on activating telomerase activity in both normal and cancerous human cells.

TERT-3A counteracts telomere-length shortening and extends the proliferation life-span

It is well-known that ectopic expression of TERT can counteract the progressive telomere-length shortening and significantly extend the proliferation life-span in BJ fibroblasts (Bodnar et al., 1998). Therefore, our final investigation was to determine whether nucleolar localization of TERT is required for these functions. Telomere-length analysis at the indicated time points (PDs, Fig. 6A) showed that both GFP-TERT- and GFP–TERT-3A-expressing BJ cells had stable telomere length during their successive cultural propagations, whereas a progressive telomere-length shortening was observed for the vector-control BJ cells with the extension of their cultural propagation (Fig. 6A). Growth-curve analysis (Fig. 6B) showed that control BJ cells ceased proliferation at around 65 population doublings (PDs) and became morphologically enlarged and stained positive for senescence-associated-β-galactosidase (SA-β-gal) (Fig. 6C), indicating their falling into cellular senescence. However, both GFP-TERT- and GFP–TERT-3A-expressing BJ cells still retained the robust proliferative capacity at 92 PDs (for GFP-TERT) and 86 PDs (for GFP–TERT-3A) and did not exhibit

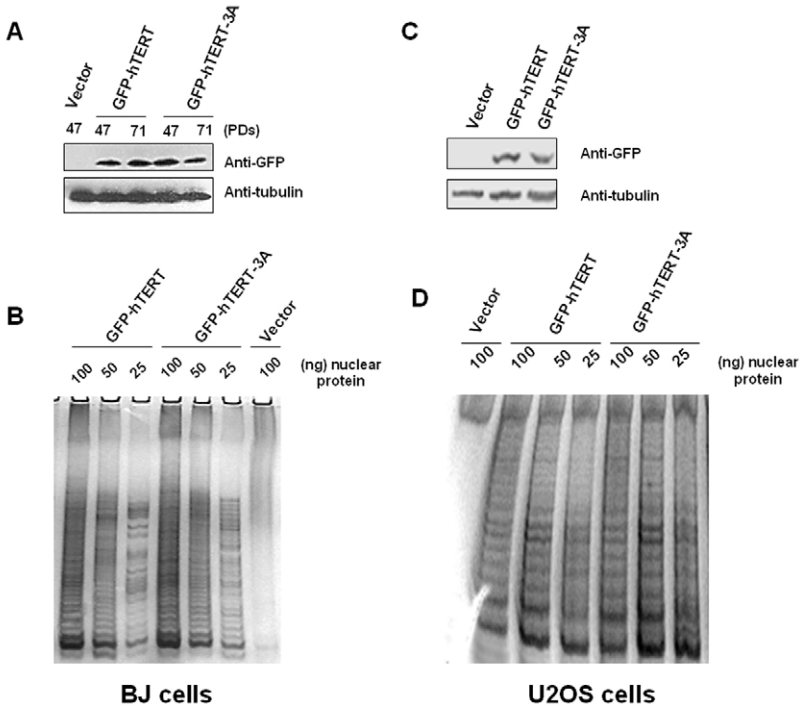

Fig. 5. Mutational disruption of TERT nucleolar localization does not affect its telomerase enzymatic activity in both normal and cancerous human cells. BJ cells (at around 45 PDs in culture) and U2OS cells were infected with recombinant lentivirus expressing GFP-TERT or GFP–TERT-3A. (A,C) Western-blotting assay with specific anti-GFP antibodies demonstrated that the stable expression of wild-type GFP-TERT and the mutant GFP–TERT-3A was at approximately the same level in corresponding stable BJ (A) and U2OS (C) cells. (B,D) TRAP assay demonstrated that stable expression of both GFP-TERT and of the GFP–TERT-3A mutant can activate telomerase activity with the same efficiency in both BJ fibroblasts (B) and in cancerous U2OS cells (D). Tubulin was used as the control to show the amounts for each loading sample in the western-blotting assays. Vector-infected BJ cells and U2OS cells were used as controls for these experiments.

any signs of senescence, as judged by the SA-β-gal staining assay (Fig. 6B,C). These results thus demonstrate that loss of nucleolar localization does not affect the ability of TERT to maintain telomere length or to extend the cellular proliferative life-span in BJ cells.

