The role of telomerase in brain during ageing and under dietary restriction

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Abstract

Telomerase is best known for its nuclear telomere maintenance function via its enzymatic activity requiring the two major components TERT (protein) and TERC (RNA). However, telomere and TERC-independent functions for TERT have been described recently.

Our group and others have previously shown that telomerase shuttles to mitochondria improving their function and decreasing cellular oxidative stress. Oxidative stress and mitochondrial dysfunction are well known to increase during ageing and have been implicated as a cause for age-related neurodegenerative diseases.

I report that TERT protein localises in mammalian brain mitochondria specifically in neurons. Moreover, although telomerase activity is negligible in brain, I found considerable amounts of the telomerase protein TERT in mouse and human brain by independent techniques.

Dietary restriction (DR) is known as a condition that improves mitochondrial function, delays or prevents age related diseases and improves cognitive function. In this study mTert was detected in brain mitochondria under basic conditions, and it becomes elevated in brain mitochondria of animals in three independent experiments of short term (3-6 months) DR.

Decreased signalling through mTOR has been described as a major mechanism of the DR response. Accordingly, I found that mTOR phosphorylation was down regulated in brains from DR animals. Moreover, to analyse whether decreased mTOR signalling causes mitochondrial redistribution of mTert, mTert wild type and mTert knock-out mice were treated with rapamycin what resulted in increased mTert protein in brain mitochondria and correlated with decreased mitochondrial ROS production. The conclusion is that down-regulation of mTOR is a possible mechanism to increase mTert protein levels within mitochondria under DR.

Together, this data shows that mitochondrial mTert might be an important new player in the protection of neurons and improve brain function during ageing.
Declaration

I, the undersigned, hereby declare that this thesis contain my own work and I have correctly acknowledged the work of collaborators. Any part of this work has not been submitted for fulfilment of a degree at this or any other university, and that all the sources I have used here have been indicated or acknowledged by means references.

Rafal Czapiewski

Signature:
Acknowledgements

This work would not be completed without support of my supervisors, colleagues, friends and family.

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<th>Definition</th>
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<tr>
<td>AA</td>
<td>antimycin A</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
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<tr>
<td>AL</td>
<td><em>ad libitum</em></td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DDR</td>
<td>DNA damage respond</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DR</td>
<td>dietary restriction</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immuno sorbent assay</td>
</tr>
<tr>
<td>EPI-NCSC</td>
<td>epidermal neural crest stem cells</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transport chain</td>
</tr>
<tr>
<td>f1</td>
<td>first generation knock-out</td>
</tr>
<tr>
<td>f4</td>
<td>fourth generation knock-out</td>
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FADH₂  flavin adenine dinucleotide
FBS  foetal bovine serum
FCCP  carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone
FSC  forward scatter
GAPDH  glyceraldehyde 3-phosphate dehydrogenase
HBSS  Hank's balanced salt solution
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP  horseradish peroxidase
hTERC  human telomerase RNA component
hTERT  human Telomerase Reverse Transcriptase
IF  immunofluorescence
IMS  inner membrane space
MEaF  mouse ear fibroblasts
MEF  mouse embryonic fibroblasts
MEM  minimum essential media
mRNA  messenger ribonucleic acid
mtDNA  mitochondrial DNA
mTerc  mouse telomerase RNA component
mTert  mouse telomerase reverse transcriptase
mTOR  mammalian target of rapamycin
MTS  mitochondrial-targeting signal
NADH  nicotinamide adenine dinucleotide
NBTR  Newcastle Brain Tissue Resource
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>NES</td>
<td>nuclear export signal</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NSC</td>
<td>neural stem cells</td>
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<tr>
<td>OCR</td>
<td>oxygen consumption rate</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>oxidative phosphorylation</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PD</td>
<td>Parkinson's disease</td>
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<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PGC1α</td>
<td>peroxisome proliferator-activated receptor-γ coactivator 1α</td>
</tr>
<tr>
<td>PM</td>
<td>pyruvate:malate</td>
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<tr>
<td>POLG</td>
<td>mutated mitochondrial DNA polymerase γ</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction (Real-Time PCR)</td>
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<tr>
<td>RCR</td>
<td>respiratory control ratio</td>
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<tr>
<td>RMRP</td>
<td>processing endoribonuclease</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
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<tr>
<td>rTert</td>
<td>rat telomerase reverse transcriptase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
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<tr>
<td>SASP</td>
<td>senescence-associated secretory phenotype</td>
</tr>
<tr>
<td>SA-β-Gal</td>
<td>senescence associated β-galactosidase</td>
</tr>
<tr>
<td>SFM</td>
<td>serum free media</td>
</tr>
<tr>
<td>SIPS</td>
<td>stress-induced premature senescence</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
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<tr>
<td>SSC</td>
<td>sideway scatter</td>
</tr>
<tr>
<td>STS</td>
<td>staurosporine</td>
</tr>
<tr>
<td>TA</td>
<td>telomerase activity</td>
</tr>
<tr>
<td>TBST</td>
<td>tris-buffered saline with Tween</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid cycle (Krebs cycle)</td>
</tr>
<tr>
<td>TIM</td>
<td>translocator of the innermembrane</td>
</tr>
<tr>
<td>TMB</td>
<td>tetramethylbenzidine</td>
</tr>
<tr>
<td>TOM</td>
<td>translocator of the outermembrane</td>
</tr>
<tr>
<td>TRAP</td>
<td>telomeric repeat amplification protocol</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet radiation</td>
</tr>
<tr>
<td>WB</td>
<td>Western blotting</td>
</tr>
<tr>
<td>γH2A.X</td>
<td>histone H2AX phosphorylated at serine 139</td>
</tr>
<tr>
<td>ΔΨm</td>
<td>mitochondrial membrane potential</td>
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Chapter 1 – Introduction

1.1. Cellular senescence and organismal ageing

Ageing research is one of the fastest growing fields within biomedical science - mainly due to the impact it has on the costs of public health care. Advanced age remains the highest risk factor for chronic diseases such as Alzheimer’s and diabetes. Reduction in mental and physical ability of older people is also important in the perspective of demographic challenges in aged western societies [1]. Moreover, it is thought that improvements in health of older people will increase their general life satisfaction and comfort. Despite improvements in human longevity in the last decades, disabilities and age related disorders are still unsolved challenges for biomedicine. Moreover, basic mechanisms of ageing still remain elusive.

Cell senescence was originally defined in vitro as the irreversible loss of replicative potential without loss of metabolic capacity [2-4]. This definition is used to describe ageing-associated molecular changes in human cells and might have relevance to the ageing process in vivo [5]. Ageing of an organism is described as the decreased ability to respond to stress, increased homeostatic imbalance and increased risk of diseases accompanied by decreased fertility and increased mortality, all correlated with chronological age [6].

A proposed evolutionary function of senescence is tumour suppression since the main feature of cell senescence is cell cycle arrest via the p53 and p16INK4a pathways [3]. Internal and external factors may induce/accelerate senescence. It is believed that senescence is closely linked with damage. Macromolecules like DNA and proteins are exposed to damage from factors like UV and Reactive Oxygen Species (ROS). The origin and role of ROS will be discussed in the following chapter. To initiate cellular senescence a DNA damage response is required and it can be triggered by uncapping of telomeres or by DNA damage in non-telomeric regions of chromosomes [7]. Among the manifestations of a senescent cell, the most apparent is shortening of telomeres (ends of chromosomes) and accumulation of DNA damage [3, 8]. Furthermore, senescence is associated with a variety of phenotypic changes including growth arrest, altered gene expression, apoptosis resistance and autophagy inhibition [3, 9-11]. Moreover, recent research by Judith Campisi’s group shows that cells when they senesce exhibit a Senescence-Associated...
Secretory Phenotype (SASP) which involves the secretion of specific signalling factors [12, 13]. For instance, it was shown that senescent cells secrete interleukins (*i.e.* IL-6, IL-7), inflammatory cytokines (*i.e.* IL-8), and growth factors (*i.e.* bFGF) that can affect surrounding cells and are mostly associated with inflammation and cancer development [13]. Furthermore, a new term SIPS (Stress-Induced Premature Senescence) was used to define senescence induced by exogenous oxidative factors which leads to irreversible growth arrest of proliferative cells. However, there is still open discussion whether SIPS shows the same characteristics as replicative senescence [4, 14].

