Mitochondrial Telomerase Reverse Transcriptase Binds to and Protects Mitochondrial DNA and Function From Damage

Judith Haendeler, Stefan Dröse, Nicole Büchner, Sascha Jakob, Joachim Altschmied, Christine Goy, Ioakim Spyridopoulos, Andreas M. Zeiher, Ulrich Brandt, Stefanie Dimmeler

Objective—The enzyme telomerase and its catalytic subunit the telomerase reverse transcriptase (TERT) are important for maintenance of telomere length in the nucleus. Recent studies provided evidence for a mitochondrial localization of TERT. Therefore, we investigated the exact localization of TERT within the mitochondria and its function.

Methods and Results—Here, we demonstrate that TERT is localized in the matrix of the mitochondria. TERT binds to mitochondrial DNA at the coding regions for ND1 and ND2. Binding of TERT to mitochondrial DNA protects against ethidium bromide–induced damage. TERT increases overall respiratory chain activity, which is most pronounced at complex I and dependent on the reverse transcriptase activity of the enzyme. Moreover, mitochondrial reactive oxygen species are increased after genetic ablation of TERT by shRNA. Mitochondrially targeted TERT and not wild-type TERT revealed the most prominent protective effect on H2O2-induced apoptosis. Lung fibroblasts from 6-month-old TERT−/− mice (F2 generation) showed increased sensitivity toward UVB radiation and heart mitochondria exhibited significantly reduced respiratory chain activity already under basal conditions, demonstrating the protective function of TERT in vivo.

Conclusion—Mitochondrial TERT exerts a novel protective function by binding to mitochondrial DNA, increasing respiratory chain activity and protecting against oxidative stress–induced damage. (Arterioscler Thromb Vasc Biol. 2009;29:929-935.)

Key Words: aging • apoptosis • mitochondrial functions • mitochondrial DNA • reactive oxygen species • telomerase reverse transcriptase

To date several theories exist to explain the phenomenon of normal and pathological aging. The free radical theory of aging1 proposes that reactive oxygen species (ROS) in biological systems attack molecules and thereby cause functional decline of organ systems that eventually leads to death. This damage accumulates over time and may contribute to diseases associated with aging like atherosclerosis, neurodegeneration, or cataracts.2 Recently, Schriner et al produced transgenic mice that overexpressed human catalase localized to the peroxisome, the nucleus, and the mitochondria. Only mice overexpressing mitochondrially targeted catalase showed a significant increase in life span and a reduction in oxidative damage to DNA and consequently in apoptosis.3 Thus, these data define the mitochondria as compartment of ROS formation, which contributes to aging processes. Further evidence supporting the importance of mitochondria and formation of ROS in the mitochondria comes from findings that overexpression of mitochondrially localized antioxidant enzymes lengthens lifespan of Drosophila4,5 and that deletion of manganese superoxide dismutase results in the age-related decline of mitochondrial function, culminating in increased apoptosis.6 Recent studies using isolated complex I of the respiratory chain clearly demonstrated that superoxide production into the mitochondrial matrix is predominantly dependent on flavine-mononucleotide within complex I.7,8

The enzyme telomerase counteracts the shortening of the physical ends of chromosomes and, thereby, prevents the onset of replicative senescence and genetic instability.9–12 The catalytic subunit, which antagonizes telomere-shortening, is the telomerase reverse transcriptase (TERT).9 Several studies suggested that TERT exerts functions independently of its net telomere lengthening (for review see13). TERT increased cell survival and resistance against oxidative stress after short term incubation of cells with different stimuli, which did not affect telomere shortening.14–18 TERT has been shown to have a genuine mitochondrial leader sequence which targets this protein to the mitochondria.19,20 However the exact role of mitochondrial TERT remains
controversial, with 1 group showing that it exacerbates oxidative injury\textsuperscript{19,20} and another group demonstrating a protective effect after oxidative stress.\textsuperscript{21} Taking into account that aging processes are associated with dysfunctional mitochondria, the goal of the present study was to elucidate the localization of TERT within the mitochondria and its functional role in the mitochondria with specific respect to respiratory chain activity, apoptosis, and formation of ROS.