Discussion

Recent studies reveal that TERT undergoes a dynamic nucleolar translocation that is dependent on the cell cycle, cellular transformation and DNA-damage response (Wong et al., 2002). However, the molecular mechanisms underlying TERT nucleolar localization and the corresponding cellular biological significance of this process are still unclear. It was previously shown that the N-terminal section of the TERT polypeptide contains two independent putative nucleolar-targeting elements (Etheridge et al., 2002; Yang et al., 2002). However, individual mutation of these two N-terminal NTSs does not completely destroy TERT nucleolar targeting, suggesting that they do not play the dominant role in this process. In the present study, we show that the aa965-981 sequence of the TERT C-terminal polypeptide is an active NTS with functional conservation in both human and mouse cells. We further demonstrate that mutational disruption of the C-terminal NTS not only completely prevents TERT from localizing to the nucleolus in both normal and cancerous human cells, but also completely abolishes the response of TERT to DNA-damage-induced nucleolar accumulation in cancer cells. We therefore conclude that the C-terminal NTS is a crucial determinant essential for TERT nucleolar localization under either the physiologic cellular growth condition or the stress condition of DNA damage. The fact that TERT contains multiple nucleolar-localizing signals suggests that the process of TERT shuttling between the nucleoplasm and the nucleolus might be regulated by different molecular mechanisms.

It appears that the activity of the C-terminal NTS identified here might be regulated by its N-terminal upstreamed amino acid sequence in the TERT protein molecule. This can be reflected by the fact that expression of TERT C-terminal fragments containing this NTS exhibited complete nucleolar concentration, whereas expression of the full-length TERT displayed diffuse nucleoplasmic distribution in cancer cells. Because the expressed TERT displays the dominant nucleolar-localization behavior in normal cells, we further suggest that cancerous mutations may establish a particular negative mechanism on the suppression of the activity of the C-terminal NTS of TERT in malignant cells, possibly by altering specific protein-protein interactions at regions nearby or within this NTS site. Identification of these putative regulatory factors involved in modulating the C-terminal NTS activity will certainly help to elucidate the mechanisms underlying the process of TERT nucleolar translocation in human cells.

The cellular biological significance of TERT nucleolar localization is another issue waiting to be addressed. Because disrupting TERT nucleolar localization by mutations on the C-terminal NTS does not affect its abilities to activate telomerase enzymatic activity, maintain telomere length or block the telomere-shortening-induced cellular senescence in BJ fibroblasts, our data disfavor the previous speculation that the nucleolar transportation of TERT is a step in the formation of a functional telomerase RNP complex in human cells (Yang et al., 2002). It has also been proposed that TERT nucleolar transportation might provide negative regulation on telomerase by sequestering this enzyme and preventing its contact with its default substrate telomeric DNA (Lin and Blackburn, 2004; Blackburn, 2005). However, this statement could be argued by the observation that, as compared with wild-type TERT, stable expression of the TERT-3A mutant does not cause telomere over-elongating effects in BJ cells (Fig. 6A). Alternatively, the regulation of TERT nucleolar localization might be functionally linked to its other biological activities that are unrelated to its well-known telomerase actions. It is now clear that TERT can facilitate human cellular tumorigenesis through pathways other than telomere-length maintenance (Stewart et al., 2002), and that it possesses an anti-

