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***Saccharomyces cerevisiae* as a model for the study of extranuclear functions of mammalian telomerase**

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Abstract

The experimental evidence from the last decade made telomerase a prominent member of a family of moonlighting proteins performing different functions at various cellular loci. However, the study of extratelomeric function(s) of the catalytic subunit of mammalian telomerase (TERT) is often complicated by the fact that it is sometimes difficult to distinguish them from its role(s) at chromosomal ends. Here we describe an experimental model for studying extranuclear function(s) of mammalian telomerase in the yeast *Saccharomyces cerevisiae*. We demonstrate that the catalytic subunit of mammalian telomerase protects the yeast cells against oxidative stress and affect the stability of mitochondrial genome. The advantage of using *S. cerevisiae* for the study of mammalian telomerase is that (i) mammalian TERT does not interfere with its yeast counterpart in the maintenance of telomeres, (ii) yeast telomerase is not localized in mitochondria and (iii) it does not seem to be involved in the protection of the cells against oxidative stress and in the stabilization of mtDNA. Thus yeast cells can be used as a ‘test tube’ for reconstitution of mammalian TERT extranuclear function(s).

Keywords

telomerase; mitochondria; yeast; oxidative stress

Introduction

Telomerase is one of the most fascinating enzymes involved in DNA replication. It carries its own RNA template (TR) and by employing an RNA-dependent DNA polymerase activity of its protein catalytic subunit (telomerase reverse transcriptase, TERT) it extends 3′ single stranded overhangs present at the termini of nuclear chromosomes thus preventing the shortening of DNA due to the end-replication problem (Blackburn 2010). Besides its major role in telomere maintenance, telomerase is also involved in cellular processes not associated with these specialized nucleoprotein structures at the ends of linear chromosomes (for review see (Chung et al. 2005; Bollmann 2008; Majerska et al. 2011; Chiodi and

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Mondello 2012; Ding et al. 2013; Saretzki 2014; Li and Tergaonkar 2014; Jaiswal et al. 2013; Sung et al. 2014)). Telomerase was shown to be involved in the control of genes whose products participate in cell cycle, cell signaling, metabolism and apoptosis (Xiang et al. 2002; Smith et al. 2003; Perrault et al. 2005; Rahman et al. 2005; Choi et al. 2008; Yang et al. 2008; Park et al. 2009; Wege et al. 2011; Shkreli et al. 2012; Iannilli et al. 2013; Listerman et al. 2013), repair of DNA double-strand breaks (Shin et al. 2004), DNA damage response (Masutomi et al. 2005; Singhapol et al. 2013), chromatin remodeling (Maida et al. 2014), multidrug resistance phenotype (Ling et al. 2012) and cell reprogramming (Kinoshita et al. 2014). Interestingly, some of the extratelomeric activities of telomerase are performed in mammalian mitochondria (for review see (Saretzki 2009; Gordon and Santos 2010; Ale-Agha et al. 2014)). For example, TERT associates with an RNA subunit of mitochondrial RNA-processing endoribonuclease forming an RMRP complex (RNA component of mitochondrial RNA processing endoribonuclease) possibly involved in the regulation of gene expression (Maida et al. 2009). In addition, mitochondrial TERT can act as a TR-independent reverse transcriptase and is associated with several organellar transfer RNAs *in vivo* (Sharma et al. 2012). Importantly, while under normal conditions only about 10-20% of TERT is present in mitochondria, in cells exposed to oxidative stress almost 80% of the protein localizes in these organelles (Haendeler et al. 2003; Haendeler et al. 2004; Santos et al. 2004; Santos et al. 2006; Ahmed et al. 2008; Singhapol et al. 2013). Mammalian TERT contains a putative mitochondrial targeting sequence (MTS) (Santos et al. 2004) as well as nuclear export signal (Seimiya et al. 2000) that seem to be required for the re-localization of the protein from nucleus to mitochondria in cells exposed to oxidative stress (Kovalenko et al. 2010). In addition to the regulated protein transport, mitochondrially localized TERT is regulated by phosphorylation catalyzed by the protein tyrosine kinase Src (Buchner et al. 2010). However, mitochondrial role(s) of TERT in stressed cells are not clear. It was shown that the protein binds to defined regions of mitochondrial DNA (mtDNA), but as it is not associated with the RNA component of telomerase (Haendeler et al. 2009; Sharma et al. 2012), its role in mtDNA metabolism is therefore apparently different from that played at telomeres. Furthermore, there are contradictory results on the effect of the mitochondrial TERT on mtDNA stability. On one hand, there is an evidence indicating that the re-localization of TERT to mitochondria sensitizes mtDNA to oxidative damage resulting in apoptosis (Santos et al. 2004; Santos et al. 2006). On the other hand, other experimental data demonstrate that TERT protects mtDNA against oxidative damage (Ahmed et al. 2008; Haendeler et al. 2009). The protective role of mitochondrial TERT is in agreement with an observation that cells expressing TERT lacking nuclear export sequence are more sensitive to reactive oxygen species (ROS; Kovalenko et al. 2010). Similarly, cells with a mutant form of TERT unable to enter mitochondria due to an absence of MTS exhibit various mitochondrial dysfunctions (Sharma et al. 2012). How TERT affects mitochondria is a matter of debate. The possibilities include effects on respiratory chain (Ahmed et al. 2008; Haendeler et al. 2009), enhancement of antioxidant defense system (Indran et al. 2011), binding and protection of mtDNA (Haendeler et al. 2009), participation in mtDNA repair (Haendeler et al. 2009; Sharma et al. 2012) and mtDNA replication (Haendeler et al. 2009; Maida et al. 2009; Mukherjee et al. 2011; Sahin et al. 2011; Sharma et al. 2012). In general, elucidation of the mechanism might provide new insights on anti-telomerase therapy of diseases associated with telomerase-positive cells.

