BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

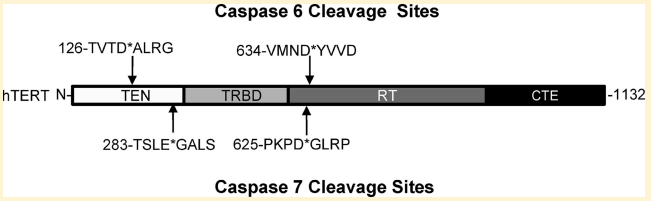
pubs.acs.org/biochemistry

The Catalytic Subunit of Human Telomerase Is a Unique Caspase-6 and Caspase-7 Substrate

Joana Soares, Margaret M. Lowe, and Michael B. Jarstfer*

Division of Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, CB 7568, Chapel Hill, North Carolina 27514, United States

ABSTRACT: Telomerase is a ribonucleoprotein complex that is essential for persistent cellular proliferation. The catalytic subunit of human telomerase, hTERT, functions as a reverse transcriptase and promotes vitality by maintaining telomeric DNA length. hTERT is tightly regulated with complex but poorly understood positive and negative regulation at several levels including transcription, protein−protein interactions, and post-translation modifications. Because evidence implicates hTERT as an apoptosis inhibitor and because telomerase activity tends to decrease during apoptosis, we hypothesized that hTERT is a caspase substrate leading to down regulation during apoptosis. Caspases are proteases that initiate and execute apoptosis by cleaving target proteins. Indeed, we found that caspases-6 and -7 cleave hTERT during apoptosis in cultured cells. Caspase-6 cleaves at residues D129 and D637, and caspase-7 cleaves at E286 and D628. Three of the caspase cleavage sites are unique motifs. All four caspase motifs appear conserved in TERTs from old world monkeys and apes, and the caspase-6 sites appear conserved in all primates. The caspase site that cleaves at D129 appears conserved in amniotes. hTERT fragments generated by cleavage were remarkably persistent, lasting hours after caspase activation. These results reveal a new biologically relevant mechanism for telomerase down regulation through caspase-mediated cleavage of hTERT and expand the list of known caspase motifs.



Telomerase is a ribonucleoprotein complex that extends the 3′ strand of each telomere in order to maintain the proper length of chromosome ends and ensure chromosome stability.[1,2] In addition to this essential role, telomerase functions away from telomeres appear to affect transformation, stem cell biology, proliferation, and survival.[3,4] Telomerase activity is generally absent from normal somatic cells but present in malignant tumors, consistent with its critical function in cellular immortalization and carcinogenesis.[5−8] In addition to roles in cancer, telomerase deficiencies have been linked to premature aging and age related disorders.[9,10]

Telomerase consists minimally of two subunits: telomerase RNA (TER), which serves as a template for telomeric DNA synthesis, and telomerase reverse transcriptase (TERT), a reverse transcriptase that adds telomeric repeats onto telomeres.[11−14] Human TER expression is regulated, but constitutive, whereas hTERT expression is upregulated in proliferative cells and repressed in nonproliferative cells.[15,16] Because of its importance, telomerase regulation has been the subject of several studies[16,17] but remains poorly understood. Understanding the basic roles of telomerase in cellular biology and physiology requires a more complete understanding of its activation and repression, and it is likely that the mechanisms of telomerase regulation vary with its biological functions.

The canonical biochemical telomerase activity, telomere maintenance by primer extension, requires both TERT and TER.[14,18,19] There is evidence for noncanonical telomerase functions away from the telomere, and some of these appear to require telomerase activity, whereas others do not.[10,20] For example, a suggested role of telomerase in DNA repair appears dependent on its catalytic activity but not on telomere lengthening.[21] Other noncanonical telomerase functions, such as inhibition of apoptosis[22−24] and activation of Wnt signaling,[4] do not appear to require telomerase catalytic activity. Ectopic expression of hTERT has been shown to increase resistance to apoptosis and necrosis,[22,25,26] and hTERT has been shown to prevent apoptosis independently of hTER and telomere extension.[27−29]

Defects in apoptosis regulation are associated with a variety of conditions including neurodegenerative,[30] cardiovascular,[31] and immune system disorders[32] and cancer.[33,34] Apoptosis is initiated and executed by caspases, a family of cysteine proteases that cleave substrates at specific tetrapeptide sites (P4−P3−P2−P1) where P1 is usually aspartate and occasionally glutamate.[35,36] Caspases can be separated into two groups: initiator caspases and effector caspases.[35,37] Initiator caspases, such as caspases-2, -8, -9, and -10, are activated by various signaling pathways and activate effector caspases-3, -6, and -7. Effector caspases ensure apoptosis by cleaving cellular substrates.[22,47] The cleavage of a substrate by a caspase can result in several effects including loss of function, gain of function, and changes in subcellular localization.[38,39]

Because telomerase and caspases play pivotal but opposite roles in the regulation of apoptosis, we hypothesized that

Received: July 6, 2011
Revised: September 12, 2011
Published: September 21, 2011

telomerase is negatively regulated by caspases. Here, we demonstrate that hTERT is both a caspase-6 and -7 substrate in apoptotic cells. Remarkably, cleavage occurs at noncanonical motifs that are conserved in primate TERTs, and cleavage results in the formation of stable hTERT-derived fragments.

MATERIALS AND METHODS

Materials. The following antibodies were used: rabbit anti-hTERT (Abcam, Ab32020, recognizes a C-terminal epitope), mouse anticaspase-6 (Cell Signaling), mouse anticaspase-7 (Cell Signaling), mouse anti-β-actin (Sigma, St. Louis, MO), HRP-conjugated anti-rabbit secondary antibody (GE biosciences), and HRP-conjugated goat anti-mouse secondary antibody (Thermo-Scientific). Peptides z-VAD-fmk, z-VEID-fmk, and ac-DEVD-fmk were purchased from Calbiochem. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), vincristine, etoposide, and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma-Aldrich. Active recombinant caspases (caspases-1−10) were from BioVision.

Cells and Cell Culture. The human chronic myelogenous leukemia K562 cell line was cultured in Dulbecco's modified Eagle/F12 medium (DMEM/F12) (Gibco) supplemented with 10% FBS. The transformed human embryonic kidney HEK293T cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. The peripheral T-cell leukemia Jurkat cell line was maintained in RPMI 1640 supplemented with 10% FBS. All cells were from ATCC.

Caspase Cleavage Reactions. Recombinant telomerase complexes were reconstituted in rabbit reticulocyte lysates and immunopurified using anti-Flag antibody beads as we previously described.[40] 10 μL of immunopurified, [^{35}S]-labeled, recombinant hTERT containing a C-terminal Flag epitope (Flag-hTERT), Flag-hTERT-hTER complex, or Flag-hTERT mutants were incubated with caspase (1 unit) in caspase buffer (50 mM Hepes, pH 7.2, 50 mM NaCl, 0.1% CHAPS, 10 mM EDTA, 5% glycerol, and 10 mM DTT) for 1 or 6 h at 37 °C. A general caspase inhibitor z-VAD-fmk (10 μM) was preincubated with caspase-7 for 30 min prior to incubation with Flag -hTERT-hTER as a control. Reaction products were resolved by SDS-PAGE and analyzed by phosphorimaging.

Mutagenesis. hTERT mutants were generated using a standard PCR-based site-directed mutagenesis protocol,[41] and mutations were verified by DNA sequencing.

Detection of Proteins by Western Blot. Cell lysates containing 50−100 μg of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with nonfat dry milk (5% w/v) in Tris-buffered saline containing 0.1% Tween-20 for 1 h, blotted with a primary antibody for hTERT, caspase-6, or caspase-7 for 1 h at room temperature, and then blotted with a HRP-conjugated secondary antibody for 1 h at room temperature. Blots were developed using ECL chemiluminescence detection reagent (Amersham Bioscience). Membranes were reprobed with anti-β-actin antibody as a loading control.