Although a common molecular marker for all senescent cells has not been found, a wide range of senescent cells show DNA damage foci with activated γH2A.X, increased p16*INK4a* expression and often are Senescence Associated β-Galactosidase (SA-β-Gal) – positive [15].

Apart from molecular mechanisms and manifestations of cell senescence, there is still an unanswered question as to how these processes contribute to age-related diseases and what is their influence on tissue decline as well as organ dysfunction *in vivo?* It was shown recently in a mouse model by Baker *et al.*, (2011) that clearance of p16*INK4a* –positive (senescent) cells might delay age-related phenotypes in various tissues *i.e.* skeletal muscle or bone marrow [16]. Furthermore, it is thought that senescence of stem cells might significantly contribute to age-related decline in tissue renewal and homeostasis [17]. During ageing, a deterioration of stem cell function and sometimes a decline in quantity has been observed [18]. For example, it was shown in a mouse model that depletion of the Neural Stem Cells (NSCs) pool in brain is responsible for age-related phenotypes, such as loss of olfactory and cognitive functions [19-22]. Molecular manifestations of stem cell senescence are similar to somatic cells; however, telomere shortening is significantly slower due to reactivation of telomerase in ageing stem cells [23, 24]. The dynamic of telomerase in stem cells will be discussed in the forthcoming chapter.
1.2. Mitochondria

1.2.1. Structure and function of mitochondria

A close connection between mitochondria and ageing has been discussed and studied for decades. It was shown that defects in mitochondria function may be a cause and an effect of accelerated senescence and ageing at the same time [25].

Mitochondria are “the powerhouse of the cell” and are responsible for production of ATP during OXPHOS (Oxidative phosphorylation), cell signalling regulation, Ca²⁺ storage and cellular pH maintenance. Mitochondria are enclosed by an outer- and inner-membrane, which forms numerous cristae. In addition, mitochondria contain nucleic acids like RNA and mitochondrial DNA (mtDNA) coding some of the mitochondrial proteins as well as rRNAs and tRNAs. Since only 13 proteins are coded in mitochondria, most of the polypeptides necessary for mitochondria function are transported from the cytoplasm to the mitochondrial matrix in an unfolded form by complexes of the TOM and TIM protein family (Translocator of the Inner- and Outer-Membrane). The signal sequence on the N-terminus of polypeptides synthesized in the nucleus is recognized by TOM complex. Next, the transported polypeptide chain is inserted in the TOM complex, then TIM complex, which is linked at sites of contact between two membranes, translocates molecules into the matrix of mitochondria [25].

The main function of mitochondria is the production of easily accessible energy in the form of ATP (Adenosine Triphosphate) through OXPHOS. The machinery for oxidative phosphorylation contains the Electron Transport Chain (ETC), which includes five large protein complexes. Each complex contains multiple polypeptides, and changes in their expression are currently analysed for their role in mitochondria bioenergetics and potential impact on ageing [25]. Complex I (properly referred to as NADH-ubiquinone oxidoreductase) is a structure of 46 protein subunits and 7 of them are encoded by mtDNA. Complex I is a provider of electrons from oxidation of NADH carried by ubiquinone to complex II and protons H⁺ which are released to IMS (Inner Membrane Space). Complex II (succinate - ubiquinone oxidoreductase) is the simplest polypeptide in the ETC consisting of only four peptides coded by nuclear DNA. This complex catalyses the oxidation of succinate to fumarate, which is another source of electrons carried by ubiquinone. The receiver of electrons from complex I and II is ubiquinone (Q) which
passes electrons to complex III. Also known as ubiquinone-cytochrome c oxidoreductase, complex III is assembled from 11 peptides in mammals (only one – cytochrome b is encoded by mtDNA) and its role is to pass electrons through cytochrome c to complex IV. Cytochrome c oxidase, as complex IV is often referred to, catalyses the reaction where cytochrome c is a terminal acceptor of electrons which results in H₂O and H⁺ release to the IMS. Proton efflux to IMS coupled with reactions catalysed by complex I, III and IV causes an electrochemical gradient also referred as mitochondrial membrane potential (ΔΨm). This potential is a source of energy for the ATP synthase complex (also called complex V). Reflux of protons accumulated in the IMS through complex V releases energy for the phosphorylation of ADP. This reaction is coupled with redox reactions in complex I, III and IV as well as with electron transport (Figure 1.1 A.) [25].

The electron transport chain is coupled with the Tricarboxylic Acid Cycle (TCA) also known as the Krebs cycle or citric acid cycle. The role of these series of chemical reactions occurring in the mitochondrial matrix is to convert pyruvate obtained from glucose to carbon dioxide. Furthermore, TCA is a source of protons carried in molecules of NADH and FADH₂ used in the ETC (Figure 1.1 A.)[25].
Figure 1.1. Electron transport chain and superoxide generation sites. A. Electron transport chain with forward electron transport. B. Superoxide production during reverse electron flow is caused by excess of succinate (substrate) and accumulation of electrons. Reverse electron flow in vitro might be increased by rotenone which blocks ubiquinone binding site of complex I and inhibit electron passing.
1.2.2. The role of mitochondria in senescence and ageing

There is a plethora of published data describing the role of mitochondria in the senescence process [26-29]. It has been shown that mtDNA mutation, disruption in the electron transport chain and decrease in membrane potential may lead to cell dysfunction, senescence and – potentially – death. A wide discussion about the role of mitochondria in senescence started in 1954 with the work of Denham Harman and his “free radical theory” where he suspected that ageing is a result of molecular damage accumulation generated by ROS [30].

Reactive oxygen species include the superoxide radical (O$_2^-$), hydroxyl radical (OH$^-$), and hydrogen peroxide (H$_2$O$_2$). Mitochondria are responsible for the majority of overall cellular ROS production [31, 32]. The majority of free radicals are generated due to “leakage” of electrons from the ETC (mostly from complex I and III) and by deriving them from molecular oxygen [33-35]. Complex I appears to be the prime producer of ROS in cells. It can form superoxide during forward electron flow (NADH – oxidizing) described in the subchapter 1.2.1 as well as during reverse electron transport (NAD$^+$ – reducing) which is caused by accumulation of electrons from complex II (due to excess of substrate – succinate) during the resting state of mitochondria (Figure 1. 1 B.) [36]. Moreover, significant amounts of ROS are also produced in the cytosol during metabolic reactions which use oxygen as a substrate [25].