With the aim to develop a simple experimental system for addressing the above questions, we expressed mammalian TERT in the yeast *Saccharomyces cerevisiae*. In contrast to mouse or human TERT, yeast protein subunit of telomerase (encoded by the gene *EST2*) does not contain MTS (Santos et al. 2004) and is not associated with mitochondria (Sickmann et al. 2003). At the same time, the expression of mammalian TERT in yeast should not interfere with the maintenance of nuclear telomeres, as it is unable to form a functional complex with the RNA subunit of yeast telomerase (Bah et al. 2004). Thus, if targeted to yeast mitochondria, we should be able to assess mitochondria-specific function(s) of mammalian TERT. We reasoned that if we were able to recapitulate the effect(s) of TERT on yeast mitochondria similar to those observed in mammalian cells, *S. cerevisiae* might become a suitable *in vivo* test tube for tackling questions related to the mitochondrial functions of telomerase in higher eukaryotes.

Materials and Methods

Microbial strains and growth conditions

S. cerevisiae W303-1A (*MATa ade2-1; ura3-1; his3-15; trp1-1; leu2-3,1,2; can 1-10*) was used in most of the experiments. The strains BY4741 (*MATa; his3⁰; leu2⁰; met15⁰; ura3⁰*) and YLR318W_BY4741 (*est2*) (*MATa; his3⁰; leu2⁰; met15⁰; ura3⁰; est2⁰*) from the EUROSCARF library of deletion mutants were employed in the experiment described in Fig. S1. For routine purposes yeast cultures were grown at 28°C in a complex medium (1% (w/v) yeast extract, 2% (w/v) bacto-peptone) supplemented with the corresponding carbon source (2% (w/v) glucose (YPD) or 3% (v/v) glycerol plus 0.05% (w/v) glucose (YPDG)). Alternatively, synthetic medium (0.17% (w/v) yeast nitrogen base without amino acids and ammonium sulfate, 0.5% (w/v) ammonium sulfate) supplemented with appropriate amino acids, bases and corresponding carbon source (2% (w/v) glucose (SD), or 2% (w/v) galactose (SGal)) was used. To induce the expression of genes that were cloned under the *GAL1* promoter (*P_{GAL1}*) into the pYES2/CT plasmid (Life Technologies), the cells were grown overnight at 28°C with shaking (225 rpm) in 5 ml of SD medium, then diluted to 4×10⁶ cells/ml into either SD (repressible conditions), or SGal (inducible conditions) medium and cultivated for 20 h at 28°C with shaking. The cells were subsequently washed with water and diluted to 5×10⁶ cells/ml in 1× phosphate-buffered saline (PBS). To induce oxidative stress menadione was added to a final concentration of 65 mM (SGal) or 100 mM (SD), followed by incubation for 1 h at 28°C with shaking. 10-fold dilutions were then spotted on solid media as indicated and cultivated at 28°C. For ethidium bromide (EtBr) treatment, the cells were pre-cultivated as above and diluted to 1×10⁶ cells/ml in SD medium. EtBr was added to a final concentration of 25 μM of EtBr followed by incubation for 8 h at 28°C. The cells were then spread on solid YPDG media and incubated at 28°C for 3-5 days. Respiratory-deficient cells were distinguished from wild-type cells by colony size (*petite* vs. *grande*) as well as using 2,3,5-triphenyltetrazolium chloride (TTC) test (Ogur et al. 1957). The differences in the frequency of *petite* colonies were statistically evaluated using unpaired Student's t-Test by the Microsoft Excel 2010 T.TEST function. P values < 0.05 were considered significant. Transformation of *S. cerevisiae* was done as described by (Gietz et al. 1995). *Escherichia coli* DH5α (F⁻, *φ80dlacZ M15*, (*lacZYA-argF*) *U169*, *deoR*, *recA1*, *endA1*, *hsdR17* (*rk⁻, mk⁺*), *λ*, *thi-1*,