Treatment of Cells with Apoptosis Inducing Agents. HEK293T cells and K562 cells were incubated with CCCP (50 μM) or etoposide (50 μM) for various time points. Jurkat cells were incubated with vincristine (50 nM) for 12 or 24 h. In experiments with caspase inhibitors, cells were treated with 100 μM of z-VAD-fmk (a general caspase inhibitor), ac-DEVD-fmk (caspase-7 inhibitor), or z-VEID-fmk (caspase-6 inhibitor) for 1 or 3 h prior to treatment. Cells were lysed using Chaps lysis buffer (10 mM TrisHCl pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.5% CHAPS, 10% glycerol, supplemented before use with protease inhibitor cocktail III (Calbiochem) and 5 mM β-mercaptoethanol). hTERT cleavage products were analyzed by Western blot. Apoptotic cells were quantified by flow cytometry and cell viability was measured using the MTT assay. Telomerase activity was detected by TRAP assay in wild-type cells or direct assay in cells expressing recombinant telomerase as previously described.[40]

Transient Expression of Telomerase. HEK293T cells or K562 cells were transfected in 6-well plates using Lipofectamine 2000 (Invitrogen) and 4 μg of total plasmid DNA following the manufacturer's protocol: 0.66 μg of pVan107 to express hTERT and 3.33 μg of pBS-U1-hTER to express hTER were used.[42] Control transfections included empty vector, hTERT or mutant expressing plasmid only, and hTER expressing plasmid only. 24 h post-transfection, cells were trypsinized, transferred to a 25 cm^2 flask, and grown 24 h (HEK293T) or 48 h (K562). 48 or 72 h post-transfection, cells were trypsinized, washed once in PBS, and lysed in 400 μL of Chaps lysis buffer. After incubation at 4 °C for 30 min on a rotating wheel, cell debris was removed by centrifugation at 4 °C for 10 min at 13000*g*. The supernatant (~4 mg total protein/mL) was aliquoted, flash frozen in dry ice, and stored at −80 °C. Telomerase activity was detected by direct assay,[40] and hTERT was detected by Western blot.

Generation of Stable Caspase-6 and Caspase-7 Knockdown Cell Lines. K562 cells were plated in six-well plates and infected with shRNA-lentiviral vectors targeting either caspase-6 or -7 (Sigma-Aldrich, caspase-7: TRCN0000003519, caspase-6: TRCN0000003516). Cultures were grown to confluence, and knockdown of caspase-6 or -7 was confirmed by Western blot (anticaspase-6 or anticaspase-7, Cell Signaling). K562 transfected with shGFP was used as control. Lentivirus for caspase-6 and caspase-7 were produced by the UNC Viral Vector Core Facility.

RESULTS

Telomerase Is a Caspase Substrate in Vitro. To test the hypothesis that hTERT is a caspase substrate, we incubated immuopurified, recombinant telomerase (Flag-hTERT-hTER) with recombinant human caspases-1−10. Remarkably, caspases-6 and -7 each generated three major proteolytic fragments, demonstrating for the first time that hTERT is a caspase substrate (Figure 1A). Other caspases, 1, 5, and 8, appear to have some, if limited, ability to cleave hTERT. We did not follow up on these as we expect that these are nonspecific reactions owing to the excess of caspase used in the experiments and the long incubation times. The robust, rapid, and complete cleavage by caspases-6 and -7, by contrast, led us conclude the cleavage was specific. To ensure that cleavage of hTERT was not due to nonspecific degradation, we showed that cleavage with caspase-7 can be blocked with the general caspase inhibitor z-VAD-fmk (Figure 1A, compare lane 12 to lane 8). We also found that cleavage of hTERT was independent of hTER using affinity purified Flag-hTERT (data not shown). These results indicate that both caspase-6 and -7 cleave hTERT in vitro, cleavage is independent of assemblage of hTERT in the telomerase complex, and each caspase appears to cleave hTERT at two sites to generate three fragments.

hTERT Is a Caspase-6 and -7 Substrate in Apoptotic Cancer Cells. To determine if hTERT is a caspase substrate

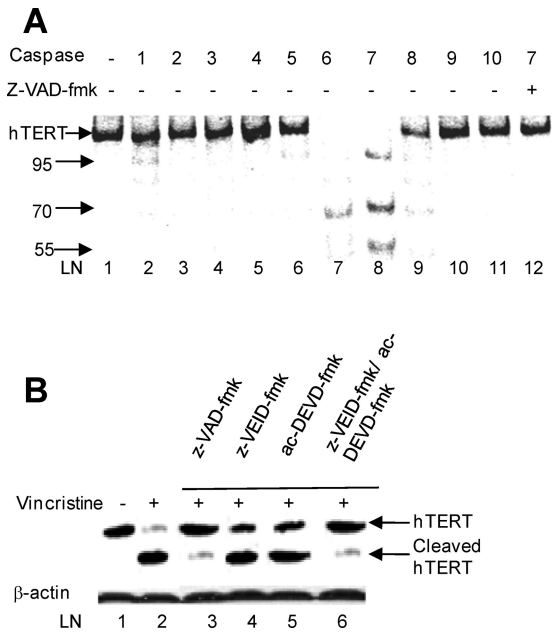


Figure 1. Recombinant [^{35}S]-Flag-hTERT is cleaved by caspase-6 and caspase-7. (A) Recombinant hTERT is a caspase substrate in vitro. Affinity purified recombinant Flag-hTERT-hTER complex was incubated with 1 unit of the indicated caspase. Caspases are identified above the gel, and the position of full-length hTERT is labeled hTERT. Molecular weight markers are approximate and indicated by arrows on the right. Caspase inhibitor z-VAD-fmk was added in one caspase-7 reaction (lane 12). (B) Endogenous hTERT is a cellular caspase substrate. Jurkat cells were treated with vincristine and hTERT was detected in whole cell extracts by Western blotting. Cells were preincubated with a general caspase inhibitor (lane 3), with a caspase-6 or -7 inhibitor (lanes 4 and 5, respectively), or with a combination of caspase-6 and -7 inhibitors (lane 6). Full length and hTERT fragments are indicated with arrows to the right of the blots. *β*-Actin was used as a loading control.

in apoptotic cells, we treated Jurkat cells with vincristine in the presence or absence of the general caspase inhibitor z-VAD-fmk, the caspase-6 inhibitor z-VEID-fmk, the caspase-7 inhibitor ac-DEVD-fmk, or with a combination of both caspase-6 and -7 inhibitors. As predicted from our in vitro studies, cellular hTERT was cleaved in cells treated with vincristine and the general caspase inhibitor z-VAD-fmk prevented cleavage (Figure 1B). Preincubation with either a caspase-6 inhibitor or a caspase-7 inhibitor only partially protected hTERT from caspase cleavage, but a combination of caspase-6 and -7 inhibitors afforded complete protection (Figure 1B, compare lanes 4 and 5 to lane 6). These data suggest that hTERT is a substrate for both caspase-6 and -7 in apoptotic cells.

To determine if hTERT was a caspase substrate in other cell types, we treated HEK293T and K562 with etoposide or with the mitochondrial depolarizing agent CCCP and probed hTERT cleavage by Western blot. Full length hTERT levels decreased concomitant with the appearance of new hTERT-antibody reactive peptides, as expected if hTERT is cleaved by a caspase (Figure 2). Notably, in each cell line tested cleavage of hTERT was rapid and resulted in stable hTERT peptide fragments that remained for several hours.