There is a continuous discussion on role of ROS in senescence and ageing [37, 38]. Most importantly, ROS generated in mitochondria is thought to be responsible for generating mtDNA damage leading to mitochondrial dysfunction. It has been shown that point mutations and deletions in mtDNA accumulate with age in mammalian tissue causing respiratory disruption that leads to dysfunction of somatic stem cells and premature ageing [39, 40]. Interestingly, a very recent study from our institute, shows that ROS production in mitochondria and nuclear DNA damage can be amplified by a positive feedback loop through the p21 pathway [7]. According to this theory, repair mechanisms and defence systems become overwhelmed with accumulating damage, leading to mitochondrial damage causing permanent senescence changes. Direct evidence for the connection between mitochondria dysfunction and ageing was provided by Trifunovic et al., (2004) and Kujoth et al., (2005) using a mouse model. Both groups showed elegant experiments on a
progeroid effect of proofreading deficiency by mutated mitochondrial DNA polymerase γ (POLG) causing a number of age related changes like reduced life span, hair loss, anaemia, osteoporosis, reduced fertility and induction of apoptotic markers in different tissues (no senescence, mainly apoptosis) [41, 42]. Furthermore, it was shown that mitochondrial lipid oxidation (i.e. cardiolipin) may affect membrane fluidity and disrupt the electron chain transport, thus impairing respiration and as a consequence, ROS generation might be accelerated [43]. Interestingly, there is evidence that ROS can act as a signalling molecule, enabling adaptation to stress [44]. For example, Hekimi and colleagues have formulated an interesting hypothesis in which ROS generation is not a cause of ageing, but rather characterises a stress signal in response to damage [45].

Abundance of ROS in healthy cells is continuously controlled by antioxidant enzymatic defence. Antioxidants like superoxide dismutase, catalase or peroxidases are able to inactivate free radicals, however, with age their activity might be reduced and oxidative stress becomes more apparent [46-48]. Additionally, it was shown that over-expression of antioxidant genes might increase – while its deletion might reduce lifespan of mouse models [49-51]. However, on the other hand, diet supplementation with antioxidants did not show a clear pro-survival effect in many models so far [52]. Furthermore, it was shown by Perez and colleagues that over-expression of different combinations of SOD1, SOD2 or catalase decreases oxidative damage in transgenic mice without extending lifespan [51]. In addition, similar studies on fruit flies and C. elegans also showed that over-expression or deletion of antioxidant genes may have no influence on the survival of these animals leading to a conclusion that the role of ROS in ageing is still not explained sufficiently [53, 54].

In humans, there is evidence that ATP production and respiratory capacity of mitochondria decrease with age [55]. Moreover, mitochondrial dysfunction is associated with many age related brain diseases (encephalopathy) and muscular disorders (myopathy) [56, 57]. However, there is still no agreement in the role of mitochondria in neurodegenerative diseases. It was shown that disruption of complex I in mitochondria in the substantia nigra of the human brain can lead to the death of dopaminergic neurons and may be a cause of Parkinson’s disease (PD) [58, 59]. In contrast, the role of mitochondrial dysfunction in death of cholinergic neurons in Alzheimer’s disease (AD) is still not fully eluded. Observed mutations of mtDNA
and mitochondrial dysfunction in AD are recognised as an effect of the disease rather that one of primary causes [60]. To conclude, damage of mtDNA and respiration deficiency appear as a main contributor to age related dysfunction of mitochondria. Nonetheless the cause-effect relationship between malfunction of mitochondria and neurodegenerative diseases still needs to be elucidated [27-29, 61].
1.3. Dietary restriction and related signalling pathways

1.3.1. Dietary restriction

Dietary restriction (DR) is the only non-genetic manipulation able to significantly increase life span of many animal models. Thus, DR is an attractive intervention to analyse lifespan, ageing and age-related diseases.

It was proposed for the first time in 1935 by McCay that DR may be able to prolong lifespan. He observed that laboratory rats, who were fed a reduced calorie diet while maintaining micronutrient levels, had extended life spans when compared to controls [62]. There is a plethora of reports from yeast, throughout invertebrate animals such as worms (C. elegans) and flies (D. melanogaster) to a variety of mammalian species, including rodents, dogs and monkeys that DR is beneficial for health improvement and lifespan extension [63-70]. The term Dietary Restriction or caloric restriction (CR) refers to the limitation of food intake by an organism without malnutrition or starvation. CR is a category of DR where only caloric food components are reduced. Other dietary modifications used in ageing research include alterations in protein intake (or some amino acids), and changes of fat content in the diet. For example, experiments on mice with reduction of selected amino acids (tryptophan, methionine) in their diets, increased life span of rodents but not to the same extent as DR [71-74].

The most important physiological benefits of DR include body mass reduction, fat content decrease, lower blood glucose and improved insulin sensitivity [75, 76]. It was shown on many animal models that DR improves mitochondrial function and biogenesis [77]. An important feature of DR is the significant reduction of the mitochondrial ROS production and decreased level of oxidative stress in liver, muscle and brain in rodents and primates [32, 78-81]. Furthermore, recently Miwa et al., (2008) showed that dietary restriction significantly increases mitochondrial turnover rate in mouse liver compared to ad libitum (AL) feed controls, this may help explain improved mitochondrial function after DR [82]. Another example of the beneficial effect of DR on mitochondria was described by Jang and colleagues who have shown that DR can reduce oxidative damage in rodent muscle and protects against sarcopenia even with a deletion of a key antioxidant enzyme (Sod1-/-) [83]. On the other hand, it has been shown that DR may actually increase transient ROS
production. For instance, Shultz et al., (2007) reported that reduced nutrients in *C. elegans* increased production of ROS which induced catalase activity, leading to increased oxidative stress resistance and survival [84]. The authors propose the explanation that DR transiently promotes ROS production, which leads to ignition of stress defence mechanisms, resulting in long term reduction of oxidative damage [84]. It is thought that many of these mitochondrial and metabolic benefits of DR are observed due to increased expression of the transcriptional co-activator PGC-1α, which modulates activity of transcription factors [85, 86]. PGC-1α was shown to increase mitochondrial biogenesis under DR due to its ability to modulate the activity of several transcription factors and co-activators involved in mitochondrial respiration and biogenesis e.g nuclear respiratory factor 1 (NRF-1), NRF-2, PPARc, steroid receptor coactivator-1, and mitochondrial transcription factor A [87, 88].

Interestingly, DR was shown to have a protective effect on the brain. For example, short term DR resulted in a delayed occurrence of neurodegenerative syndromes in animal models of Parkinson’s and Alzheimer’s disease [89, 90]. Several studies suggested that inflammation might be an important cause of the age related cognitive decline and neurodegeneration. It was reported that the anti-inflammatory effect of DR might suppress age related deterioration of the central nervous system [91]. Furthermore, Morgan et al., (1999) showed that DR is able to attenuate the activation of astrocytes and microglia, which are recognised as factors related to synaptic atrophy, demyelination and neurodegeneration [92]. In addition, several studies have revealed that generation of new neurons can be stimulated by dietary restriction. It has been shown that DR is efficient with restoration of neuronal plasticity and improves cognitive abilities of ageing animals [93]. Moreover, DR may improve immune function of T-lymphocytes and reduce cancer occurrence in rodent and primate models what might be another cause of average lifespan extension in restricted animals [94-96].

Surprisingly, food intake limitation is not always as efficient as life extending manipulation. Hempenstall et al., (2010) and other groups have reported that some strains of rodents do not show a life extending effect of DR. Specifically, the laboratory mouse strain DBA/2 shows some improvements in health span but there was no prolongation of lifespan under DR [97]. Furthermore, large scale studies by two independent groups on ILSXISS recombinant heterogeneous inbred mice strains,
showed inconclusive results on DR influence on longevity [98, 99]. In both studies, performed on male and female animals, an unexpected pattern was observed. For instance, DR decreased the lifespan of 20–25% strains tested, increased the lifespan of only 5–25% of strains, and had no significant result on lifespan on the majority of strains [98, 99]. Therefore, the genetic background of laboratory rodents required for a positive DR response is under intensive research [91, 97, 100, 101]. Similarly, recent research presented by Mattison et al., (2012) on Rhesus monkeys showed that caloric restriction does not extend life span [102]. Nonetheless, the control group in the study was not fed a standard *ad libitum* diet. It was stated, that AL Rhesus monkeys in this study were slightly restricted to avoid obesity [102]. However, a number of experiments on long-lived species in laboratories around the world are still not finished. Nevertheless, all data so far suggests that DR is beneficial for the health span of many animal models, including non-human primates and humans [103].