gyrA96, *relA1*, *glnV44*, *nupG*) was used for the amplification of plasmid constructs. Bacterial cultures were grown in LB medium (1% (w/v) bacto-peptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.5) containing 100 µg/ml ampicillin. Plasmids were introduced into *E. coli* cells by electroporation (Green and Sambrook 2012).

Manipulations with nucleic acids

Isolation of DNA and RNA, restriction enzyme analysis, DNA (de)phosphorylation and ligation, polymerase chain reactions, agarose gel electrophoresis, extraction of DNA from agarose gels, transfer to Nylon membranes and DNA hybridization were performed according to standard protocols (Green and Sambrook 2012) or based on the procedures recommended by suppliers of the corresponding enzymes and kits. For detection of expression of TERT-encoding gene in *S. cerevisiae*, total RNA was treated with DNase I and reverse-transcribed into cDNA using Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). The presence of mt-mTERT transcript and contamination of the samples by plasmid DNA was assessed using the primers mTERT-rtPCR-f and mTERT-rtPCR-r (sequences of all oligonucleotides are listed in Table S1) yielding a PCR fragment of 1,139 bp (Nozawa et al. 2001). The degree of contamination of the samples by genomic DNA was assessed by the primers ACT1_up2 and ACT1_down2, resulting in 117 bp fragment in case of cDNA and 426 bp fragment in case of genomic DNA. Quantitative RT-PCR (qRT-PCR) was performed on cDNA prepared as described above using Luminaris Color HiGreen High ROX qPCR Master Mix (Thermo Scientific) and primers mTERT_qpcr_f + mTERT_qpcr_r (for mTERT; Flores et al. 2006) or ACT1_rt-pcr_f + ACT1_rt-pcr_r (for ACT1; Taylor et al. 2005) and conducted with StepOne cycler (Applied Biosystems). The relative amount of RNA was determined using the method of relative standard curve method described by Livak (1997). ACT1 mRNA was used for normalization of gene expression. The differences in the relative mt-mTERT levels between cells pre-grown in SD and SGal media were statistically evaluated using Student's t-Test by the Microsoft Excel 2010 T.TEST function. P values < 0.05 were considered significant (*).

Construction of plasmids

For the gene encoding FOXC2 (www.ncbi.nlm.nih.gov/nucore/AF124843) we first needed to replace 12 TGA triplets (decoded as tryptophan in *Fusariumoxysporum* mitochondria) to TGG employed by a tryptophan codon by cytosolic ribosomes in *S. cerevisiae*. In addition, we (i) optimized the codon composition for expression of the gene in *S. cerevisiae*, (ii) placed a sequence encoding an N-terminal mitochondrial targeting signal (MTS) from subunit 9 of the F₀-ATPase (Su9) of *Neurospora crassa* (Westermann and Neupert 2000) at 5' end of the open reading frame (ORF), and (iii) placed a BssHII recognition site between MTS and FOXC2. Such engineered gene (GenBank accession number KP272109) harboring recognition sites for endonucleases HindIII and EcoRI at its 5' and 3' end, respectively, has been synthesized by MWG Operon and placed into the SmaI site of pBluescript II SK(+). The HindIII-EcoRI fragment carrying FOXC2 with MTS (mt-FOXC2) was cloned into the pYES2/CT linearized with the same restriction enzymes yielding pYES2/CT-mt-FOXC2. For construction of pYES2/CT-mt-mTERT carrying mouse TERT (mTERT) with MTS, pYES2/CT-mt-FOXC2 was digested with BssHII and EcoRI and the DNA fragment carrying mt-FOXC2 was replaced (in frame with MTS) by a PCR fragment