Identification of Caspase Cleavage Sites in hTERT. To investigate the biological importance of hTERT cleavage, caspase resistant hTERT mutants were generated. Bioinformatics analysis using PeptideCutter yielded no predictable caspase-6 or -7 sites, and CASVM[43] and Cascleave[44] predicted over 20 sites combined. To map the location of potential sites, we first analyzed the cleavage patterns of hTERT truncation mutants: hTERT fragments 1–927, 201–1132, and 401–1132.[45] On the basis of the cleavage patterns of the truncation mutants, we determined that one of the cleavage sites was between amino acids 100 and 200, another was between 250 and 400, and the other two were near amino acid 650. On the basis of these date, we mutated possible cleavage sites by changing select aspartate and glutamate residues to alanine. Both aspartate and glutamate residues were included because numerous identified substrates contain a glutamic acid residue at the P1 position.[43] Mutation at several sites predicted by CASVM[43] and Cascleave[44]: D442, D444, D516, D768, and D936 did not prevent cleavage in vitro (data not shown). However, mutation of both D129 and D637 to alanine blocked caspase-6 cleavage and mutation of both E286 and D628 to alanine blocked cleavage by caspase-7 (Figure 3). Remarkably, these are unique caspase recognition motifs; only TVTD has previously been shown to be a caspase recognition motif, and

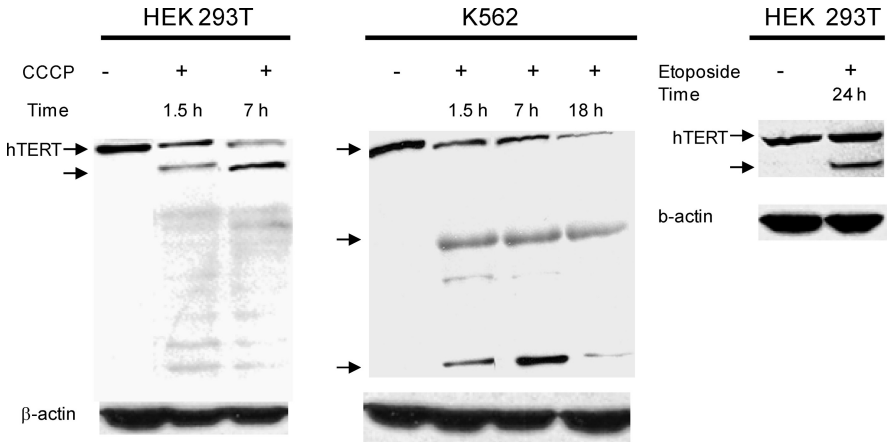


Figure 2. hTERT is a caspase substrate in HEK293T and K562 cells. hTERT was detected in cell extracts by Western blotting. (A) Western blot of HEK293T cells after treatment with CCCP for the indicated time. (B) Western blot of K562 cells after treatment with CCCP for the indicated time. (C) Western blot of HEK293T cells after treatment with etoposide for the indicated time. hTERT and stable hTERT-antibody reactive fragments are indicated by arrows. *β*-Actin was used as a loading control.

dx.doi.org/10.1021/bi2010398 | *Biochemistry* 2011, 50, 9046–9055

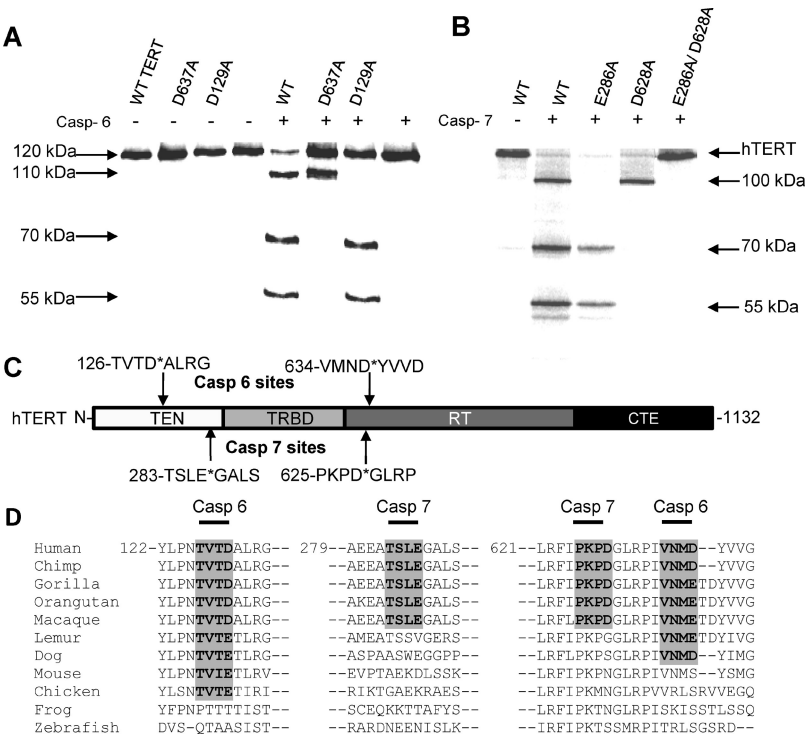


Figure 3. hTERT mutants prevent caspases-6 and -7 cleavage. Recombinant, [^{35}S]-Flag-hTERT or the indicated Flag-hTERT mutants were incubated with (A) caspase-6 or (B) caspase-7. Full-length hTERT (1132 amino acids, 120 kDaA) and cleavage products are indicated with approximate molecular weights indicated. (C) The position and sequences of the caspase recognition motifs are displayed on hTERT. The hTERT sequence is displayed as a rectangle with conserved regions shaded: the N-terminal extension or TEN domain is light gray (TEN), the RNA-binding domain is gray (TRBD), the RT domain is dark gray (RT), and the C-terminal extension is the black (CTE). (D) Sequence alignment of several vertebrate TERTs. Predicted caspase-6 and -7 motifs are shaded.

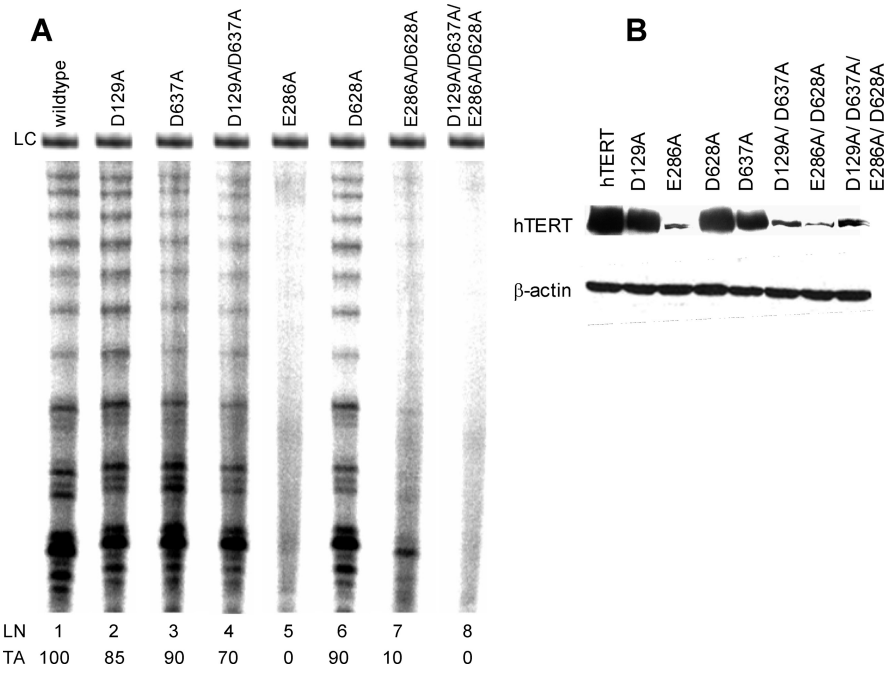


Figure 4. Caspase resistant hTERT mutants have variable telomerase activity and cellular expression levels. (A) Telomerase activity of recombinant, immunopurified mutants was determined by a direct primer extension assay and percent telomerase activity is reported with respect to wild-type hTERT. All activities were normalized to a [^{32}P]-labeled 100 nt DNA used as a loading control (LC). Lane 1, wild-type hTERT; lane 2, D129A hTERT mutant; lane 3, D637A hTERT mutant; lane 4, D129A/D637A hTERT mutant; lane 5, E286A hTERT mutant; lane 6, D628A hTERT mutant; lane 7, E286A/D628A hTERT mutant; lane 8, D129A/D637A/E286A/D628A hTERT mutant. Wild-type hTERT and hTERT mutants were assembled with hTER and telomerase activity was detected by direct assay.[30] (B) Expression levels of recombinant hTERT and caspase resistant mutants were detected by Western blot 72 h after transient transfection of K562 cells.

dx.doi.org/10.1021/bi2010398|*Biochemistry* 2011, 50, 9046–9055

the other three have not been observed to the best of our knowledge.[46] Analysis of available TERT sequences suggests that the TVTD motif has been conserved since the early amniotes, whereas the two caspase-6 sites appear to be specifically conserved in old world monkeys and apes (Figure 3C).