**1.3.2. The role of mTOR pathway in DR response**

There are several pathways known to be altered as a molecular response to DR. The most extensively analysed at the moment are the mTOR/S6K1 pathway, the insulin/IGF signalling, sirtuin regulation and the PGC-1α pathway [104-107].

Most indications so far suggest that the life span extension mechanism induced by DR could be explained by down-regulation of the mTOR pathway. Mammalian Target of Rapamycin (mTOR) is a kinase that regulates signals for cellular growth control in response to nutrients. Furthermore, the mTOR pathway is widely studied due to its key role in apoptosis and cancer development. It was shown that elevated activation of mTOR signalling reduces apoptosis and increases proliferative capacity of cancer cells [108]. Furthermore, it was shown that mTOR can be a link between nutrients and energy signalling pathways [109]. Recently, the kinase was also described as a regulator of nutritional and hormonal signals in the brain [110]. There are reports suggesting that inhibition of the mTOR pathway extends life span of yeast, worms, flies and mice [111-113].

The kinase mTOR is a catalytic subunit in two protein complexes: mTORC1 and mTORC2. The first complex, consist of mTOR kinase, Raptor protein, mLST8, PRAS40 and DEPTOR. The function of mTORC1 is to regulate protein synthesis
and to coordinate nutrient/energy/redox signalling. This complex is stimulated by insulin, growth factors, amino acids and oxidative stress. The downstream targets of mTORC1 are S6K1 (activated) and 4E-BP1 (suppressed), which are both known for the regulation of protein synthesis and cell proliferation [114, 115]. The second complex – mTORC2 – is formed by mTOR, Rictor, GβL and mSIN1. Its main function is the phosphorylation of Akt protein kinase, which is known for its role in the regulation of glucose metabolism and cell proliferation [116].

It is known that the downstream targets of mTOR are engaged in the reduction of oxidative stress as well as the response to DNA damage and induction of apoptosis. Schieke et al., (2006) reports that mTOR co-purifies with mitochondrial fraction and plays a significant role in oxidative capacity and mitochondrial oxygen consumption [117, 118]. In the study of Schieke and colleagues, treatment with rapamycin resulted in the disruption of the mTOR-raptor complex that caused lower mitochondrial membrane potential (ΔΨm), oxygen consumption and decreased ATP synthesis [117]. Furthermore, Anisimov et al., (2011) showed that inhibition of mTOR by rapamycin slows ageing and increases lifespan in mice [119]. More recently, Wilkinson et al., (2012) confirmed that rapamycin supplementation could increase lifespan in mice, decrease tumour incidence as well as decrease degenerative age related changes in liver and heart [120]. However, in the same study animals fed with rapamycin had an increased cataract severity and increased testicular degeneration – both not reported before in comparable studies [120]. Direct evidence that mTOR/S6K1 pathway is involved in mammalian lifespan regulation was provided by Selman et al., (2009) [113]. The authors showed in a mouse model that deletion of the S6K1 kinase, a downstream target of mTOR pathway, increased lifespan; improved insulin sensitivity and increased blood glucose clearance. Moreover, in S6K1β−/− neuromuscular function was improved in comparison to the wild type litter mates. Furthermore, S6K1β−/− animals had reduced body weight and total body fat content compared to control animals which all together makes the mTOR pathway a prime candidate for further study on longevity and dietary restriction [113]. Interestingly a recent publication by Fok et al., (2012) suggests that indeed treatment with rapamycin and dietary restriction may have a similar effect on mice [121]. In the experiments mice were dietary restricted or supplemented with rapamycin, the authors reported a similar inhibition of the S6K1 – mTOR pathway.
downstream target as well as similar increase in autophagy in liver after both treatments. However, animals from the DR group and rapamycin treated group showed the opposite effect on blood glucose clearance and different insulin tolerance, showing the benefits of dietary restriction and deteriorative effect of rapamycin [121]. Thus, it is still debatable as to whether inhibition of mTOR pathway might mimic the effects of dietary restriction on health and lifespan.
1.4. Structure and canonical function of telomerase

1.4.1. TERT and TERC complex

Telomerase is a ribonucleoprotein and reverse transcriptase, well known for protecting the ends of chromosomes (telomeres) during cell proliferation and for preventing senescence [122]. Telomerase was first described in the ciliate *Tetrahymena* by Nobel Prize winners Elizabeth Blackburn, Carol Greider and Jack Szostak in 1985 [123]. It attracted the attention of the public and scientists from different fields of molecular biology and medicine due to the popular belief that telomerase may hold the secrets of human immortality. Telomerase contains the catalytic subunit TERT and an RNA component TERC including the template region for telomere synthesis as well as other protein components including heat-shock protein 90 (hsp90) and dyskerin (Figure 1.2) [124-126]. Both TERT and TERC elements are required and sufficient for its canonical function, which is the maintenance and elongation of telomeres (catalytic activity) [124]. Since mouse and human telomerase will be analysed in this thesis, only mammalian telomerase structure is going to be described here.

The human gene *hTERT* is located on chromosome 5 while mouse *mTert* is located on chromosome 13 [127]. Mammalian TERT mRNA consists of 16 exons and 15 introns extending over 35kb [128]. There are a number of publications showing that mouse and human TERT gene have a similar promoter region, although are differently regulated [129]. The sequence of the *hTERT* promoter comprises a core region from -330bp to +37bp numbered from transcription start codon ATG of the *hTERT* gene and contains E-boxes (CACGTG) but does not contain TATA or CAAT boxes and is comparable to the *mTert* promoter [127, 128]. It was shown that only *hTERT* but not *mTert* transcription is activated by c-Myc – a well-known oncogene. Furthermore, *hTERT* expression is up-regulated by SP1, AP-2, and HIF-1 transcription factors [128, 130-132]. Human TERT gene is repressed by a number of factors including: MEN1, WT1, Mad1 (by blocking c-Myc), E2F, AP-1, HDAC1, and p53 (by repression of SP1) [133-136]. In contrast, transcription of *mTert* is not sufficiently described. Formerly, it was suggested that c-Myc might also be a *mTert* transcriptional activator, although nowadays, despite the lack of direct evidence,
proteins NFAT5, Sp1, Sp3 and NFκB appear to be the most probable regulators of mTert transcription [137-140].

On the protein level, human TERT consists of 1132 amino acids, encoding a ~127 kDa polypeptide [125, 127, 141]. The structure of the protein revealed four conserved domains: the telomerase essential N-terminal domain (TEN), the telomerase RNA-Binding Domain (TRBD) including T-motif, the RT repeats and the C-terminal extension (CTE) (Figure 1. 2 A.). The tertiary structure of the TERT protein consists of fingers, palm and thumb which allow telomerase to attach by the TEN domain to single stranded telomeric DNA (Figure 1. 2 B.) [124, 125, 142].