representing mTERT amplified from pGRN188 plasmid containing mTERT cDNA ((Greenberg et al. 1999); kindly provided by Ronald A. DePinho, The University of Texas, USA) using the primers mTERT-mtpYES-F and mTERT-mtpYES-R. Mouse TERT lacking MTS-coding sequence amplified from pGRN188 using primers mTERT-pYES-F and mTERT-mtpYES-R was cloned into pYES2/CT-Su9 digested with *Bss*HII and *Eco*RI resulting in pYES2/CT-mTERT. The plasmid pUG35-mTERT_yEGFP3 was prepared by inserting the PCR fragment carrying mTERT amplified from pGRN188 using the primers mTERT_up and mTERT_dn_noSTOP in to the *Sma*I site of pUG35-yEGFP3 ((Niedenthal et al. 1996) provided by Johannes H. Hegemann (Heinrich-Heine-Universität, Düsseldorf, Germany)). For all plasmid constructs, the regions containing the inserted DNA were verified by DNA sequencing (Macrogen, South Korea).

Fluorescence microscopy

S. cerevisiae strain W303-1A carrying pUG35_yEGFP3 or pUG35_mTERT_yEGFP3, was grown overnight in 5 ml of SD media, then inoculated into a fresh SD to a final concentration of 5×10^6 cells/ml and cultivated for another 3 h. Cells were then washed with $1 \times$ PBS, resuspended in the same buffer and incubated for 30 min at room temperature in the presence of 0.5 μ g/ml of 4',6-diamidino-2-phenylindole (DAPI). 5 μ l of the cell suspension was inspected using *Zeiss Spinning Disc* (Zeiss) microscope.

Results

Regulated expression of mitochondrially targeted mammalian TERT in *S. cerevisiae*

Before investigating effects of expression of mouse TERT (mTERT) we tested if the yeast catalytic subunit of telomerase Est2p does not affect cellular sensitivity to oxidative stress. We compared the growth of wild-type and *est2* mutant cells after the treatment with H₂O₂ and observed similar sensitivity to this agent in both strains (Fig. S1). This result together with the fact that Est2p lacks MTS (Santos et al. 2004) and is not associated with purified *S. cerevisiae* mitochondria (Sickmann et al. 2003) indicates that yeast telomerase does not play a role in protecting mitochondria against oxidative damage. To express mTERT in *S. cerevisiae* and ensure its mitochondrial localization, we constructed a plasmid pYES2/CT-mt-mTERT carrying an ORF for mTERT fused with the first 69 amino acids of the *N. crassa* Su9 that is used to target heterologously expressed proteins into *S. cerevisiae* mitochondria (Westermann and Neupert 2000). The fusion gene was placed under *P_{GALI}* promoter (Fig. 1A) enabling its conditional expression in the cells grown in SGal media containing 2% galactose. The expression of mt-mTERT in SGal media was verified by RT-PCR analysis (Fig. 1B). To obtain quantitative data on the expression of mt-mTERT under repressible and inducible conditions, we performed qRT-PCR analysis and found that the levels of mt-mTERT RNA is nearly 50-fold higher in cells grown on galactose compared with cells cultivated on glucose (Fig. 1C). Next, we investigated, if heterologously expressed mTERT is targeted to yeast mitochondria. We expressed the protein in fusion with yEGFP3 at C-terminus and assessed its subcellular localization. Indeed, when inspected by fluorescence microscopy, we observed distinct mTERT-GFP foci. Although we cannot exclude a possibility that some of these foci are represented by mTERT-GFP aggregates,

their co-localization with mitochondria stained with DNA-intercalating dye DAPI indicates that the fusion protein is present mostly in the organelles (Fig. 2).