Telomerase was reconstituted in vitro with the caspase resistant hTERT mutants. We found that caspase-6 resistant mutants D129A, D637A, and D129A/D637A were active in telomerase assays (Figure 4A, lanes 2–4). Caspase-7 resistant mutant D628A retained telomerase activity whereas E286A and the double mutant E286A/D628A were inactive (Figure 4A, lanes 5–7). The caspase-6 and -7 resistant mutant D129A/D637A/E286A/D628A was completely devoid of telomerase activity as expected since it contained the inactivating E286A mutation (Figure 4A, lane 8).

Both Caspase-6 and -7 Cleave hTERT in Apoptotic Cells. To confirm that caspase-6 and -7 are responsible for hTERT cleavage in cells, we transfected K562 cells with wild-type or caspase resistant hTERT mutants and analyzed hTERT cleavage after treatment with CCCP. We explored transient transfection as a simple system to examine the behavior of the caspase resistant mutants in cells. Western blot analysis revealed that hTERT expression was decreased for the enzymatic-inactive mutants that contained E286 as well as D129A/D637A (Figure 4B). This suggests that these mutations are destabilizing. Importantly, we found that recombinant hTERT was a target of caspase cleavage, and its activity decreased during apoptosis (Figure 5). Cells expressed similar

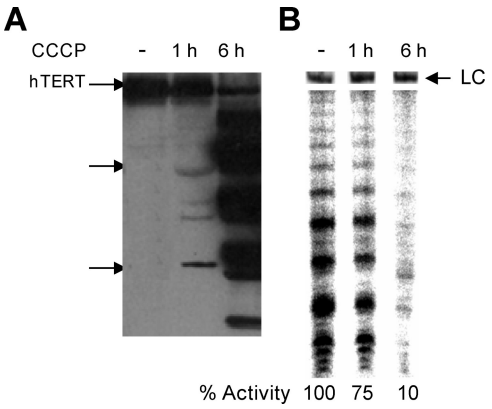


Figure 5. Recombinant hTERT is a cellular caspase substrate. (A) Proteolytic cleavage of recombinant hTERT expressed in transiently transfected K562 cells after CCCP treatment. (B) Activity of recombinant telomerase in transiently transfected K562 cells after CCCP treatment. Telomerase activity was detected by primer extension assays using crude cell extracts.[30]

levels of recombinant hTERT or hTERT mutants when transfected in the absence of the telomerase RNA-expressing plasmid pBS-U1-hTER (see Figure 6 lanes marked with a minus sign), so experiments below were generally performed with only hTERT-expressing plasmid. In addition, cells expressed significantly more recombinant hTERT than endogenous hTERT. We estimate ~100-fold higher expression of recombinant hTERT based on telomerase activity and Western blot (see Figure 6A for example, where the lane showing the per-caspase resistant mutant no cleavage products from endogeneous hTERT are visible). These studies demonstrate that transient transfection of hTERT expressing

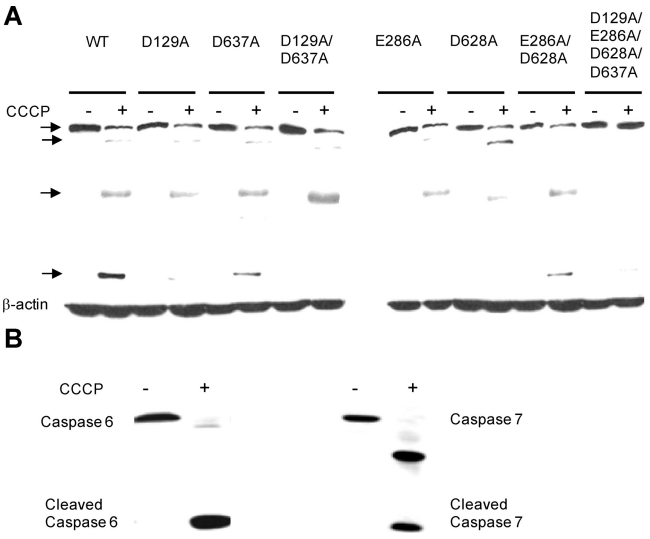


Figure 6. Mutation at predicted caspase cleavage sites protects hTERT in cells. (A) Proteolytic cleavage of transiently transfected hTERT or hTERT mutants in K562 cells 1.5 h after treatment with CCCP. (B) Caspase-6 and caspase-7 are activated 1.5 h after incubation of K562 cells with CCCP.

plasmids is sufficient for a preliminary examination of the molecular consequences of caspase activity on hTERT and hTERT mutants in cells.

We then examined cleavage patterns of caspase resistant hTERT mutants after CCCP treatment. Mutants were predictably resistant to caspsase cleavage at the sites of mutation, and only the hTERT mutant D129A/D637A/E286A/D628A was completely resistant to cleavage in cells, as expected (Figure 6A). The K562 cell line expresses both caspase-6 and -7, and both caspase-6 and -7 were activated after CCCP treatment, indicating that hTERT cleavage occurred concomitantly with caspase activation (Figure 6B). To further confirm that both caspase-6 and -7 contribute to hTERT cleavage, we transiently transfected hTERT or hTERT mutants into K562 cells that stably express caspase-6 or -7 shRNA and examined the effect of CCCP treatment on hTERT cleavage. We found that the caspase-6 resistant hTERT mutant D129A/D637A remained intact in caspase-7 knockdown cells and the caspase-7 resistant hTERT mutant E286A/D628A remained intact in caspase-6 knockdown cells (Figure 7). Taken together, our results confirm that caspase-6 and caspase-7 cleave hTERT at four unique sites (Figure 3C).

Effect of Caspase Resistant hTERT Mutants on Cell Viability. Several studies have demonstrated that telomerase can inhibit or activate apoptosis depending upon cell type and experimental conditions.[3,23,25,47] We anticipate that caspase resistant hTERT mutants will be useful for investigating the role of telomerase in apoptosis and its regulation by caspases. To explore this possibility, we examined if transient transfection of wild-type or caspase resistant hTERT mutants affected cell viability of CCCP-treated K562 cells. Control experiments showed that transient transfection of hTERT or hTER-expressing plasmids alone or in combination had no effect on CCCP-induced apoptosis (Figure 8 and data not shown). Since equivalent levels of hTERT mutants were expressed in the absence of telomerase RNA overexpression (see the untreated, i.e., CCCP minus, lanes in Figure 6A and data not shown), experiments were conducted with cells transfected with hTERT

dx.doi.org/10.1021/bi2010398 | Biochemistry 2011, 50, 9046–9055

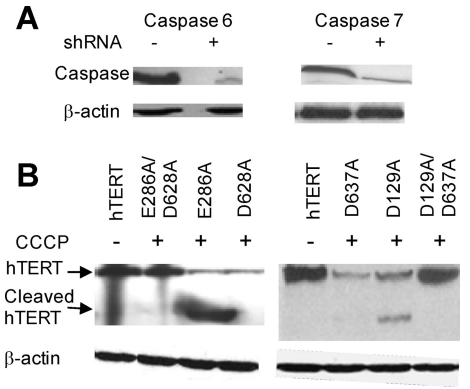


Figure 7. Caspase-6 resistant hTERT is fully protected in caspase-7 knockdown cells and caspase-7 resistant hTERT is fully protected in caspase-6 knockdown cells. (A) K562 cells were engineered to stably express caspase-6 or caspase-7 shRNA, and knockdown was confirmed by Western blot. (B) Cleavage products of caspase-7 resistant hTERT mutants were analyzed by Western blot in caspase-6 knockdown cells after CCCP treatment. (D) Cleavage products of caspase-6 resistant mutants were analyzed by Western blot in caspase-7 knockdown cells after CCCP treatment.