The non-coding RNA component of telomerase (TERC or TR) also shows a high degree of similarity between mouse and human, and both hTERC and mTerc are located on chromosome 3. Mature mammalian TERC contains 451 nucleotides and its template part contains 11 nucleotides (5'-CUAACCCUAAC-3') complementary to the telomeric sequence [143, 144]. Apart from high structural similarity, Martin-Rivera et al., (1998) have shown a high functional similarity between mouse and human telomerase. In their experiments mTerc knockout mouse cells (telomerase activity deficient) were transfected with hTERC that fully restored telomerase activity, indicating that mouse and human telomerase components are functionally interchangeable and thus structurally conserved [141]. Interestingly, in most adult human tissues there is a very low expression level of hTERT protein and negligible telomerase activity, while in mouse the enzyme is expressed in a wide range of adult tissues [127]. It was shown that altered post-translational regulation is responsible for the different in vivo expression of hTERT and mTert proteins [145]. Furthermore, in mammals, tissue specific alternative splicing variants are possibly responsible for a lack of telomerase activity even when both TERT and TERC are detected [146, 147]. Interestingly, accumulating evidence suggests that alternative splicing of hTERT mRNA is involved in the carcinogenesis of gastric cancer, melanoma and kidney cancer [148-150].
Figure 1.2. The structure of human telomerase. A. Functional domains of the hTERT protein. Drawing based on Cong et al., (2002) [133] B. Model of telomerase. Both subunits TERT and RNA template TERC are required for catalytic/ enzymatic activity. Picture adapted from Lingner et al., (1997) [124].
1.4.2. Canonical function of telomerase

To understand the canonical function of telomerase it is important to explain the biology of telomeres – the ends of chromosomes. In mammals, telomeres are specific 5-15 kb repetitive DNA sequences (TTAGGG), and are bound by a multi protein complex, known as shelterin. This protein complex comprised of TRF1, TRF2, Rap1, TIN2, TPP1 and POT1 [151-153]. The function of these proteins is to arrange structures to stabilise the end of a chromosome known as T-loop and D-loop. The proteins TRF1 and TRF2 associate with Rap1, and bind to the double stranded telomeric sequence, catalysing the folding of telomere DNA into the centromere direction forming a large telomere loop (T-loop) [154, 155]. The heterodimer POT1/TPP1 binds to the 3’ end of single stranded DNA to bind to the double stranded telomeric sequence of the 5’end and form a displacement loop (D-loop). The TIN2 protein is a linchpin of telomeric loops by facilitating assembly of TPP1/POT1 to TRF1 and TRF2 (Figure 1. 3 A.) [153, 156-159].

With each cell division chromosomes must be replicated to share nuclear DNA between two daughter cells. The end of chromosomes (telomeres) become shorter due to the “end of replication problem”, which occurs when during replication the DNA of the lagging 3’ – strand cannot be elongated by DNA polymerase continuously and gets shorter than the leading 5’ – strand [160]. To clarify, on the lagging strand, replication cannot be continuous at the end of chromosomes due to the semiconservative replication of DNA (which occurs only in the 5’ to 3’ direction). On the 3’ to 5’ strand DNA polymerase α uses short RNA sequences as primers, and initiates replication of short DNA sequences from a 3’ hydroxyl group on the lagging strand (Okazaki fragments). Afterwards the RNA primers are removed and DNA ligase connects the DNA of the Okazaki fragments (Figure 1. 3 B.). However, at the very end of the 3’ strand in the telomeric region, the RNA primer is dissociated and the DNA polymerase cannot fill the gap leaving a 3’ overhang what results in the shortening of the telomere in the next cell division. Thus, a short fragment of the telomere (~20bp) is lost during each cycle of replication. Once the length of the telomeric DNA reaches a critical level the cell is facing replicative senescence and cell cycle arrest [161]. Telomerase has the ability to recognise the strand (3’ – G-rich) and elongate it with telomeric repeats 5’
TTAGGG which are complementary to the RNA template on the TERC component (Figure 1.3 C.) [162].

Interestingly, experimentally measured loss of telomere length with each cell division in mammalian somatic cells without telomerase is higher than would be expected from the “end replication problem”. It was shown by von Zglinicki et al., (1995, 2000) that telomere shortening is significantly accelerated with each cell division by oxidative stress (~50-100 bp) [163, 164].

Telomerase through its primary function (telomere elongation) protects against erosion of telomeres, instability of chromosomes, and against chromosome end fusion. Hence, an active enzyme is necessary to keep an indefinite potential of proliferation in immortal cells (i.e. cancer cells, germ cells, embryonic stem cells and in vitro immortalized cells) [127, 141, 165]. In the absence of telomerase (i.e. in human somatic cells) telomeres become shorter and activate, at a critical point, a p53-dependent DNA damage response (DDR) in the telomeric region and may lead to cell cycle arrest and senescence or apoptosis [166-168]. The DDR is activated by the sensor kinases as ATM/ATR or DNA-PK, phosphorylation of histone γH2A.X, and as a result, activation of p53/p21 pathway or Chk1 and Chk2 – cell cycle check point proteins which leads to a cell-cycle arrest [169]. Thus, telomerase through its telomere dependent function is involved in a number of processes in cells; most importantly in development, carcinogenesis and senescence.

The level of telomerase activity is tissue specific in mammals. In mice it remains active in different cells and tissues (i.e. lymphocytes, intestine, muscle, testes, and liver) throughout life. Furthermore, mus musculus maintain significantly longer telomeres (~50-70kb) than in humans (~10kb). Telomerase is active during human embryonic development and decreases gradually after 20 weeks of gestation in the embryo. However, in adult humans, telomerase activity is still detectable in lymphocytes, endothelial cells, germ cells and adult stem cells. [170, 171]. Telomerase activity during differentiation of stem cells was shown to be regulated by epigenetic modifications including: methylation/demetylation as well as acetylation/deacetylation of histone H3 and H4 in the promoter region of hTERT and histone H3 at the hTERC promoter [172-174].

Interestingly, telomerase is a crucial factor in ageing through its role in rejuvenating stem cells by telomere maintenance. Age-related tissue degeneration
might be enhanced by stem cell ageing that diminishes the regeneration potential of tissues. Interestingly, it was shown that adult stem cells are telomerase positive and are characterised by longer telomeres than the surrounding tissue [175]. Reduced telomerase activity and shortening of telomeres in senescent stem cells is a crucial cause of tissue atrophy and age-related phenotypes [176]. There is direct evidence from mTert/- mice that telomere shortening impairs proliferation capacity and differentiation of somatic and germ cell lines [177]. Furthermore, in vitro experiments revealed that over-expression of hTERT in embryonic stem cells improved differentiation, enhanced proliferation and enriched resistance for apoptosis and oxidative stress [172, 178].

Mutations or changes in expression of telomerase genes are linked to several diseases. For example, telomerase was found active in over 90% of human malignancies and is responsible for indefinite proliferative capacity of cancer cells. Therefore, a plethora of in vitro and in vivo studies suggests that telomerase might be a major pharmacological target for new cancer therapies [179, 180]. However, about 10% of tumours exhibit telomere stabilization without detectable telomerase activity, via the alternative lengthening of telomeres (ALT) mechanism [181]. In addition to cancer, a number of diseases are related to dysfunctional telomerase genes. For example, dyskeratosis congenita syndrome, which shows some features of premature ageing, is a human phenotype of an autosomal dominant deletion (821bp) in the hTERC gene with accelerated telomeres shortening [182, 183]. Other examples of diseases where mutations in telomerase genes plays a role of risk factors include: aplastic anaemia, pulmonary fibrosis and hepatic cirrhosis, which are all characterised by abnormally short telomeres [184].