**Methods**

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**Cell Culture**

Human umbilical vein endothelial cells (HUVECs; Lonza, Cologne, Germany) were transfected with Superfect as previously described.\textsuperscript{22} After detachment with trypsin, cells were grown for at least 18 hour prior to all further manipulations.

**Isolation of Primary Mouse Lung Fibroblasts**

A small piece of lung tissue was minced with 2 scalpels in a culture dish and incubated in HEK 293 medium at 37°C. After 5 days the fibroblasts grown out from tissue fragments were passaged to culture flasks.

**Proteinase K Digestion of Mitochondria**

To determine whether TERT is localized in the mitochondrial matrix or the intermembrane space, 700 μg of mitochondria was distributed in 3 equal aliquots. Mitochondria were pelleted and incubated at 4°C on a shaker in 40 μL of 3 different buffers for 20 minutes. Buffer 1 (isotonic buffer): 250 mmol/L sucrose, 1 mmol/L EGTA, 10 mmol/L HEPES, pH 7; Buffer 2 (hypotonic buffer): 1 mmol/L EGTA, 10 mmol/L HEPES, pH 7, 25 μg/mL proteinase K; Buffer 3 (hypotonic buffer with detergent): 1 mmol/L EGTA, 10 mmol/L HEPES, pH 7, 1% (v/v) Triton-X100, 25 μg/mL proteinase K. After 20 minutes, digestion was stopped by adding phenylmethylsulfonyl fluoride to a final concentration of 2 mmol/L and incubated for further 5 minutes with shaking. Aliquot 3 was boiled for 5 minutes in Laemmli-buffer. Aliquot 1 and 2 were washed once with Buffer 1 and resuspended in 40 μL RIPA-buffer (50 mmol/L Tris/HCl pH 8, 1% [v/v] Nonidet, 150 mmol/L NaCl, 0.1% [wt/vol] SDS, 0.5% [wt/vol] Desoxycholate) and boiled for 5 minutes in Laemmli-buffer. Western blot analyses were performed using antibodies against endogenous TERT (Epitomics, 1:500), TOM40 (Santa Cruz, 1:250), and TIM23 (BD Transduction laboratories 1:1000).

**Results**

**TERT Is Localized in Mitochondria**

It has been demonstrated that mitochondria contain measurable telomerase enzymatic activity,\textsuperscript{19} suggesting that a fraction of cellular TERT is localized in these organelles. After having previously shown that telomerase activity is found in mitochondria of HUVECs,\textsuperscript{23} we confirmed that this is also true for HEK cells. As shown in Figure 1A, telomerase enzyme activity was detectable in all 3 compartments under basal conditions. Because commercially available antibodies against endogenous TERT could not be used for immunoprecipitation, we next tested whether overexpressed TERT wild-type protein containing a myc-tag at the C terminus (TERTwt-myc) is detectable in mitochondria. From our previous studies, we knew that TERTwt-myc is exported from the nucleus on stress and thereby behaved identical to endogenous TERT in HEK cells and HUVECs.\textsuperscript{17} Overexpressed TERTwt-myc was detected in the mitochondria by immunoblot (supplemental Figure 1A). The biochemical fractionation was always controlled by proteins, which are known to be located in the nucleus or in the mitochondria.

To determine the exact localization of TERT, which has a genuine mitochondrial leader sequence at the N terminus, within these organelles, we digested the outer mitochondrial membrane with proteinase K. With this approach we could demonstrate that a large portion of endogenous TERT is localized in the mitochondrial matrix as indicated by its presence in the proteinase K resistant fraction (Figure 1B). This requires an import into the mitochondria, as the protein is encoded by the nuclear genome and thus translated in the cytoplasm. The best-characterized pathway to import a protein into the mitochondria of higher eukaryotes is by binding to a complex containing the translocases of the outer membrane 20 and 40 (TOM20 and TOM40) and the translocase of the inner membrane 23 (TIM23). We therefore investigated
whether TERT can associate with this complex by coimmunoprecipitation studies. As stated above, TERTwt-myc was detected in the mitochondria, and all antibodies directed against endogenous TERT could not be used for immunoprecipitation. Therefore, we performed all further experiments with overexpressed TERTwt-myc. The fact that TERTwt-myc physically interacts with the TOM20/TOM40 complex (supplemental Figure IB) and with TIM23 (Figure 1C) strongly suggests a transport mechanism involving these proteins. However, these experiments do not exclude participation of other translocases, like for instance TIM22.