expressing plasmids only. We found that cells expressing D628A, D637A, or D129A/D637A/E286A/D628A hTERT mutants were less sensitive to CCCP treatment when compared to cells transfected with wild-type hTERT (Figure 8A,C). Interestingly, expression of hTERT D129A resulted in modestly increased sensitivity to CCCP (Figure 8A). The observed effects of hTERT mutants on CCCP-induced cell death does not appear to be dependent on telomerase activity as inactive mutants, for example D129A/D637A/E286A/D628A hTERT, protected cells despite not reconstituting telomerase activity.

To explore the individual contributions of hTERT cleavage specifically at caspase-6 and -7 sites to cell viability, we expressed caspase-6 resistant hTERT mutants in caspase-7 knockdown cells and caspase-7 resistant hTERT mutants in caspase-6 knockdown cells and analyzed cell viability after CCCP treatment (Figure 8B,D). Overall, the effects were modest, though under some condition as much as a 2-fold increase in survival was observed (Figure 8D, 100 μM CCCP). In addition, some interesting trends were observed. For example, in wild-type cells, only mutants D129 and E286 failed to provide any greater protection against CCCP than

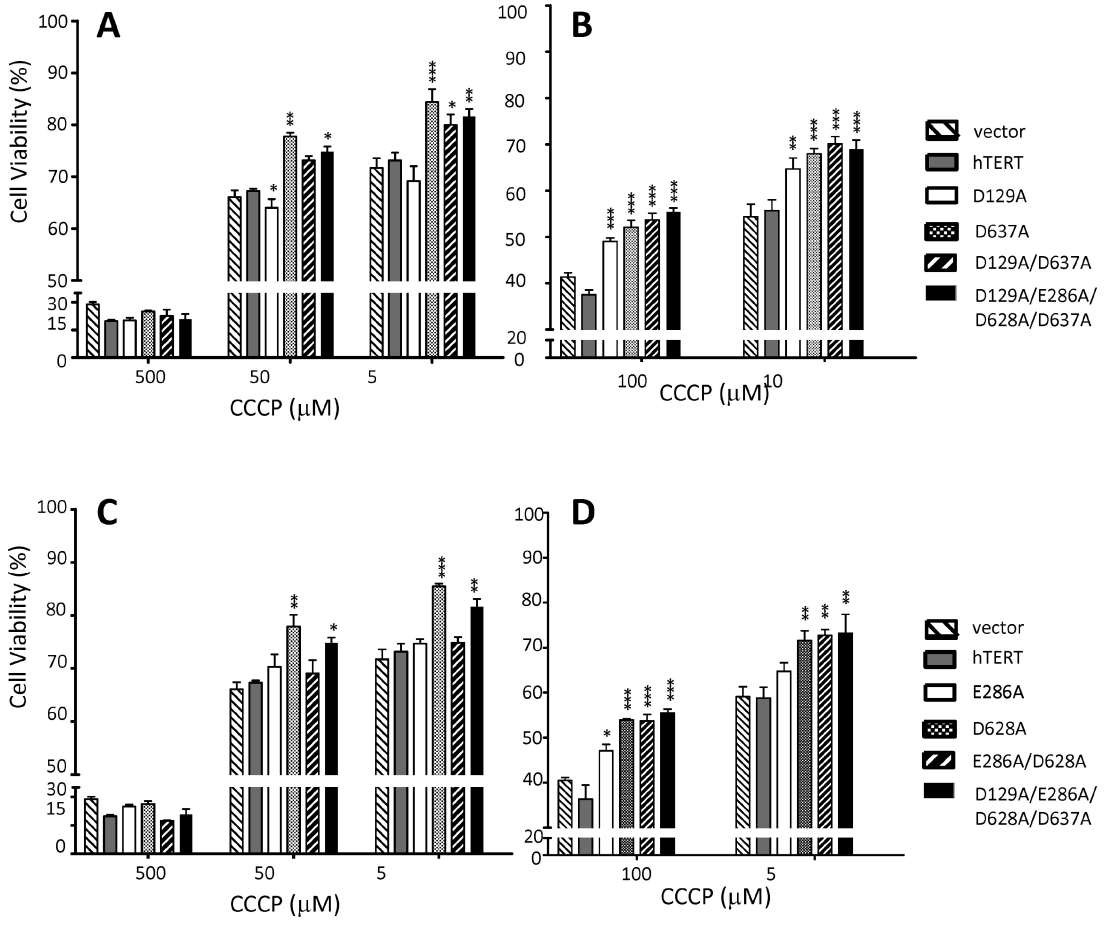


Figure 8. Caspase-6 and -7 resistant mutants affect viability of CCCP-treated K562 cells. K562 cells or K562 cells stably transfected with caspase-6 or -7 shRNA were transiently transfected with hTERT or caspase resistant mutants followed by treatment with CCCP (50 μM) for 72 h. Cell viability was determined after 72 h of treatment using the MTT assay. Results represent the mean ± SEM of three independent experiments performed in triplicates. Statistical significance: *P*-values are (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ for cells transfected with caspase resistant mutants versus cells transfected with wild-type hTERT. (A) Wild-type K562 cells transfected with wild-type hTERT or caspase-6 resistant mutants were treated with CCCP. (B) K562 cells stably transfected with caspase 7 shRNA were transfected with wild-type hTERT or caspase-6 resistant mutants and treated with CCCP. (C) Wild-type K562 cells transfected with wild-type hTERT or caspase-7 resistant mutants were treated with CCCP (D) K562 cells stably transfected with caspase 6 shRNA were transfected with wild-type hTERT or caspase-7 resistant mutants and treated with CCCP.

dx.doi.org/10.1021/bi2010398|*Biochemistry* 2011, 50, 9046–9055

wild-type hTERT (Figure 8A,C). In caspase knockdown cells, however, each caspase resistant hTERT mutant was protective (Figure 8B,D). Interestingly, in contrast to the results observed with wild-type K562 cells, the D129A mutant was more resistant to apoptosis than cells overexpressing wild-type hTERT in caspase-7 knockdown cells (compare Figures 8A and 8B). These results further validate the hypothesis that caspases cleave hTERT as part of the apoptosis pathway.

DISCUSSION

Telomerase enhances cellular proliferation and promotes genetic stability by helping maintain telomeres as well as other putative noncanonical functions.[4,10,20] Because of its role in proliferation, telomerase activity is upregulated in most cancer cells and stem cells and during the proliferative phase of a variety of cell types including activated lymphocytes.[16,48−50] Given its prominent role in preserving proliferative potential, it is not surprising that antiproliferative agents, for example p53, downregulate telomerase.[51] To date, several mechanisms for telomerase downregulation have been reported including decreased hTERT transcription,[16,52,53] altered cellular localization,[54] changes in hTERT phosphorylation status,[55] and altered splicing of hTERT mRNA.[56,57] Here, we show for the first time that hTERT, the catalytic subunit of telomerase, is downregulated by caspase-6 and -7. We identified four caspase cleavage sites, two sites for caspase-6 and two for caspase-7 (Figure 3C). Remarkably, three of the caspase recognition motifs are unique: TSLE, VNMD, and PKPD. Only the caspase cleavage motif TVTD had been previously identified as a cleavage site in another protein: the antiapoptotic baculovirus p49 protein, which contains a TVTD motif that functions as a suicide substrate-type caspase inhibitor.[46] It is interesting that mutation of the TVTD motif in hTERT was modestly sensitizing to CCCP (Figure 8), which is the expected result if the hTERT TVTD motif inhibits caspase activity.