Apart from the influence of telomerase on rejuvenation and disease, as predicted just after discovery, lack of telomerase expression and telomere shortening is an essential contributor to cellular senescence and ageing [171]. It is established that telomerase activity is a key factor for senescence of cells through maintenance of telomeres [26]. However, first generation mTert or mTerc knock-out mice do not show reduced lifespan and health span or significant shortening of telomeres [185-187]. Interestingly, late generation telomerase knock-outs show an increased incidence of age-related diseases and premature tissue degeneration that affects tissues with high cellular turnover such as the bone marrow or intestine [188]. It was
shown on late generation mTert-/ mice that lack of functional telomerase decreases fertility, reduces testis mass and increases intestinal cell apoptosis [177, 189, 190]. Furthermore, late generation mTerc knock-out mice when compared to wild type littermates show shorter lifespan, impaired regeneration of organs (i.e. liver, spleen, skin), decreased body weight, kyphosis and skewed differentiation of stem cells [186, 191, 192].

More recently, Sahin et al., (2010) have shown in vivo that lack of telomerase activity (generation f4 of mTert or mTerc knock-out mice), causes telomere dysfunction, that is associated with impaired mitochondrial biogenesis and function. Furthermore, in this study mTert-/ animals showed decreased gluconeogenesis, cardiomyopathy, and increased reactive oxygen species production in liver, heart and hematopoietic stem cells. These results were explained by a telomere dependent activation of p53 that contributes, through a PGC-1α – dependent pathway, to accelerated failure of organs and metabolism [193]. Interestingly, the same group engineered a reversible mTert knock-out by introducing an allele encoding a 4-OHT – inducible telomerase reverse transcriptase-oestrogen receptor (TERT-ER) and showed that telomerase reactivation by 4-OHT (4-Hydroxytestosterone) treatment in the f4 generation of mTert knock-out mice was sufficient to reduce DNA damage signalling and restore tissue function, mostly due to the re-established population of stem cells e.g. in brain or in intestinal crypts [194].

Currently, there is a number of laboratories working on reactivation of telomerase by gene therapy or natural and chemical telomerase activators in animal models. For instance, recent data published by Maria Belasco’s group shows that telomerase gene therapy might substantially reduce or delay age-related decline of mouse health-span. In this study, introduction of an adeno-associated virus (AAV) carrying mTert to mature and old mice resulted in improved insulin sensitivity, better neuromuscular coordination, reduced biomarkers of ageing and, most importantly, this gene therapy increased median lifespan. Interestingly, all these beneficial effects were observed without increase in the incidence of cancer, making telomerase gene therapy a strong candidate to combat age-related diseases [195, 196].

Apart from genetic manipulation, there is accumulating data on compounds enhancing telomerase. For example, de Jesus et al., (2011) have shown that TA-65 (a natural extract isolated from Astragalus membranaceus) was able to induce
telomerase activity in vitro and increase mRNA of mTert in vivo in adult and old mice. In the study, supplementation with TA-65 resulted in elongation of short telomeres and increased health-span without increase in cancer incidence [197]. More recently, Eitan et al., (2012) reported that a novel polyaryl compound – AGS-499 – increased telomerase activity and protein abundance in mouse forebrain, brain stem and spinal cord. The authors of this publication show that treatment with AGS-499 has a neuroprotective effect on motor neurons after treatment with N-methyl-D-aspartic acid (NMDA) and delays onset of amyotrophic lateral sclerosis (ALS) in a transgenic mutant SOD1 mice [198]. However, there are no reports so far explaining the molecular mechanism of telomerase activation neither by TA-65 nor AGS-499.
Figure 1.3. The structure of telomeres and canonical function of telomerase. A. Shelterin complex protects the telomere by forming t-loop and d-loop from telomeric DNA, (picture adapted from Deng et al., (2008) [153]; B. Scheme of the DNA replication fork. Synthesis of the Okazaki fragments on the lagging strand causes the “end of replication problem”; C. Telomere elongation by telomerase. 1) Telomerase binds to 3’-overhang. 2) Telomerase extends 3’ – end strand with TTAGGG repeats. 3) DNA polymerase synthesise new lagging 5’ – end strand of telomeres.
1.4.3. Non-canonical functions of telomerase

In addition to the mentioned canonical function of telomerase, there is a growing number of studies for new non-telomeric functions of TERT [26, 61].

TERT was shown to act as a transcriptional co-activator and integrator of signalling pathways like Wnt/β-catenin and Myc [199, 200]. For example, Choi et al., (2008) have shown that TERT controls tissue progenitor cells by transcriptional regulation of a developmental program controlled by the Myc and Wnt pathways [199]. Furthermore, Park et al., (2009) have shown that TERT plays a role as a co-factor at the promoters of Wnt downstream genes (i.e. SWI/SNF family) [201]. Interestingly, more recently Hoffmeyer et al., (2012) suggested that β-catenin (effector in Wnt signalling) might be a direct regulator of mTert expression, showing a regulatory feedback loop between the two genes [202].

Evidence for a telomere independent function of telomerase in cell survival was shown by Lee et al., (2008) in an mTert knock-out mouse model. The authors in this study analysed the role of mTert and telomerase activity on apoptosis induced by staurosporine (STS) and on excitotoxicity caused by NMDA. Interestingly, in this study the induction of apoptosis was only efficient on first generation mTert knock-out mice/cells while there was no sensitivity to apoptosis induction on mTert wild type or mTerc knock-out mice both in vitro (MEFs) and in vivo (animal survival). Thus, along with no changes in telomere length in mTert/- animals, this data strongly supports a non-telomeric, protective function of mTert [203].

Another elegant study on a double knock-out (mTert-/- and ATM-/-) mouse model published by Nitta et al., (2011), demonstrated that expression of mTert is required for stem cell (HSCs) function and tissue renewal during ageing. Moreover, they showed increased senescence markers (i.e. p16 and γH2AX), occurrence of apoptosis and increased oxidative stress in hematopoietic stem cells from double knock-out (mTert-/- and ATM-/-) mice, not observed in single knock-out animals mTert/- or ATM-/- . Furthermore, the authors showed no decrease of telomere length and no increase in damage foci in telomeric areas of hematopoietic stem cells, which strongly supports a protective function of mTert against apoptosis and oxidative stress independently of telomere maintenance [204].

An additional function of telomerase in the nucleus, independent of telomere elongation, was provided previously by Maida et al., (2009) [205]. Authors of this
publication showed that hTERT is able to interact with the RMRP (mitochondrial RNA processing endoribonuclease), the gene that is mutated in the inherited pleiotropic syndrome cartilage-hair hypoplasia. However, the name “mitochondrial” may be misleading since its function is in processing ribosomal RNA in the nucleus. Experiments in vitro revealed that the complex hTERT-RMRP has the ability to produce dsRNA molecules which might be used as a substrate for production of siRNA that regulates the level of the RMRP in mitochondria by a negative feedback control mechanism. Thus, authors suggest a critical role of the hTERT-RMRP complex in cartilage-hair aplasia [205]. In addition, it shows the great flexibility of functions which TERT can have independent of TERC.
1.5. TERT in mitochondria

1.5.1. TERT shuttling and localisation

Functions of telomerase related to its nuclear localisation are well described and are reviewed in chapter 1.4.1. However, recent studies report cytosolic and mitochondrial telomerase localisation and show new non-telomeric functions of telomerase outside the nucleus [61, 206, 207]. Controversially, there is still a strong group of scientists who claim that the reported telomere-independent functions are de facto linked to pathways responding to telomere length (i.e. p53) [187, 193, 208].