**TERT Binds to mtDNA**

One important component of the mitochondrial matrix is the mtDNA. This prompted us to test whether TERTwt-myc can associate with the genome of the organelle. Therefore, we performed a modified chromatin-immunoprecipitation assay, using primers amplifying segments of mtDNA. After careful examination of the different regions of the circular mitochondrial genome, we identified 2 regions containing the coding sequences for NADH-ubiquinone oxidoreductase (complex I) subunit 1 and 2 (ND1, ND2) as interaction sites for overexpressed TERTwt-myc protein. In contrast, another region of the mtDNA containing the coding sequence for ATP synthase subunit 6 (ATP6) did not show interaction with TERTwt-myc (supplemental Figure II).

**TERT Protects mtDNA From Damage**

It has previously been shown that telomerase can protect mitochondrial function under oxidative stress. As a number of mitochondrial proteins are encoded by the mitochondrial genome it was suggestive to analyze whether TERT bound to mtDNA could protect it against damage. Therefore we first assessed in vitro whether TERT can prevent UV-induced destruction of mtDNA. We treated isolated total cellular DNA (containing nuclear and mtDNA) in vitro in the presence or absence of TERTwt-myc for 3 minutes with short-wave UV light (254 nm) and analyzed intactness of mtDNA by long-range PCR. Indeed, preincubation of the DNA with in vitro translated TERTwt-myc effectively protected mtDNA against UV-induced degradation, whereas immediate irradiation showed only weak protection. Preincubation with β-galactosidase, a protein of comparable size, had no effect (Figure 2A).

To assess whether this protection of mtDNA is of relevance in living cells, we irradiated primary lung fibroblasts from homozygous TERT knockout (TERT−/−) mice and their wild-type littermates with different doses of UVB irradiation and determined MTT conversion as a measure for mitochondrial activity. We demonstrated that TERT−/− fibroblasts are more sensitive to UV-irradiation with regard to their mitochondrial activity (Figure 2B), indicating that TERT has a protective function for mitochondria in vivo. In addition we could show that TERTwt-myc directly protects mtDNA in HEK cells (Figure 5A).

**TERTwt Protects the Respiratory Chain and Specifically Complex I in Cells and in Mice**

To further investigate whether TERT binding to mtDNA has an impact on mitochondrial function, we next measured respiratory chain activity in TERT-transfected cells. We included a TERT mutant (TERTD868A-myc) lacking reverse transcriptase activity, from which we know that it can still bind to mtDNA (data not shown), to elucidate whether catalytic activity of the protein is required for maintaining mitochondrial functions. Measuring the respiratory chain activity in general, we discovered a 30% reduction in succinate-dependent respiration in cells overexpressing TERTD868A-myc (Figure 3A and supplemental Figure III), suggesting that not only the mtDNA binding but also the activity of TERT plays a role for mitochondrial functions. Besides the overall decrease found in respiratory chain activity, we wanted to determine whether the effect was most pronounced for complex I. To emphasize the latter, we normalized all other values to the same rate of succinate-respiration that was set to 1 (Figure 3A) and found a significant reduction in complex I activity. To further underscore these findings, we used a second method to measure complex I activity. Indeed, a significant increase in complex I activity was only found in cells overexpressing TERTwt-myc. (Figure 3B). Of note, the data shown in Figure 4b demonstrate that although all respiratory rates were decreased in TERTD868A, the mitochondria showed no differences in respiratory chain control factors ie, in the ratios of state 4/state 3 or state 4/uncoupled rates, which essentially also excludes changes in membrane permeability. To determine whether our results obtained by overexpression of TERT are of relevance in vivo, we next analyzed the respiratory chain activity of intact heart and liver mitochondria isolated from TERT−/− mice and wild-type animals. Indeed, we found that heart mitochondria from TERT−/−...
mice showed a significant reduction of the complex I–dependent respiration with the NADH-generating substrates malate and glutamate, which was most obvious in state 3 (ADP-stimulated) respiration (Figure 4a). In contrast, no difference between TERT−/− mice and their wild-type littermates was observed in liver mitochondria. This indicated that the protective effect of TERT in vivo might be more important in tissues with a high respiratory rate like the myocardium and with no or weak regenerative capacity.