Mitochondrially targeted mTERT protects *S. cerevisiae* against oxidative stress

The results indicating that we can conditionally express mTERT and target it to mitochondria of *S. cerevisiae* enabled us to assess its effect on the yeast cells exposed to oxidative stress. In the preliminary experiments, we observed that the expression of mt-mTERT protected yeast cells against 30 mM H₂O₂ (Fig. S2). However, we observed some variation in sensitivity of the control cells between the experiments. Namely, the effect of H₂O₂ treatment of control cells resulted in only a mild decrease of survival in some cases and thus the protective effect of mt-mTERT was not that evident. Therefore, we induced oxidative stress by alternative means and treated the cells with menadione (2-methyl-1,4-naphthoquinone). Menadione is a redox-cycling drug that can be reduced in vivo to semiquinone. This in turn reduces oxygen to superoxide anion radical, regenerating the oxidized quinone thus inducing acute generation of ROS (Hassan and Fridovich 1979; Criddle et al. 2006). We found that whereas *S. cerevisiae* transformed with an empty vector were sensitive to the drug (Fig. 3A), expression of mt-mTERT protected the cells against menadione (Fig. 3A). This protective effect was largely diminished when mTERT lacked MTS (Fig. 3B). Furthermore, rescuing growth of menadione-treated cells was dependent on the pre-cultivation of the cells carrying pYES2/CT-mt-mTERT in media containing galactose. On the other hand, pre-cultivation in repressible SD media did not result in better growth of the cells carrying the plasmid (Fig. 3C) indicating a dependence of the rescuing effect on the mt-mTERT expression. Importantly, the effect of mt-mTERT on yeast cells exposed to oxidative stress is not accompanied by changes in telomere length (Fig. S3). This implies that, similarly to mammalian cells (Sharma et al. 2012), the mt-mTERT-mediated protection against oxidative damage is independent of its function at nuclear telomeres.

Mitochondrial reverse transcriptase FOXC2 from *F. oxysporum* also renders *S. cerevisiae* resistant to oxidative stress

Next, we investigated, if the protective effect on yeast mitochondria is limited to mTERT, or can be mediated by another reverse transcriptase. To address this question we engineered a gene for FOXC2, a reverse transcriptase encoded by a mitochondrial plasmid of *F. oxysporum* (Galligan et al. 2011; Walther and Kennell 1999). We found that, similarly to mammalian TERT, expression of mt-FOXC2 in *S. cerevisiae* increased their resistance to both menadione and H₂O₂ (Fig. 3A and Fig. S2). These results demonstrate that presence of TERT and FOXC2 is beneficial for cells exposed to oxidative stress and indicate that it might be a general feature of mitochondrially localized reverse transcriptases.

Expression of TERT in *S. cerevisiae* affects the stability of mitochondrial DNA

S. cerevisiae is a facultative anaerobic *petite*-positive yeast species thus allowing testing the effect of TERT expression on the stability of mtDNA. Cells lacking a part or entire mtDNA molecule are able to grow on glucose (where they form small (*petite*) colonies), whereas they lose the ability to grow on nonfermentable carbon source such as glycerol. We observed that the expression of mt-mTERT and mt-FOXC2 harboring Su9 MTS in yeast

cells significantly increases the frequency of spontaneously formed *petite* colonies (Fig. 4A). This effect was not observed in the case of mTERT without the Su9 MTS (Fig. 4A). On the other hand, expression of mt-mTERT and mt-FOXC2 in cells exposed to EtBr reduced the frequency of respiratory-deficient mutants compared with the EtBr-treated cells lacking TERT (Fig. 4B). Conversely, a presence of mTERT without Su9 MTS did not affect the frequency of EtBr-induced *petite* mutants (Fig. 4B). Although it is difficult to explain these apparently conflicting effects of TERT on stability of mitochondrial genome it is clear that it directly or indirectly interacts with mtDNA and affects its replication, recombination and/or segregation.

Discussion

There is a growing number of so called moonlighting proteins performing different functions at various cellular loci (Huberts and van der Klei 2010). The experimental evidence from the last decade made telomerase a prominent member of this list. Although extra telomeric functions of mammalian telomerase are investigated for several years, the studies very often report conflicting results. In our paper we established a simpler experimental system allowing the analysis of mitochondrial functions of mammalian TERT in *S. cerevisiae* cells taking advantage of the fact that yeast catalytic subunit of telomerase (Est2p) is not localized in mitochondria and it does not seem to be involved in the protection of the cells against oxidative stress. On one hand it is difficult to envisage that the rest of the machinery involved in TERT-associated oxidative stress response is also present in yeast. On the other hand, this is not necessarily a limitation, because it would enable additions of other mammalian proteins and testing their effect on mTERT-mediated protection of the cells against oxidative stress. For example, similar approach has been successfully employed for reconstitution of Bax-induced cell death in *S. cerevisiae* taking advantage of the fact that there is no Bax-like protein in yeast (Polcic and Forte 2003).