Haendeler et al., (2003) showed for the first time that the enzymatic (catalytic) subunit of telomerase hTERT localised in the cytoplasm of HEK293 cells (human embryonic kidney cells) after treatment with H$_2$O$_2$[207]. The cytoplasmic as well as mitochondrial localisation of hTERT was later confirmed independently by other groups in a number of human cell lines including fibroblasts and cancer cells [61, 209, 210]. Data from Haendeler’s group suggests that upon oxidative stress nuclear TERT is phosphorylated by Src kinase on tyrosine 707. This activation leads to binding of the GTPase Ran to TERT protein [207]. After that, TERT is exported through nuclear pores into the cytoplasm what is controlled by protein 14-3-3 which promotes TERT interaction with the nuclear exportin CRM1 [211, 212]. The whole mechanism of TERT shuttling became more complete after identification of a Mitochondrial-Targeting Signal (MTS) at the enzyme N-terminus and a Nuclear Export Signal (NES) at the C-terminus of hTERT, which play crucial roles in regulating the subcellular localisation of telomerase protein component [211, 213, 214]. Furthermore, Kovalenko et al., (2010) showed that a mutation in the NES disrupts nuclear export of TERT [215]. The mechanism by which hTERT enters the mitochondrial matrix was recently described by Haendeler et al., (2009). The authors of this report showed that hTERT physically interacts with the mitochondrial outer- and inner membrane translocator complexes TIM23 and TOM20/TOM40 – the main channels for transporting proteins in and out of mitochondria (Figure 1.4) [206]. Interestingly, it was reported that hTERT localises in the cytoplasm also under basal conditions in CD4$^+$ T-lymphocytes as well as in human ovarian cancer cells, and upon activation it might be shuttled into the nucleus through pathways involving estrogen and Akt kinase [212, 216]. Therefore, it is possible that TERT localisation can be different in various cell types.
The only factor that triggers shuttling of TERT into mitochondria described to date is oxidative stress. Accumulating data supports the finding that treatment with H$_2$O$_2$ or cultivation of cells in 40% O$_2$ conditions are able to activate telomerase shuttling out of the nucleus *in vitro* [61, 206]. Furthermore, it was shown that irradiation and chemical treatments with etoposide or paraquat also affect mitochondrial localisation of hTERT. The potential function of TERT in mitochondria will be reviewed in the next subchapter.
Figure 1.4. Shuttling of TERT protein into mitochondria. Upon oxidative stress (1) TERT protein is phosphorylated by Src kinase on Tyr 707 (2) and is shuttled into the cytoplasm (3). Next, it interacts with TIM and TOM proteins and enters the mitochondrial matrix (4). Picture adapted from Saretzki, (2010) [217].
1.5.2. Functions of TERT protein in mitochondria

To date, there exists some conflicting data about TERT function in mitochondria. Early studies from Santos et al., (2004 and 2006) showed that hTERT shuttling into mitochondria increases mtDNA damage and promotes apoptosis after H$_2$O$_2$ treatment [210, 213]. In contrast, Ahmed et al., (2008) and Haendeler et al., (2009) reported a protective function of hTERT against mtDNA damage [61, 206]. Ahmed and colleagues showed that in MRC5 hTERT-over-expressing cells the amount of mtDNA damage is reduced after hydrogen peroxide treatment and under hyperoxia, when compared with telomerase negative MRC5 cells. In addition, hTERT over-expressing cells showed improved resistance to apoptosis when compared to normal MRC5 cells. Furthermore, hTERT-over-expressing cells displayed a decrease of ROS production and increased mitochondrial membrane potential [61]. Similarly, Haendelers’ group showed that localisation of telomerase in mitochondria not only reduced ROS production but also improved respiration in HEK cells. Furthermore, the same study showed that silencing of endogenous TERT via shRNA increases mitochondrial ROS formation. Moreover, the same group showed in an mTert+/+ and mTert-/- mouse model that mTert improves respiration of heart mitochondria and protects against UV-induced degradation of mtDNA in isolated primary lung fibroblasts [206].

Interestingly, recent studies from Santos’ group brought further evidence for a protective function of TERT in mitochondria. This group reported that cells with mutated NES (nuclear export signal for TERT) were unable to shuttle TERT protein from the nucleus while maintaining telomerase activity. Mutation in NES in this study resulted in increased mitochondrial ROS production, elevated oxygen consumption and increased mtDNA damage. [215]. This is additional evidence for a protective function of telomerase shuttling into mitochondria.

To test directly whether localisation of TERT in mitochondria reduces oxidative stress, our group used organelle specific vectors to express TERT protein in different subcellular compartments. This method enabled us to deliver TERT on a plasmid construct directly into mitochondria or the nucleus using the multiple NLS (nuclear localisation signal) or MLS (mitochondrial localisation signal) [218]. In this recent study Singhapol et al., (2013) showed, in cancer cells, that only mitochondrial TERT but not nuclear was efficient to reduce oxidative stress induced by ionising
irradiation and H₂O₂ - providing direct evidence for the protective function of TERT in mitochondria [218].

It is still debatable what the exact working mechanism of TERT in mitochondria is. Haendeler and colleagues demonstrated that the catalytic subunit of telomerase binds to mtDNA at regions coding for polypeptides of the ETC complex I (ND1 and ND2). Subsequently, proposing that the function of TERT is vital in the protection and/or repair of mammalian mtDNA [206].

Another proposed function of hTERT in mitochondria is its role as a reverse transcriptase independent from hTERC. Sharma *et al.*, (2012) showed in NHF cells that hTERT binds to mtDNA, various mitochondrial RNAs and tRNAs. Interestingly, this newly discovered mitochondrial complex, hTERT-tRNA’s, showed reverse transcriptase enzymatic activity [219]. Moreover, in this study the authors showed that TERT is localised in liver mitochondria of mouse and rat. The authors also confirmed previous findings that lack of mitochondrial TERT indeed increases the amount of mtDNA mutations and raises ROS production. However, there is still no direct evidence for the hypothesis that TERT is a part of the mitochondrial DNA replication system or mtDNA repair machinery [219].

A list of proposed functions of telomerase related to its cellular localisation is summarised in Figure 1.5.
Figure 1.5. Function of telomerase depends on its localisation. Adapted from Saretzki, (2010) [217].
1.6. Telomerase in mammalian brain and its role in neurodegenerative diseases

The brain is a central organ of the nervous system in all vertebrates. Its functions include the control of all the organs in the body, processing of perception and co-ordinating motor functions [220]. Age is a major risk factor for neurodegenerative diseases like Alzheimer's and Parkinson's. The general phenotype of these conditions includes neuronal death and synaptic alterations [1]. When conducting in vivo research on the brain it has to be considered that this organ consists of different cell types which play different roles in the healthy organ and during neurodegeneration. On the cellular level brain primarily consist of neurons and glial cells. The role of glia is as a structural and metabolic support, while neurons process and transmit information via electrical and chemical signals [220]. Although broadly discussed, it was shown recently that there is a close to 1:1 ratio of glial cells to neurons in the central nervous system of humans and primates. However, this ratio might be different in particular brain areas. For instance, it was shown in human and rat, that in the frontal cortex glia cells outnumber neurons three fold while in the cerebellum there is three fold more neurons than glia [221, 222].

Telomerase shows a high enzymatic activity in brain during development in mammals along with a high abundance of TERT protein [223, 224]. However, in the postnatal brain the activity of telomerase decreases sharply, although, TERT mRNA and TERT protein are still detected in different brain areas in adult mice, mostly in neuronal progenitors, stem cells, pyramidal neurons of the hippocampus and Purkinje neurons of the cerebellum [223, 225, 226]. Furthermore, it was shown recently by Eitan et al., (2012) that abundance of mTert protein is reduced in mouse brain with age [226].