**TERT Reduces Formation of ROS in Mitochondria**

Recent studies from Santos et al using a retroviral approach showed that reintroduction of human TERT into human fibroblasts increased oxidative stress, mtDNA lesions, and apoptosis.19,20 In contrast, Ahmed et al showed that TERT protects mitochondria from damage and reduces oxidative stress.21 Therefore, we investigated the effect of TERT under basal and EtBr-induced conditions on ROS formation. Overexpression of TERTwt-myc reduced ROS formation compared to LacZ- and TERTD868A-myc–overexpressing cells (Figure 4B). This is in contrast to Santos19 but in agreement with Ahmed et al.21 Moreover, ablation of endogenous TERT by shRNA (Figure 4C and supplemental Figure IVA) revealed increased mitochondrial ROS formation as measured with Mitosox, a specific compound, which is imported into the mitochondria for ROS detection (Figure 4D and supplemental Figure IVB), suggesting that the presence of TERT in the mitochondria protects from respiratory chain dysfunction.

Mitochondrially Targeted TERT Is More Protective Than TERTwt Against mtDNA Depletion and Oxidative Stress–Induced Apoptosis

Because mitochondrial damage importantly contributes to apoptosis induction, we cloned TERT into a mitochondrial target vector (mitoTERT-myc) to specifically investigate the effect of the mitochondrial localization of TERT on mtDNA protection and apoptosis inhibition. After having shown that TERT binds to mtDNA and protects cells against UVB-induced decrease of mitochondrial function, we first assessed the effect of mitoTERT-myc on mtDNA protection in living cells. Therefore we reduced mtDNA content in HEK cells transfected with an empty control vector, TERTwt-myc, or mitoTERT-myc by treating...
cells with low doses of ethidium bromide (EtBr). Expression of TERTwt-myc completely rescued the loss of mtDNA content observed in control transfected cells. This effect was even more pronounced in cells expressing mitoTERT-myc (Figure 5A).

We previously described that H$_2$O$_2$ induces apoptosis in endothelial cells. Overexpression of TERTwt-myc in HUVECs showed a slight but significant inhibition of H$_2$O$_2$-induced apoptosis, whereas mitoTERT-myc completely abrogated H$_2$O$_2$-triggered apoptosis (Figure 5B). These data suggest that protection of mtDNA integrity, mitochondrial function, and apoptosis protection by TERT can be mainly ascribed to the mitochondrial function of this protein.

### Discussion

Our results presented here demonstrate that a large portion of endogenous mitochondrial TERT is localized in the mitochondrial matrix. TERT binds to mtDNA and thereby protects mtDNA from damage. Concomitantly we observe a reduction in the formation of mitochondrially produced ROS. Overexpression of mitochondrially targeted TERT is more protective than TERT wild-type against EtBr-induced DNA damage and ROS-induced apoptosis. Strikingly, the protective function of TERT was also evident in vivo from the fact that heart mitochondria from TERT$^{-/-}$ mice exhibited significantly reduced respiratory chain activity.

Previous studies have shown that TERT contributes significantly to processes of aging by protecting telomeres from critical shortening and by inhibiting apoptosis and decreasing oxidative stress. At the same time, oxidative stress and mitochondrial dysfunction have been demonstrated to be important players of aging processes. Our study now shows that mitochondrial TERT inhibits formation of mitochondrially produced ROS and can contribute to increased energy metabolism. Thus, it is tempting to speculate that not only nuclear TERT has an impact on aging processes, but also mitochondrially localized TERT. A reduction in mitochondrially produced ROS has recently been demonstrated to increase the lifespan of mice. Schriner et al produced mice that overexpressed mitochondrially targeted catalase as well as nuclear targeted catalase and showed that in both mice strains catalase was able to reduce H$_2$O$_2$, but only mice which overexpress mitochondrially targeted catalase exhibited an increased lifespan compared to their wild-type littermates. Likewise, overexpression of manganese superoxide dismutase, a mitochondrially localized antioxidative enzyme reducing O$_2^-$ to H$_2$O$_2$, increased the lifespan of adult *Drosophila melanogaster*. However, recent studies on mice deficient for the mitochondrially localized DNA polymerase $\gamma$ (Pol-$\gamma$) did not find an increase in oxidatively damaged proteins or a reduction in mitochondrial aconitase activity, a classic marker for oxidative damage of proteins. Nevertheless, an accumulation of mitochondrial mutations and the development of many of the phenotypes characteristic of human premature aging were reported. Thus, it was concluded that the Pol-$\gamma$-deficient mice exhibited an aging phenotype in the absence of enhanced ROS production. However, it was also demonstrated that embryonic fibroblasts of the Pol-$\gamma$-deficient mice show a 95% reduction in oxygen consumption, which would suggest that the extensive mutations lead to a complete breakdown of the respiratory chain. This would exclude the formation of mitochondrially produced ROS in these mice. Therefore, these data do not prove that mitochondrially produced ROS are not involved in aging processes.