One question this study provokes is why would caspases target hTERT? Caspases are involved in both apoptotic and nonapoptotic functions and are critical players in development and inflammation.[58] Telomerase is also involved in apoptosis, acting primarily as an inhibitor of apoptosis through its ability to extend telomeric DNA and potentially through noncanonical activities.[20] Moreover, positive and negative regulation of hTERT coincides with proliferation during development, lymphocyte activation, and stem cell expansion and differentiation, and new roles of hTERT in these pathways continue to be revealed.[53,59] The observation that hTERT is a caspases-6 and -7 substrate identifies a previously unknown mechanism of telomerase regulation with implications for a number of biologically important processes. In addition, the observation that hTERT mutants that prevent caspase cleavage have modest but statistically significant effects on apoptosis is consistent with a biological role for this interaction.

An intriguing biological overlap between telomerase and caspase activity is in neuronal apoptosis induced by ischemic brain injury[28,60] and Alzheimer's disease (AD).[61−63] The p10 fragment corresponding to active caspase-6 is detected in human brain tissue and is elevated in brain samples from AD patients,[61] and mounting evidence suggests that decreased telomerase activity and telomere shortening in neurons also correlates with neurodegenerative disorders.[64,65] In addition, studies have shown that hTERT expression is decreased in neurons of AD patients.[66] Since caspase cleavage is a major factor in neuronal degeneration in AD and caspase-6 is the main caspase implicated in neuronal dysfunction and AD pathogenesis, it is possible that the decreased telomerase activity, hTERT levels, and the loss of nuclear localization of hTERT in AD are due in part to proteolytic cleavage of hTERT by caspase-6. Our results provide a basis for further studies linking neuronal damage, telomere deterioration, caspase activation, and hTERT cleavage.

An important consideration is the biochemical consequences of hTERT cleavage by caspases. hTERT is cleaved at several key positions within the polypeptide chain. D129, a site of caspase-6 cleavage, is located in GQ motif of the conserved N-terminus, also known as the TEN or DAT domain. The N-terminus is critical for telomere recruitment, is involved in telomerase RNA-binding by hTERT, and may promote telomerase processivity.[67−70] Perhaps more germane to apoptosis is a mitochondrial localization leader sequence in hTERT located within the first 20 amino acids of its N-terminus.[47] The role of telomerase in the mitochondria is poorly understood, and many reports demonstrate that hTERT inhibits apoptosis, reduces oxidative stress, and provides stress resistance,[71−73] though there is evidence that under some conditions hTERT may have a pro-apoptotic role.[47] We showed that blocking cleavage at D129 lead to a modest increase in sensitivity to CCCP treatment. Perhaps, mutation of D129 affects localization of hTERT fragments to the mitochondria. For example, since the proteolytic fragment generated from the D129A hTERT is a non-natural fragment of hTERT, it might have a dominant negative effect on the hTERT protective functions associated with mitochondrial localization.

The other N-terminal caspase cleavage site, E286, is located in the flexible linker between the N-terminal TEN domain and the RNA binding domain, TRBD.[74] hTERT-E286A was found to be inactive when reconstituted with hTER both in vitro and in cells. Perhaps hTERT containing the E286A mutations is less stable than wild-type as indicated by its decreased expression in cells compared to wild-type hTERT when coexpressed with the telomerase-RNA expressing plasmid (see Figure 4B). The other hTERT caspase cleavage sites, D628 and D637, are surrounded by highly conserved residues and are located in the reverse transcriptase (RT) domain. D628 is located at the end of motif 1 and D637 is located in motif 2. Both D628A and D637A hTERT mutants provided moderate resistance to CCCP toxicity and both were catalytically active. Importantly, the hTERT mutant D129A/E286A/D628A/D637A, which is catalytically inactive, conferred protection when compared to cells transfected with wild-type hTERT. This indicates that the increased cell viability we observed in Figure 8 was directly related to hTERT cleavage, independent of telomerase activity.

The effects of the various caspase resistant hTERT mutants on viability in wild-type and caspase knockdown cells suggest that the products of hTERT cleavage have variable effects depending on the site(s) of cleavage. For example, in caspase-7 knockdown cells, the hemi caspase-6 resistant D129A mutant resulted in decreased sensitivity to CCCP, whereas in wild-type K562 cells, the D129A mutant conferred increased sensitivity to CCCP. A substantial portion of caspase substrates are cleaved into stable domains, suggesting that physical separation of functional domains can lead not only to protein inactivation but also to production of dominant negative fragments or fragments with new activities.[36,75] hTERT appears to generate stable domains after caspase cleavage (see Figure 2). Combined with the different effects of the caspase mutants, it suggests that

each hTERT fragment may have unique biological activity. For example, it is possible that each caspase-cleavage product could differentially impact the formation of the telomerase holoenzyme complex, apoptosis, or inhibit (or activate) proposed noncanonical hTERT functions. The fact that several hTERT fragments persist after cleavage in cells portends a gain of function. Future studies will reveal the phenotypes, if any, of these fragments.

In conclusion, we have shown that hTERT is a caspase-6 and -7 substrate and hTERT cleavage attends apoptosis. Caspase-6 and -7 cleavage of hTERT results in stable fragments, and cleavage occurs at four separate sites that appear conserved in old world monkeys and apes. Three of the cleavage sites are unique, and one is a noncanonical site previously observed in only one other protein. Finally, we generated caspase resistant hTERT mutants that confirmed the location of cleavage sites and will allow the biological roles of hTERT cleavage to be carefully examined in future experiments.

AUTHOR INFORMATION

Corresponding Author

*Phone: +1 919 966-6422. Fax: +1 919 966-0204. E-mail: jarstfer@unc.edu.

Funding

This work was supported by grants from the National Science Foundation, MCB-0751372, and the American Federation of Aging Research.

ACKNOWLEDGMENTS

We thank Lea Harrington for plasmids expressing the hTERT truncation mutants and Joachim Lingner for pVan107 and pBS-U1-hTER.

ABBREVIATIONS

TERT, telomerase reverse transcriptase; TER, telomerase RNA; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AD, Alzheimer's disease; TRAP, telomere repeat amplification protocol; CHAPS, 3[(3-cholamidopropyl)-dimethylammonio]propanesulfonic acid; DTT, (2*S*,3*S*)-1,4-bis(sulfanyl)butane-2,3-diol; PBS, phosphate buffered saline; shRNA, short-hairpin RNA; HRP, horse radish peroxidase; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

REFERENCES

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

(2) Marion, R. M., and Blasco, M. A. (2010) Telomere rejuvenation during nuclear reprogramming. *Curr. Opin. Genet. Dev. 20*, 190−196.

(3) Cong, Y., and Shay, J. W. (2008) Actions of human telomerase beyond telomeres. *Cell Res. 18*, 725−732.

(4) Park, J. I., Venteicher, A. S., Hong, J. Y., Choi, J., Jun, S., Shkreli, M., Chang, W., Meng, Z., Cheung, P., Ji, H., McLaughlin, M., Veenstra, T. D., Nusse, R., McCrea, P. D., and Artandi, S. E. (2009) Telomerase modulates Wnt signalling by association with target gene chromatin. *Nature (London) 460*, 66−72.

(5) Artandi, S. E., and DePinho, R. A. (2010) Telomeres and telomerase in cancer. *Carcinogenesis 31*, 9−18.