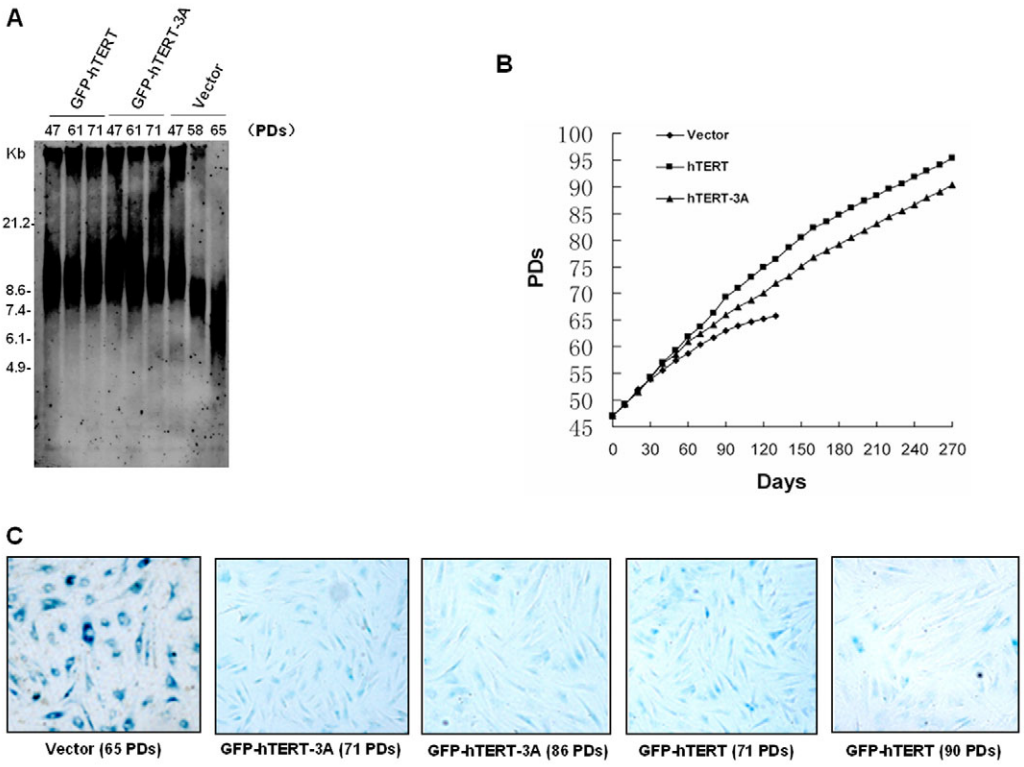

Fig. 6. Mutational disruption of TERT nucleolar localization does not affect its biological effects on maintaining telomere-length and extending replicative life-span in BJ fibroblasts. (A) Telomere lengths of corresponding stable BJ cells were analyzed at the indicated time points and showed that the telomere length of vector-infected BJ cells underwent a progressive shortening with the process of cultural propagation, whereas the telomere lengths both of GFP-TERT- and GFP–TERT-3A-expressing BJ cells were stably maintained during their successive propagation in culture. (B) Growth-curve analysis showed that vector-infected BJ cells ceased proliferation at around 65 PDs, whereas both GFP-TERT- and GFP–TERT-3A-expressing BJ cells still retained a robust proliferation rate at 92 and 86 PDs, respectively. (C) SA-β-gal assay showed that vector-infected BJ cells at 65 PDs became morphologically enlarged and displayed strong positive results of SA-β-gal staining, whereas both GFP-TERT- and GFP–TERT-3A-expressing BJ cells at the indicated time points were only faintly stained by the SA-β-gal assay and were without morphological changes. 100× magnification.

apoptotic effect independent of its telomerase catalytic activity (Fu et al., 1999; Rahman et al., 2005). There is also compelling evidence suggesting that the nucleolus not only provides a site for the completion of biogenesis of RNP complexes such as ribosomes, but also actively participates in regulating multiple cellular processes, including apoptosis (Horky et al., 2002). The successful identification in this study of the essential structural element for directing TERT nucleolar translocation will certainly facilitate the further elucidation of the molecular mechanisms and cellular biological significances of TERT nucleolar localization in human cells.

Materials and Methods

Cell culture

BJ, HeLa, H1299, U2OS and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (HyClone) and 20 μg/ml antibiotics (ampicilin and kanamycin), and maintained in an incubator with 5% CO_2 at 37°C.

Recombinants construction

DNA sequences encoding wild-type forms of the full-length TERT polypeptide and its indicated fragments (Fig. 1A) were prepared by PCR from the template plasmid pCI-neo-TERT (kindly provided by R. A. Weinberg, Whitehead Institute, MA). The resultant DNA fragments were further used as templates to construct their corresponding mutant forms containing the indicated point mutation (Fig. 2A) by the PCR method. For transient transfection, the indicated *TERT* cDNA fragments were inserted individually into the vector pLEGFP-C1 (Clontech) so as to be expressed as GFP-tagged fusion proteins. For stable transfection, GFP-tagged TERT and the mutant TERT-3A were cloned into the lentiviral vector pSL6 (Chen et al., 2007) (a kind gift from YangChao Chen, The Chinese University of Hong Kong, Hong Kong). The correction of these constructs was confirmed by DNA sequencing. Cellular transfection was performed with Lipofectimin-2000 reagent (Invitrogen) according to the manufacturer's instruction.

Subcellular localization analysis

The subcellular distribution patterns of ectopically expressed GFP-tagged TERT recombinants in their corresponding transfected cells were observed directly by fluorescence microscopy. Microscopic images were recorded by a cooled charged-coupled device camera and processed by the Photoshop software.