Recently, it has been reported that flavine-mononucleotide in complex I of the respiratory chain is the major site where ROS are produced. Our data now demonstrate that TERT protects the genes for subunits ND1 and ND2 in the mtDNA specifically. Thus, it is tempting to speculate that an increase in ROS production by complex I leads to more damaged mtDNA spurring a vicious cycle finally resulting in dysfunctional respiratory chain and mitochondria. Mitochondrial TERT may counteract ROS production by complex I by binding to the ND1 and ND2 genes, thereby increasing the synthesis of functional complex I subunits which in turn can reduce the formation of damaged complex I that leaks electrons onto oxygen. Unfortunately, too little is still known about the structure and function of complex I. Therefore, we do not know whether protection of ND1 and ND2 subunit genes by TERT is a key prerequisite for reducing complex I produced ROS.
Several studies discuss an important role for apoptosis in the process of aging and age-related diseases. Here, we demonstrate that TERT protects mtDNA from damage and that mitochondrial targeted TERT is protective against oxidative stress induced damage. Our data could implicate that mitochondrial TERT also plays an important role in apoptotic processes leading to pathological aging. Key processes during the onset of apoptosis are outer mitochondrial membrane permeabilization, release of cytochrome c into the cytosol, and activation of caspases. Of note, Bayir et al demonstrated that a mitochondrial pool of cytochrome c exists which acts as a cardiolipin oxygenase. This cytochrome c/cardiolipin oxygenase is activated during apoptosis, uses ROS, and causes selective oxidation of cardiolipin. The oxidized cardiolipin is required for the release of proapoPotic factors from mitochondria to the cytosol. Thus, a reduction of ROS by mitochondrial TERT could result in a reduced activation of the cytochrome c/cardiolipin oxygenase, reduced oxidized cardiolipin, and therefore inhibition of the release of proapoptotic factors into the cytosol. Taking this into account, it is not surprising that nuclear as well as mitochondrial TERT can inhibit apoptotic processes, because one may speculate that mitochondrial TERT inhibits the formation of ROS by complex I, blocks cardiolipin oxidation and release of proapoptotic factors from the mitochondria, whereas nuclear TERT protects telomeres from shortening, interacts with the antiapoptotic proteins PARP and Akt and is regulated by p53, a known gatekeeper of apoptotic processes.

Moreover, it has been shown that cells depleted of mtDNA (P cells) are resistant to apoptosis induction by several stimuli. Dissipation of mitochondrial potential and release of cytochrome c into the cytosol was absent in these cells. These data clearly demonstrate that mitochondria are necessary for the onset of apoptosis. Similar to these findings, it has been demonstrated that mitochondrial dysfunction is a prerequisite for the onset of aging processes. Therefore, it is tempting to speculate that apoptosis is also a prerequisite for aging processes or that apoptosis and aging processes are tied together. This hypothesis is strengthened by data from Kujoth et al who found that many tissues of Pol− mice show at early age a phenotype of accelerated aging with increased levels of caspase-3 activity and TUNEL-positive cells. Similar to this study in mice, patients who carry a high load of mitochondrial mutations show an increase in TUNEL-positive muscle fibers. We show here that protection of mtDNA by TERT is also associated with a higher protection from apoptosis.

**Conclusion/Perspectives**

Therefore, one may consider revising the old dogma that only the nuclear function of TERT is important for aging and apoptotic processes. Mitochondrial TERT may also exert a significant contribution to the antiaging and antiapoptosis function of TERT.

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**Disclosures**

None.