Our expression system provides advantage of conditional expression of mt-mTERT as demonstrated by qRT-PCR analysis (Fig. 1C). Our attempts to confirm the expression of mt-mTERT on protein level resulted in varying results indicating that the levels of the protein are too low to be reproducibly detected by Western blot using available antibodies (data not shown). This is consistent with the previously published studies on expression of mammalian telomerase in yeast cells (Bah et al. 2004). It is possible that translation of mRNA for mt-mTERT is not efficient enough to result in detectable amount of the protein. Yet the fact that the pre-cultivation of mt-mTERT expressing cells on galactose is required for its protective effect (Fig. 3C) indicates that even a small amount of telomerase can protect *S. cerevisiae* against oxidative stress.

Both mouse and human TERTs contain an N-terminal MTS that was shown to mediate its localization into mammalian mitochondria (Santos et al. 2004). When expressed in their native forms, at least a fraction of mTERT was shown to co-localize with *S. cerevisiae* mitochondria (Fig. 2). However, to fully mediate the protective effect on menadione-treated cells, it was important to attach Su9 MTS at N-terminus of the mTERT. It is possible that, given the overall low abundance of heterologously expressed mTERT in *S. cerevisiae* (see above), its mitochondrial targeting via a more robust MTS enables its mitochondrial

accumulation above the threshold concentration needed to protect the organelles against oxidative stress.

Mitochondrially targeted mTERT and FOXC2 had a complex effect on the stability of mitochondrial genome in control and EtBr-treated cells, respectively (Fig. 4). Importantly, the complexity of this effect is similar as that observed in mammalian cell. Namely, Haendeler et al. (2009) showed that human cells containing TERT directed exclusively to mitochondria exhibit an increased resistance to EtBr-induced reduction of mtDNA copy number, which is consistent with our results. These two opposing effects of TERT possibly cause the seemingly contradictory outcomes: (1) destabilization of mtDNA (by direct or indirect means) and (2) decreasing the accessibility of mtDNA to EtBr. At present it is difficult to speculate about potential molecular mechanisms involved in these opposing effects of TERT on the stability of mtDNA. One possibility is based on the observations of Sharma et al (2012), who have shown that mitochondrially localized mammalian TERT binds various mitochondrial RNAs, suggesting that reverse transcriptase activity in the organelle is reconstituted with mitochondrial RNAs. Reversed transcription of mitochondrial RNAs might result in generation of a heterogeneous population of DNA molecules, which may interfere with mtDNA replication machinery thus causing instability of mitochondrial genome. On the other hand, it was shown that RNA converted into DNA by a reverse transcriptase activity could be used to repair DNA double strand breaks (Storici et al. 2007). Therefore, DNA molecules formed from mitochondrial RNAs by telomerase reverse transcriptase might mediate repair of defective mtDNA molecules generated in EtBr-treated cells. To test this possibility it would be important to investigate, if mutant version of TERT lacking reverse transcriptase activity lose or retain their protective ability. Furthermore, it was shown that binding of EtBr and its derivatives to DNA affects telomerase activity (Koeppel et al. 2001). Hence it is also possible that intercalation of EtBr into mtDNA may affect its interaction with TERT and change its biochemical properties compared to the untreated cells thus resulting in different effects of TERT on mtDNA stability in EtBr-treated versus control cells. A complex effect of TERT on stability of mitochondrial genome observed in our experiments also goes in line with conflicting reports demonstrating that re-localization of TERT to mammalian mitochondria can either sensitize (Santos et al. 2004; Santos et al. 2006) or protect (Ahmed et al. 2008; Haendeler et al. 2009) mtDNA against damage. These opposing outcomes are most likely caused by the fact that mitochondrially-targeted TERT may affect multiple events including mtDNA turnover, modulation of mtDNA replication, recombination and repair, processing of DNA double-strand breaks, or alteration of antioxidant defenses (reviewed in Gordon and Santos 2010) and the final effect on mtDNA stability will depend on circumstances specific for the given experimental set-up. Perhaps exploiting the experimental system described in our study will eventually shed more light on the puzzling and contradictory results.