(6) Blasco, M. A. (2005) Telomeres and human disease: ageing, cancer and beyond. *Nat. Rev. Genet. 6*, 611−622.

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

(8) Oulton, R., and Harrington, L. (2000) Telomeres, telomerase, and cancer: life on the edge of genomic stability. *Curr. Opin. Oncol. 12*, 74−81.

(9) Aubert, G., and Lansdorp, P. M. (2008) Telomeres and aging. *Physiol. Rev. 88*, 557−579.

(10) Moon, I. K., and Jarstfer, M. B. (2007) The human telomere and its relationship to human disease, therapy, and tissue engineering. *Front. Biosci. 12*, 4595−4620.

(11) Collins, K. (2006) The biogenesis and regulation of telomerase holoenzymes. *Nat. Rev. Mol. Cell Biol. 7*, 484−494.

(12) 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.

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

(14) Nakamura, T. M., Morin, G. B., Chapman, K. B., Weinrich, S. L., Andrews, W. H., Lingner, J., Harley, C. B., and Cech, T. R. (1997) Telomerase catalytic subunit homologs from fission yeast and human. *Science 277*, 955−959.

(15) Cong, Y. S., Wright, W. E., and Shay, J. W. (2002) Human telomerase and its regulation. *Microbiol. Mol. Biol. Rev. 66*, 407−425.

(16) Cairney, C. J., and Keith, W. N. (2008) Telomerase redefined: integrated regulation of hTR and hTERT for telomere maintenance and telomerase activity. *Biochimie 90*, 13−23.

(17) Cerone, M. A., Burgess, D. J., Naceur-Lombardelli, C., Lord, C. J., and Ashworth, A. (2011) High-throughput RNAi screening reveals novel regulators of telomerase. *Cancer Res. 71*, 3328−3340.

(18) Weinrich, S. L., Pruzan, R., Ma, L., Ouellette, M., Tesmer, V. M., Holt, S. E., Bodnar, A. G., Lichtsteiner, S., Kim, N. W., Trager, J. B., Taylor, R. D., Carlos, R., Andrews, W. H., Wright, W. E., Shay, J. W., Harley, C. B., and Morin, G. B. (1997) Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTERT. *Nature Genet. 17*, 498−502.

(19) Beattie, T. L., Zhou, W., Robinson, M. O., and Harrington, L. (1997) Reconstitution of human telomerase activity *in vitro*. *Curr. Biol. 8*, 177−180.

(20) Martinez, P., and Blasco, M. A. (2011) Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins. *Nat. Rev. Cancer 11*, 161−176.

(21) Masutomi, K., Possemato, R., Wong, J. M., Currier, J. L., Tothova, Z., Manola, J. B., Ganesan, S., Lansdorp, P. M., Collins, K., and Hahn, W. C. (2005) The telomerase reverse transcriptase regulates chromatin state and DNA damage responses. *Proc. Natl. Acad. Sci. U. S. A. 102*, 8222−8227.

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

(23) Cao, Y., Li, H., Deb, S., and Liu, J. P. (2002) TERT regulates cell survival independent of telomerase enzymatic activity. *Oncogene 21*, 3130−3138.

(24) Del Bufalo, D., Rizzo, A., Trisciuoglio, D., Cardinali, G., Torrisi, M. R., Zangemeister-Wittke, U., Zupi, G., and Biroccio, A. (2005) Involvement of hTERT in apoptosis induced by interference with Bcl-2 expression and function. *Cell Death Differ. 12*, 1429−1438.

(25) Gorbunova, V., Seluanov, A., and Pereira-Smith, O. M. (2002) Expression of human telomerase (hTERT) does not prevent stress-induced senescence in normal human fibroblasts but protects the cells from stress-induced apoptosis and necrosis. *J. Biol. Chem. 277*, 38540−38549.

(26) Holt, S. E., Glinsky, V. V., Ivanova, A. B., and Glinsky, G. V. (1999) Resistance to apoptosis in human cells conferred by telomerase function and telomere stability. *Mol. Carcinog. 25*, 241−248.