**References**


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Mitochondrial telomerase reverse transcriptase binds to and protects mitochondrial DNA and function from damage

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Running title: Functions of mitochondrial TERT

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**Methods**

**Cell Culture**

Primary human umbilical vein endothelial cells (HUVEC) were cultured in endothelial basal medium supplemented with hydrocortisone (1 µg/ml), bovine brain extract (12 µg/ml), gentamicin (50 µg/ml), amphotericin B (50 ng/ml), epidermal growth factor (10 ng/ml), and 10 % fetal calf serum (FCS). HEK 293 cells (HEK cells) were maintained in DMEM containing 10 % FCS and were transfected with Lipofectamine/Plus according to the manufacturer's protocol (GibcoBRL) as previously described \(^1\) with a transfection efficiency of 90 ± 4 %.

**cDNA cloning, plasmids and PCRs**

A human cDNA TERT construct was kindly donated by Dr. Weinberg \(^2\). Human TERT cDNA was subcloned into pcDNA3.1myc-his vector (TERTwt-myc) or into mitochondrial pShooter vector, which targets TERT into the mitochondria (mitoTERT-myc) (Invitrogen). The mitochondrial pShooter mammalian expression vector incorporates signal sequences into TERT to direct TERT to its localization. TERTD868A-myc was generated by site-directed mutagenesis (Promega). Moreover, we cloned shRNA TERT under the control of an U6 promoter into a GFP-containing vector to control the transfection efficiency. shRNA TERT target sequence: 5'`TCCTGCCTTGGGATGATT-3`.

cDNA was prepared from total cellular RNA using reverse transcriptase and used as template for gene specific PCRs with intron spanning primers. TERT specific PCRs were performed with primers derived from exon 6 (5'-CCGCTGAGCTGTACTTTGT-3`) and exon 7 (5'-ATGTACGGCTGGAGGTCTGT-3`). As housekeeping gene L32 was used (primer sequences: 5'-GTGAAGCCCAAGATCGTCAA-3` and 5'-TTGTTGCACATCAGCAGC-3`).

**Detection of oxidative stress**

Living cells were incubated for dye uptake with 20 µM 2',7' dichlorodihydrofluorescein diacetate (H\(\_2\)DCF-DA) or 5 µM Mitosox for 30 min (Molecular Probes, Germany). Cells were trypsinized for 2 min and reaction was stopped with PBS containing 10 % FCS and cells were pelleted by centrifugation. Cells were resuspended in PBS and measured using FACS analysis.

**Cellular fractionation and Immunoprecipitation**
Cellular fractionation was performed as described previously \(^3\) and nuclear, cytosolic or mitochondrial lysates were immunoprecipitated with 5 \(\mu\)g TOM20-antibody, 5 \(\mu\)g TIM23-antibody or 5 \(\mu\)g myc-antibody overnight at 4°C in the corresponding lysis buffers \(^3\). After incubation with A/G Plus agarose (Santa Cruz) for 2 h at 4°C, resulting beads were washed three times with the same buffers and subjected to SDS-PAGE.

**Measurements of TERT-mitochondrial DNA binding**

Living cells were incubated with 1% formaldehyde in cell culture medium for 1h at 4°C. Cross-link reaction was terminated by adding 0.125 M glycine and cells were scraped off the plate. DNA-protein immunoprecipitation was performed according to the manufacturer’s instructions (Upstate Biotechnology) and after separating of protein and DNA, PCR was performed with primers specific for human ND1 \((5’-\text{ATACCCATGGCCAACCTCCTACTCTATT}-3’),\)

\(\text{CCCTGATCAGAGGTAGTAACGGCTAG-3’})\),

ND2 \((5’-\text{CCCTGATCAGAGGTAGTAACGGCTAG-3’})\),

CGACTGATTTCCTACCTGAAGGCTTAG-3’),

AGATTAGCCGCTAGGTAGTAGAGGTGAAG-3’),

and ATP6 \((5’-\text{AGATTAGCCGCTAGGTAGTAGAGGTGAAG-3’})\) and ATP6 \((5’-\text{AGATTAGCCGCTAGGTAGTAGAGGTGAAG-3’})\)

Coding regions.