Immunoblotting and telomerase-activity assays

Cell extracts from stable BJ cell lines were fractioned by SDS-PAGE and transferred onto nitrocellulose membranes. Expression of GFP-tagged TERT recombinants was detected with antibodies specific for GFP (Clontech). For cellular telomerase enzymatic-activity detection, cell extracts were subjected to the TRAP assay (Kim and Wu, 1997).

Telomere lengths analysis

Genomic DNA from indicated stable BJ cells were doubly digested with *Hin*fI and *Rsa*I, separated on agarose gel, transferred onto PVDF membrane for hybridization with digosin-labeled 5′-(TTAGGG)-3′ oligos. The hybridization signal was detected by HRP-conjugated anti-digosin antibodies (Roche) and imaged by the ECL reagent.

SA-β-gal assay

At the indicated time points, cells were fixed with 2% formaldehyde/0.2% glutaradehyde/PBS and stained for SA β-gal as previously described (Huang et al., 2003).

This work was supported by grants from Chinese NCSF funds (30370704, 35070407) to J.J.H.

References

Aisner, D. L., Wright, W. E. and Shay, J. W. (2002). Telomerase regulation: not just flipping the switch. *Curr. Opin. Genet. Dev.* **12**, 80-85.

Blackburn, E. H. (2005). Telomeres and telomerase: their mechanisms of action and the effects of altering their functions. *FEBS Lett.* **579**, 859-862.

Bodnar, A. G., Ouellette, M., Frolkis, M., Holt, S. E., Chiu, C. P., Morin, G. B., Harley, C. B., Shay, J. W., Lichtseiner, S. and Wright, W. E. (1998). Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**, 349-352.

Chen, Y., Lin, M. C., Yao, H., Wang, H., Zhang, A. Q., Yu, J., Hui, C. K., Lau, G. K., He, M. L., Sung, J. et al. (2007). Lentivirus-mediated RNA interference targeting enchancer of zeste homolog 2 inhibits hepatocellular carcinoma growth through down-regulation of stathmin. *Hepatology* **46**, 200-208.

Dez, C., Henras, A., Faucon, B., Lafontaine, D., Caizergues-Ferrer, M. and Henry, Y. (2001). Stable expression in yeast of the mature form of human telomerase RNA depends on its association with the box H/ACA small nucleolar RNP proteins Cbf5p, Nhp2p and Nop10p. *Nucleic Acids Res.* **29**, 598-603.

Etheridge, K. T., Banik, S. S., Armbruster, B. N., Zhu, Y., Terns, R. M., Terns, M. P. and Counter, C. M. (2002). The nucleolar localization domain of the catalytic subunit of human telomerase. *J. Biol. Chem.* **277**, 24764-24770.

Feng, J., Funk, W. D., Wang, S. S., Weinrich, S. L., Avilion, A. A., Chiu, C. P., Adams, R. R., Chang, E., Allsopp, R. C., Yu, J. et al. (1995). The RNA component of human telomerase. *Science* **269**, 1236-1241.

Fu, W., Begley, J. G., Killen, M. W. and Mattson, M. P. (1999). Anti-apoptotic role of telomerase in pheochromocytoma cells. *J. Biol. Chem.* **274**, 7264-7271.

Hahn, W. C., Counter, C. M., Lundberg, A. S., Beijersbergen, R. L., Brooks, M. W. and Weinberg, R. A. (1999). Creation of human tumor cells with defined genetic elements. *Nature* **400**, 464-468.

Hatanaka, M. (1990). Discovery of the nucleolar targeting signal. *Bioessays* **12**, 143-148.

Hathcock, K. S., Jeffrey, C. Y. and Hodes, R. J. (2005). In vivo regulation of telomerase activity and telomere length. *Immunol. Rev.* **205**, 104-113.

Hayflick, L. (1973). The biology of human aging. *Am. J. Med. Sci.* **265**, 675-681.

Horky, M., Kotala, V., Anton, M. and Wesierska-Gadek, J. (2002). Nucleolus and apoptosis. *Ann. N. Y. Acad. Sci.* **973**, 258-264.

Huang, J. J., Lin, M. C., Bai, Y. X., Jing D. D., Wong, B. C., Han, S. W., Lin, J., Xu, B., Huang, C. F. and Kung, H. F. (2002). Ectopic expression of a COOH-terminal fragment of the human telomerase reverse transcriptase leads to telomere dysfunction and reduction of growth and tumorigenicity in HeLa cells. *Cancer Res.* **62**, 3226-3232.