(27) Fu, W., Killen, M., Culmsee, C., Dhar, S., Pandita, T. K., and Mattson, M. P. (2000) The catalytic subunit of telomerase is expressed in developing brain neurons and serves a cell survival-promoting function. *J. Mol. Neurosci. 14*, 3−15.
(28) Kang, H. J., Choi, Y. S., Hong, S. B., Kim, K. W., Woo, R. S., Won, S. J., Kim, E. J., Jeon, H. K., Jo, S. Y., Kim, T. K., Bachoo, R., Reynolds, I. J., Gwag, B. J., and Lee, H. W. (2004) Ectopic expression of the catalytic subunit of telomerase protects against brain injury resulting from ischemia and NMDA-induced neurotoxicity. *J. Neurosci. 24*, 1280−1287.
(29) Lee, J., Sung, Y. H., Cheong, C., Choi, Y. S., Jeon, H. K., Sun, W., Hahn, W. C., Ishikawa, F., and Lee, H. W. (2008) TERT promotes cellular and organismal survival independently of telomerase activity. *Oncogene 27*, 3754−3760.
(30) Bulat, N., and Widmann, C. (2009) Caspase substrates and neurodegenerative diseases. *Brain Res. Bull. 80*, 251−267.
(31) Narula, J., Haider, N., Arbustini, E., and Chandrashekhar, Y. (2006) Mechanisms of disease: apoptosis in heart failure--seeing hope in death. *Nat. Clin. Pract. Cardiovasc. Med. 3*, 681−688.
(32) Oliveira, J. B., and Gupta, S. (2008) Disorders of apoptosis: mechanisms for autoimmunity in primary immunodeficiency diseases. *J. Clin. Immunol. 28* (Suppl. 1), S20−28.
(33) Hanahan, D., and Weinberg, R. A. (2000) The hallmarks of cancer. *Cell 100*, 57−70.
(34) Green, D. R., and Evan, G. I. (2002) A matter of life and death. *Cancer Cell 1*, 19−30.
(35) Degterev, A., Boyce, M., and Yuan, J. (2003) A decade of caspases. *Oncogene 22*, 8543−8567.
(36) Dix, M. M., Simon, G. M., and Cravatt, B. F. (2008) Global mapping of the topography and magnitude of proteolytic events in apoptosis. *Cell 134*, 679−691.
(37) Kumar, S. (2007) Caspase function in programmed cell death. *Cell Death Differ. 14*, 32−43.
(38) Demon, D., Van Damme, P., Vanden Berghe, T., Vandekerckhove, J., Declercq, W., Gevaert, K., and Vandenabeele, P. (2009) Caspase substrates: easily caught in deep waters? *Trends Biotechnol. 27*, 680−688.
(39) Timmer, J. C., and Salvesen, G. S. (2007) Caspase substrates. *Cell Death Differ. 14*, 66−72.
(40) Fu, Y. T., Keppler, B. R., Soares, J., and Jarstfer, M. B. (2009) BRACO19 analog dimers with improved inhibition of telomerase and hPot 1. *Bioorg. Med. Chem. 17*, 2030−2037.
(41) Zheng, L., Baumann, U., and Reymond, J. L. (2004) An efficient one-step site-directed and site-saturation mutagenesis protocol. *Nucleic Acids Res. 32*, e115.
(42) Cristofari, G., Reichenbach, P., Regamey, P. O., Banfi, D., Chambon, M., Turcatti, G., and Lingner, J. (2007) Low- to high-throughput analysis of telomerase modulators with Telospot. *Nature Methods 4*, 851−853.
(43) Wee, L. J., Tan, T. W., and Ranganathan, S. (2007) CASVM: web server for SVM-based prediction of caspase substrates cleavage sites. *Bioinformatics 23*, 3241−3243.
(44) Song, J., Tan, H., Shen, H., Mahmood, K., Boyd, S. E., Webb, G. I., Akutsu, T., and Whisstock, J. C. (2010) Cascleave: towards more accurate prediction of caspase substrate cleavage sites. *Bioinformatics 26*, 752−760.
(45) Beattie, T. L., Zhou, W., Robinson, M. O., and Harrington, L. (2001) Functional multimerization of the human telomerase reverse transcriptase. *Mol. Cell. Biol. 21*, 6151−6160.
(46) Guy, M. P., and Friesen, P. D. (2008) Reactive-site cleavage residues confer target specificity to baculovirus P49, a dimeric member of the P35 family of caspase inhibitors. *J. Virol. 82*, 7504−7514.
(47) Santos, J. H., Meyer, J. N., and Van Houten, B. (2006) Mitochondrial localization of telomerase as a determinant for hydrogen peroxide-induced mitochondrial DNA damage and apoptosis. *Hum. Mol. Genet. 15*, 1757−1768.
(48) Chebel, A., Rouault, J. P., Urbanowicz, I., Baseggio, L., Chien, W. W., Salles, G., and Ffrench, M. (2009) Transcriptional activation of hTERT, the human telomerase reverse transcriptase, by nuclear factor of activated T cells. *J. Biol. Chem. 284*, 35725−35734.
(49) Liu, K., Schoonmaker, M. M., Levine, B. L., June, C. H., Hodes, R. J., and Weng, N. P. (1999) Constitutive and regulated expression of telomerase reverse transcriptase (hTERT) in human lymphocytes. *Proc. Natl. Acad. Sci. U. S. A. 96*, 5147−5152.
(50) Flores, I., Benetti, R., and Blasco, M. A. (2006) Telomerase regulation and stem cell behaviour. *Curr. Opin. Cell Biol. 18*, 254−260.
(51) Xu, D., Wang, Q., Gruber, A., Bjorkholm, M., Chen, Z., Zaid, A., Selivanova, G., Peterson, C., Wiman, K. G., and Pisa, P. (2000) Downregulation of telomerase reverse transcriptase mRNA expression by wild type p53 in human tumor cells. *Oncogene 19*, 5123−5133.
(52) Cukusic, A., Skrobot Vidacek, N., Sopta, M., and Rubelj, I. (2008) Telomerase regulation at the crossroads of cell fate. *Cytogenet. Genome Res. 122*, 263−272.
(53) Marion, R. M., and Blasco, M. A. (2010) Telomere rejuvenation during nuclear reprogramming. *Curr. Opin. Genet. Dev. 20*, 190−196.
(54) Jakob, S., Schroeder, P., Lukosz, M., Buchner, N., Spyridopoulos, I., Altschmied, J., and Haendeler, J. (2008) Nuclear protein tyrosine phosphatase Shp-2 is one important negative regulator of nuclear export of telomerase reverse transcriptase. *J. Biol. Chem. 283*, 33155−33161.
(55) Kang, S. S., Kwon, T., Kwon, D. Y., and Do, S. I. (1999) Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. *J. Biol. Chem. 274*, 13085−13090.
(56) Colgin, L. M., Wilkinson, C., Englezou, A., Kilian, A., Robinson, M. O., and Reddel, R. R. (2000) The hTERTα splice variant is a dominant negative inhibitor of telomerase activity. *Neoplasia 2*, 426−432.
(57) Yi, X., White, D. M., Aisner, D. L., Baur, J. A., Wright, W. E., and Shay, J. W. (2000) An alternate splicing variant of the human telomerase catalytic subunit inhibits telomerase activity. *Neoplasia 2*, 433−440.
(58) Lamkanfi, M., Festjens, N., Declercq, W., Vanden Berghe, T., and Vandenabeele, P. (2007) Caspases in cell survival, proliferation and differentiation. *Cell Death Differ. 14*, 44−55.
(59) Andrews, N. P., Fujii, H., Goronzy, J. J., and Weyand, C. M. (2010) Telomeres and immunological diseases of aging. *Gerontology 56*, 390−403.
(60) Baek, S., Bu, Y., and Kim, H. (2004) Telomerase induction in astrocytes of Sprague-Dawley rat after ischemic brain injury. *Neurosci. Lett. 363*, 94−96.
(61) LeBlanc, A., Liu, H., Goodyer, C., Bergeron, C., and Hammond, J. (1999) Caspase-6 role in apoptosis of human neurons, amyloidogenesis, and Alzheimer's disease. *J. Biol. Chem. 274*, 23426−23436.
(62) Guo, H., Albrecht, S., Bourdeau, M., Petzke, T., Bergeron, C., and LeBlanc, A. C. (2004) Active caspase-6 and caspase-6-cleaved tau in neuropil threads, neuritic plaques, and neurofibrillary tangles of Alzheimer's disease. *Am. J. Pathol. 165*, 523−531.
(63) Cribbs, D. H., Poon, W. W., Rissman, R. A., and Blurton-Jones, M. (2004) Caspase-mediated degeneration in Alzheimer's disease. *Am. J. Pathol. 165*, 353−355.
(64) Mattson, M. P., Fu, W., and Zhang, P. (2001) Emerging roles for telomerase in regulating cell differentiation and survival: a neuroscientist's perspective. *Mech. Ageing Dev. 122*, 659−671.
(65) Harley, C. B. (2005) Telomerase therapeutics for degenerative diseases. *Curr. Mol. Med. 5*, 205−211.
(66) Franco, S., Blasco, M. A., Siedlak, S. L., Harris, P. L., Moreira, P. I., Perry, G., and Smith, M. A. (2006) Telomeres and telomerase in Alzheimer's disease: epiphenomena or a new focus for therapeutic strategy? *Alzheimer's Dementia 2*, 164−168.
(67) Armbruster, B. N., Etheridge, K. T., Broccoli, D., and Counter, C. M. (2003) Putative telomere-recruiting domain in the catalytic subunit of human telomerase. *Mol. Cell. Biol. 23*, 3237−3246.
(68) Robart, A. R., and Collins, K. (2011) Human Telomerase Domain Interactions Capture DNA for TEN Domain-Dependent Processive Elongation. *Mol. Cell 42*, 308−318.

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

(70) Jurczyluk, J., Nouwens, A. S., Holien, J. K., Adams, T. E., Lovrecz, G. O., Parker, M. W., Cohen, S. B., and Bryan, T. M. (2011) Direct involvement of the TEN domain at the active site of human telomerase. *Nucleic Acids Res. 39*, 1774−1788.

(71) Ahmed, S., Passos, J. F., Birket, M. J., Beckmann, T., Brings, S., Peters, H., Birch-Machin, M. A., von Zglinicki, T., and Saretzki, G. (2008) Telomerase does not counteract telomere shortening but protects mitochondrial function under oxidative stress. *J. Cell Sci. 121*, 1046−1053.

(72) Haendeler, J., Drose, S., Buchner, N., Jakob, S., Altschmied, J., Goy, C., Spyridopoulos, I., Zeiher, A. M., Brandt, U., and Dimmeler, S. (2009) Mitochondrial telomerase reverse transcriptase binds to and protects mitochondrial DNA and function from damage. *Arterioscler. Thromb. Vasc. Biol. 29*, 929−935.

(73) Haendeler, J., Hoffmann, J., Diehl, J. F., Vasa, M., Spyridopoulos, I., Zeiher, A. M., and Dimmeler, S. (2004) Antioxidants inhibit nuclear export of telomerase reverse transcriptase and delay replicative senescence of endothelial cells. *Circ. Res. 94*, 768−775.

(74) Sekaran, V. G., Soares, J., and Jarstfer, M. B. (2010) Structures of telomerase subunits provide functional insights. *Biochim. Biophys. Acta 1804*, 1190−1201.

(75) Mahrus, S., Trinidad, J. C., Barkan, D. T., Sali, A., Burlingame, A. L., and Wells, J. A. (2008) Global sequencing of proteolytic cleavage sites in apoptosis by specific labeling of protein N termini. *Cell 134*, 866−876.