**Preparation of mouse heart and liver mitochondria**

Intact heart and liver mitochondria from 6 months old TERT-/- mice and their wildtype littermates (F2 generation) were prepared as described earlier for mitochondria from rat organs \(^4\). Buffer volumes were reduced by a factor of ~2 due to the lower mice organ sizes and one washing step was omitted to reduce preparation dependent losses. Upon completion of the respective preparation, the mitochondrial suspensions were generally stabilized by the addition of 40 mM malate and 50 mM glutamate. The substrate addition ensured that the mitochondria retained high respiratory control factors (ratio between state 3 respiration after ADP addition and state 4 respiration; for heart mitochondria ~10; for liver mitochondria > 7) over a longer time period.

**Mitochondrial respiration**

The rate of mitochondrial respiration was monitored at 25 °C using an Oxygraph-2k system (Oroboros) equipped with two chambers and DatLab software. Mitochondria (depending on the yield of the preparations 130-338 \(\mu\)g protein of TERTwt or 72-296 \(\mu\)g protein of TERTD868A mitochondria) were added to 2 ml of a buffer containing 200 mM
sucrose, 10 mM potassium phosphate, 10 mM Tris-HCl, 10 mM MgSO₄, and 2 mM EDTA, pH 7.0. State 4 respiration was measured after the addition of the NADH-generating substrates malate (6 mM) and glutamate (7 mM). Then, 0.5 mM ADP was added to measure state 3 respiration. After determining coupled respiration, 50 nM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was added to the reaction chamber, and respiration was measured in the absence of a proton gradient. To inhibit complex I activity, a total 0.1 µM rotenone was added. Then, 25 mM of succinate were added, and complex II dependent respiration was determined. Finally, 2 mM KCN was added to inhibit complex IV activity. TERTD868A and TERTwt mitochondria were measured in parallel pairs using the same conditions (crossover design). Measurements with intact heart and liver mitochondria followed the same scheme in the identical buffer, but the concentrations of substrates/ inhibitors were: 6 mM malate + 7 mM glutamate, 1 mM ADP, 50 nM FCCP, 1 µM rotenone, 5 mM succinate and 2 mM KCN. Heart mitochondria were added in the range of 173-214 µg protein from wt mice and 180-252 µg protein from TERT⁻/⁻ mice, liver mitochondria at 452-728 µg protein from wt mice and 440-686 µg protein from TERT⁻/⁻ mice. For each preparation, duplicates (heart) or triplicates (liver) were measured.

Complex I activity

For the determination of complex I activity, mitochondria (223 – 442 µg protein of TERTwt or 147 – 356 µg protein of TERTD868A) were solubilized using sodium cholate. The final concentration in the assay was 0.01% sodium cholate. NADH:ubiquinone oxidoreductase activity was measured at room temperature in a buffer containing 2 mM Na⁺/Mops, 50 mM NaCl, and 2 mM KCN, pH 7.2, using 100 µM n-decylubiquinone (DBQ) and 100 µM NADH as substrates. Oxidation rates of NADH were recorded with a Shimadzu UV300 dual-wavelength spectrophotometer (OD₃₄₀₋₄₀₀ nm = 6.1 mM⁻¹ cm⁻¹). After ~ 10 min, 50 µM rotenone were added to inhibit complex I activity.

Measurements of mitochondrial DNA (mtDNA)

Total cellular DNA (including nuclear and mtDNA) was isolated from HEK 293 cells by standard methods. To determine the relative mtDNA content 250 ng were used as template for real-time PCR. PCR reactions were carried out on an Abi Prism 7000 (Applied Biosystems, Foster City, CA, USA) using SYBRGreen PCR Master Mix (Applied Biosystems, Darmstadt, Germany). Primers for mtDNA (5'-
GATTTGGGTACCACCAAGTATTG-3', 5'-AATATTCATGGTGCTGGCAGTA-3') amplified a 83 bp segment of the mtDNA. For normalization with the ΔΔCT method a unique genomic segment of 104 bp from the chromosomal region 9p21.3 was amplified (primers: 5'-GCAGAAACGGAGAGACATAC-3', 5'-TTTTGGGTGAACATATTG-3'). Measurements of intact mtDNA after UV-irradiation in vitro were performed with a long range PCR yielding a 6.3 kb amplification product (primers: 5'-ATACCCATGGCCAACCTCCTACTCCTCATT-3', 5'-CTAGAAGTGTGAAAAACGTAGGCTTGATTAAGGC-3'), which was analyzed by agarose gel electrophoresis.