Huang, J., Bai, Y. X., Han, S. W., Ng, S. S., Jing, D., Wong, B. C., Huang, C. F., Kung, H. F. and Lin, M. C. (2003). A human TERT C-terminal polypeptide sensitizes HeLa cells to H2O2-induced senescence without affecting telomerase enzymatic activity. *Biochem. Biophys. Res. Commun.* **301**, 627-632.

Kim, N. W. and Wu, F. (1997). Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). *Nucleic Acids Res.* **25**, 2595-2597.

Kim, N. W., Piatyzszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Weinrich, S. L. and Shay, J. W. (1994). Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**, 2011-2015.

Lin, J. and Blackburn, E. H. (2004). Nucleolar protein PinX1p regulates telomerase by sequestering its protein catalytic subunit in an inactive complex lacking telomerase RNA. *Genes Dev.* **18**, 387-396.

Lin, S. Y. and Elledge, S. C. (2003). Multiple tumor suppressor pathways negatively regulate telomerasse. *Cell* **113**, 881-889.

Masutomi, K., Yu, E. Y., Khurts, S., Ben-Porath, I., Currier, J. L., Metz, G. B., Brooks, M. W., Kaneko, S., Murakami, S., DeCaprio, J. A. et al. (2003). Telomerase maintains telomere structure in normal human cells. *Cell* **114**, 241-253.

Meyerson, M. J., Counter, C. M., Eaton, E. N., Ellisen, L. W., Steiner, P., Caddle, S. D., Ziaugra, L., Beijersbergen, R. L., Davidoff, M. J., Liu, Q. et al. (1997). hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in human tumor cells and during immortalization. *Cell* **90**, 785-795.

Mitchell, J. R., Wood, E. and Collins, K. (1999). A telomerase component is defective in the human disease dyskeratosis congenita. *Nature* **402**, 551-555.

Parkinson, K. (2005). Telomerase as a target for cancer therapy. *Curr. Opin. Investig. Drugs* **6**, 605-610.

Pogacic, V., Dragon, F. and Filipowicz, W. (2000). Human H/ACA small nucleolar RNPs and telomerase share evolutionarily conserved proteins NHP2 and NOP10. *Mol. Cell. Biol.* **20**, 9028-9040.

Rahman, R., Latonen, L. and Wiman, K. G. (2005). hTERT antagonizes p53-induced apoptosis independently of telomerase activity. *Oncogene* **24**, 1320-1327.

Stewart, S. A. and Weinberg, R. A. (2000). Telomerase and human tumorigenesis. *Semin. Cancer Biol.* **10**, 399-406.

Stewart, S. A., Hahn, W. C., O'Connor, B. F., Banner, E. N., Lundberg, A. S., Modha, P., Mizuno, H., Brooks, M. W., Fleming, M., Zimonjic, D. B. et al. (2002). Telomerase contributes to tumorigenesis by a telomere length-independent mechanism. *Proc Natl Acad Sci. USA* **99**, 12606-12611.

Tomlinson, R. L., Ziegler, T. D., Supakorndej, T., Terns, R. M. and Terns, M. P. (2006). Cell cycle-regulated trafficking of human telomerase to telomeres. *Mol. Biol. Cell* **17**, 955-965.

Wang, Y., Blandino, G. and Givol, D. (1999). Induced p21waf expression in H1299 cell line promotes cell senescence and protects against cytotoxic effect of radiation and doxorubicin. *Oncogene* **18**, 2643-2649.

Wong, J. M., Kusdra, L. and Collins, K. (2002). Subcellular shuttling of human telomerase induced by transformation and DNA damage. *Nat. Cell Biol.* **4**, 731-736.

Yang, Y., Chen, Y., Zhang, C., Huang, H. and Weissman, S. M. (2002). Nucleolar localization of hTERT protein is associated with telomerase function. *Exp. Cell Res.* **277**, 201-209.

Ye, K. (2005). Nucleophosmin/B23, a multifunctional protein that can regulate apoptosis. *Cancer Biol. Ther.* **96**, 463-472.

Zhu, Y., Tomlinson, R. L., Lukowiak, A. A., Terns, R. M. and Terns, M. P. (2004). Telomerase RNA accumulates in Cajal bodies in human cancer cells. *Mol. Biol. Cell* **15**, 81-90.