Intriguingly, the protective effect was not limited to mt-mTERT. We observed that the expression and mitochondrial targeting of another reverse transcriptase FOXC2 also renders yeast cells resistant to menadione. As this enzyme is considered to be closely related to telomerase (Walther and Kennell 1999), it would be interesting to test, if other types of reverse transcriptases or DNA-dependent DNA polymerases would have the same effect on mitochondria as telomerase and FOXC2. In our preliminary experiments we employed two

isogenic strains of *P. kluyveri* with or without a mitochondrial plasmid pPK2 encoding DNA polymerase (Blaisonneau et al. 1999) and found that both strains exhibit similar sensitivity to oxidative stress (Fig. S4). Naturally, the reliance of this experiment must be taken with caution as it was carried out in a different organism. Expression of genes encoding various RNA- and DNA-dependent DNA polymerases and their catalytically-deficient versions using our experimental system would be instrumental in addressing the above question. Furthermore, several open reading frames located within mitochondrial group II introns encode reverse transcriptases involved in splicing (Lambowitz and Zimmerly 2011). It is possible that in addition to this primary function, these enzymes may also provide a selective advantage under conditions of oxidative stress. Thus yeast cells can be used as a ‘test tube’ not only for reconstitution of mammalian TERT extranuclear function(s), but also for addressing broader evolutionary questions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Heterologous production of mTERT in *S. cerevisiae* using galactose-induced expression
 (A) Scheme of the region of the expression plasmid encoding recombinant reverse transcriptases used in this study. P_{GAL1} , *GAL1* promoter; MTS, N-terminal mitochondrial targeting signal (MTS) from subunit 9 of the F_0 -ATPase (Su9) of *Neurospora crassa*; T_{CYC1} , *CYC1* terminator. (B) The expression of mt-mTERT coding gene was tested by RT-PCR using the primers mTERT-rtPCR-f and mTERT-rtPCR-r (mTERT primers) and the level of contamination by genomic DNA was assayed using the primers ACT1_up2 and ACT1_down2 (*ACT1* primers). Specified are expected lengths of the PCR products. C, cells transformed with the control pYES2/CT plasmid. (C) Quantitative RT-PCR analysis of RNA isolated from cells grown in glucose (SD, repressible conditions) and galactose (SGal, inducible conditions) media was performed as described in Materials and Methods. Presented are results of three technical replicates. Bars represent standard errors of means (SEM). The differences in the relative mt-mTERT levels between cells pre-grown in SD and SGal media were statistically evaluated using Student's t-Test by the Microsoft Excel 2010 T.TEST function. P values < 0.05 were considered significant (*).

Figure 2. Mouse TERT co-localizes with DAPI-stained mitochondria of *S. cerevisiae* when expressed in fusion with GFP at C-terminus

C, control cells carrying pUG35-yEGFP3 plasmid.

Figure 3. Expression of mitochondrially targeted mTERT in *S. cerevisiae* protects the cells against oxidative stress

(A) Cells transformed with a control plasmid pYES2/CT ("C") or vectors carrying either mtFOXC2 or mt-mTERT were pre-cultivated in SGal (inducible conditions) medium and treated with menadione as described in Materials and Methods. Aliquots from 10-fold serial dilutions were then spotted on SD plates. (B) The same experiment as in (A) was performed with a plasmid carrying mTERT lacking Su9 MTS. (C) Cells transformed with a control plasmid pYES2/CT ("C") or plasmid carrying mt-mTERT were pre-cultivated in SGal (inducible conditions) or SD (repressible conditions) media and treated with menadione. Aliquots from 10-fold serial dilutions were then spotted on SD plates.

Figure 4. Mitochondrially localized mTERT and FOXC2 affect the stability of yeast mitochondrial genome

To induce the expression of the cloned genes, cell cultures were grown in SGal medium for 20 h followed by treatment without (A) or with (B) 25 μ MEtBr for 8 h at 28°C as described in Materials and Methods. The cells were then spread on YPDG medium and after 3-5 days of cultivation at 28°C respiratory-deficient cells were distinguished from wild-type cells by colony size (*petite* vs. *grande*) as well as using TTC overlay test (Ogur et al. 1957).

Presented are results of three independent experiments. Bars represent standard errors of means (SEM). The differences in the frequency of *petite* colonies were statistically evaluated using unpaired Student's t-Test by the Microsoft Excel 2010 T.TEST function. P values < 0.05 were considered significant (*).