**Measurement of mitochondrial activity with MTT**

10000 lung fibroblasts per well were seeded on 96well microtiter plates. 24 h later the cells were serum deprived for 24 h. Before exposure to UVB irradiation cells were washed with PBS. The cells were exposed to UVB irradiation in 100 µl PBS per well. UVB output was measured with a UV-Dosimeter Type II equipped with a UV6 sensor (Waldmann Medizintechnik, Villingen-Schwenningen, Germany) and found to be 0.56 mW/cm² at a tube to target distance of 30 cm. After irradiation PBS was exchanged to serum free medium and cells were incubated at 37°C for 24 h. Cells were incubated for four hours in medium containing 0.25 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Aldrich). Medium was exchanged to 200 µl DMSO before measurement of absorption at 550 nm.

**Apoptosis**

Apoptosis was determined by FACS analysis using annexin V-PE binding and 7-Amino-actinomycin (7AAD)-FITC staining (Pharmingen). Apoptotic cells were defined as annexin V-positive, 7AAD-negative cells. In brief, cells were trypsinized of the dish and pelleted. After washing twice with annexin binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4), cell pellets were resuspended in 50 µl of annexin binding buffer and incubated with 2.5 ng/ml annexin V-PE and 2.5 ng/ml 7AAD-FITC for 20 min. The reaction was terminated by adding 250 µl of annexin binding buffer and analyzed by FACS.

**Telomerase enzyme activity measurement**

Telomerase enzyme activity was measured using a commercially available PCR-based assay according to the manufacturer’s protocol (TRAPeze assay, Roche, Germany).
Telomeric repeat amplification protocol \(^6\) assays were performed using biotin-labelled TS primers as previously described \(^7\).

**Statistics**

All values are expressed as mean ± SEM. Statistical analysis was performed using student t-test.

**References**


**Figure Legends:**

Figure I: TERT is localized in mitochondria. (A) TERTwt-myc overexpressed in HUVEC was detected by immunoblot. Purity of fractions was controlled with topoisomerase I (nuclear fraction) and cytochrome c (mitochondrial fraction). (B) After fractionation of HEK cells transfected with TERTwt-myc into nuclear, cytosolic and mitochondrial fractions, an immunoprecipitation for TOM20 was performed. The immunoprecipitates were immunoblotted with antibodies directed against TERTwt-myc (upper panel),
TOM40 (middle panel) and TOM20 (lower panel). The absence of precipitated TOM20 in nuclear and cytosolic fractions demonstrates the purity of fractions.

Figure II: TERT binds to the region of mtDNA encoding ND1 and ND2. (A) After cross-linking protein and DNA in HEK cells transfected with TERTwt-myc or an empty control vector (EV), TERTwt-myc was immunoprecipitated. (a) PCRs were performed with the eluted DNA formerly bound to TERTwt-myc using ND1, ND2 and ATP6 specific primers. The left panel (IP: myc) shows the fragments amplified from the co-immunoprecipitated DNA, the right panel (input) the bands obtained after amplification of the material before immunoprecipitation (B) Success of immunoprecipitation assessed by immunoblot.

Figure III: TERT alters respiratory chain activity in mitochondria. Respiratory chain activity was measured in HEK cells transfected with TERTwt-myc (upper plot) or TERTD868A-myc (lower plot).

Figure IV: TERT inhibits mitochondrial derived ROS. (A and B) HEK cells were transfected with vectors expressing GFP and a TERT-specific shRNA (shRNA TERT) or a scrambled control sequence (scr). The transfection efficiency (=GFP-positive cells) was 54 +/- 9 %. Immunoblot analysis was performed out of whole cell lysates. (B) The transfected living HEK cells from (A) were incubated with Mitosox. GFP/Mitosox double positive cells were measured by FACS analyses as indicated. Panel shows representative FACS plots.
Supplemental Figure I

A

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B

IP: TOM20

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### Supplemental Figure II

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#### B

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Supplemental Figure III

TERTwt-myc

TERTD868A-myc
Supplemental Figure IV

A  scr  shRNA TERT

TERT
GAPDH

B  scr  shRNA TERT

gate R2