Cell turnover is rare in adult mammal brain. However, areas like the hippocampus and olfactory bulbs are regularly supplied with new progenitor and stem cells for neurogenesis in the adult brain. The source of these multipotent cells is localised in the subgranular zone of the dentate gyrus and in the subventricular zone of lateral ventricle [227, 228]. Neural stem cells require telomerase activity for their maintenance and differentiation potential. Thus TERT protein and TA are mostly detected in areas of brain with cell turnover [229]. However, the exact function of TERT in brain remains unclear.
An anti-apoptotic role was one of the first reported non-telomere dependent functions of telomerase in brain. There are a number of reports suggesting that telomerase can attenuate apoptosis during brain development [224, 230]. For example, Zhu and co-workers showed that over-expression of TERT in mouse protects neurons against apoptosis triggered by β-amyloid peptide and by trophic factor withdrawal, suggesting that TERT may protect against Alzheimer’s disease [231].

Furthermore, Klapper and colleagues have shown that neural stem cells gradually loose telomerase activity during differentiation into neurons, which is highly correlated with increased vulnerability of neurons to apoptosis [223]. Another report by Fu et al., (2000) showed in mice and rats that suppression of mTert expression by antisense oligonucleotides in cultured hippocampal and cortical neurons increased vulnerability for oxidative stress, increased the number of apoptotic nuclei and reduced survival of the cells, supporting a protective function of TERT [230].

Similar results were reported by Kang et al., (2004) where authors showed in mTert over-expressing transgenic mice that telomerase protects cortical neurons against NDMA and hypoxic-ischemic injury. Furthermore, in the same study authors showed an increased expression of mTert mRNA in neurons as a response to permanent occlusion of the middle cerebral artery in adult mouse brain and suggested that mTert protects against hypoxic-ischemic injury by reducing ROS-mediated neuronal death [232]. More recently, the same group presented a study by Lee et al., (2009) where it was shown that mTert have a crucial role in mouse brain function. In this study first generation of mTert/- (f1) mice showed an anxiety-like behaviour and this deficit was also significantly increased with age, suggesting that telomerase is necessary for maintaining normal brain function and behaviour during ageing. Furthermore, in the same study mTert/- animals also showed impaired olfactory function, however this was not affected with age [233]. More beneficial effects of telomerase in brain were shown recently by Jaskelioff et al., (2012) on a fourth generation of mTert/- mouse model. In this study adult telomerase deficient mice had reactivated telomerase in vivo which reversed neurodegenerative changes in brain and restored proliferation capacity and function of neural progenitor cells in subventricular zone of the brain. Furthermore, reactivation of telomerase in f4 mTert
deficient mice restored myelination of neurons and olfactory function to the level of wild type animals. However, these effects were telomere length dependent since f4 mTert-/ animals have significantly shorter telomeres [194].

Similar results were obtained by Eitan et al., (2012) by chemical activation of telomerase in mouse brain. In the reported experiments animals were treated with a new aryl compound AGS-499 which is potently increases telomerase activity in the forebrain, brain stem and spinal cord of a CD-1 mouse strain in a time and dose dependent manner. As a result animals treated with AGS-499 extended lifespan and showed higher resistance of neurons for NMDA induced neurotoxicity. Furthermore, in the same study, a mouse model of Amyotrophic Lateral Sclerosis (ALS) – transgenic SOD1 mutant showed improved neuromuscular coordination and neurological parameters after AGS-499 treatment. Interestingly, activation of telomerase by AGS-499 was also efficient to delay the onset and progression of ALS. In addition, the treatment resulted in improved motor function (improved survival of motor neurons) and increased lifespan of ALS animals [198].

In humans, the role of telomerase in brain in vivo was studied in patients with dyskeratosis congenita – a disease caused by mutation in telomerase genes and characterised by short telomeres, bone marrow failure (anaemia) and increased cancer incidents. Interestingly, individuals with dyskeratosis congenita also show neurological dysfunctions like learning difficulties, microcephaly and mental retardation, showing that telomerase and telomeres are key players in brain function and development. However, the precise mechanism of these symptoms remains unknown [234, 235].

To conclude, there is accumulating data on the beneficial and protective effect of telomerase on brain biology and function. It seems that telomerase is a strong therapeutic candidate for neurodegenerative diseases. Thus, further experiments on the molecular mechanisms of TERT function in brain are required.
1.7. Telomerase in mammalian liver

Non-telomeric functions and mitochondrial localisation of telomerase in mammalian liver have not been studied so far. However, the protective role of TERT associated with telomere lengthening and its impact on oxidative stress in disease and ageing of liver was shown previously to be crucial [236, 237]. Potentially, the enzymatic component of telomerase might be also involved in development and the ageing processes of this organ in a non-canonical way, similar to brain.

Telomerase in liver is mainly studied due to its association with hepatocellular carcinoma as a cancer marker and treatment target [238, 239]. Moreover, TERT in liver has been investigated in studies on liver regeneration in correlation with insulin signalling. For example, Tseng et al., (2009) found that in insulin-deficient rats after heptectomy, rTert mRNA level and telomerase activity was lower than in normal rats. Furthermore, liver restitution mass was lower in insulin-deficient rats, this may suggest a relation between insulin signalling and telomerase in this organ, although not directly [240].

Liver is the organ being mostly studied among all organs under dietary restriction due to its connection with insulin, fatty acids metabolism and general nutrient sensitivity [80, 241]. It was reported that DR counteracts age related changes in mouse liver. Results published by our group recently showed that DR significantly reduced the number of senescent cells in mouse liver. Moreover, in the same model Wang et al., (2010) showed a reduced ROS production and less DNA damage under DR in liver and small intestine, although changes in telomerase activity after restricted diet were not significant in this study [80].
1.8. Aims

Since dietary restriction and TERT protect mitochondria we hypothesised that part of the protective effect of DR might be an effect of TERT translocation into mitochondria. The aim of this thesis is to characterise the possible roles of telomerase expression and its cellular localisation in brain under basal and dietary restricted conditions.

Specific aims of this thesis are:

1. To analyse whether telomerase might be involved in the mechanisms and beneficial effects of dietary restriction and rapamycin supplementation in brain. The protective function of hTERT is well described in vitro, thus a natural question is whether telomerase is localised in mitochondria of mouse neurons in vitro and in the mouse and human brain in vivo? Moreover, since dietary restriction and telomerase through its telomere dependent function are able to attenuate ageing, the question arises: does DR influence expression of telomerase genes, telomerase activity and mTert protein status in vivo?

2. To study whether lack of telomerase in (f1) mTert-/ animals might influence mitochondrial function. It is known that DR protects and improves mitochondria function. Thus, in this project, I aimed to analyse oxygen consumption and reactive oxygen species generation in primary cell lines (i.e. fibroblasts, stem cells) derived from animals of different mTert status. Furthermore, respiration and ROS production by brain mitochondria after DR was analysed in different mTert genotypes of mice. In addition, an aim was to address whether mTert status influences physiological parameters known to be altered during ageing like glucose tolerance, insulin sensitivity and neuromuscular coordination under the condition of dietary restriction.

3. To investigate the potential connection between mitochondrial telomerase and mTOR signalling pathway, which is known to regulate the lifespan in response to nutrient availability? Thus, we aimed to analyse whether there are any changes in mTOR activation in the mouse brain after DR and what might be the influence of rapamycin supplementation on telomerase in mouse brain mitochondria? Furthermore, we aim to study the effect of mTOR inhibition by rapamycin on mitochondrial ROS generation and oxygen consumption in mouse brain.
Chapter 2 - Materials and methods

2.1. Mice

In the late onset dietary restriction study (experiment 1), male mouse strain ICRFa on a C57Bl/6 background was used [242]. Male mice used in experiments 2 and 3 were pure C57BL/6 strain purchased form Charles River, UK. Heterozygotes of mTert-/- (strain:B6.129S-Tert, tm1Yjc/J) and mTerc (strain: B6.Cg-Terc tm1Rdp/J) on C57BL/6 background mice were purchased form The Jackson Laboratory and were inbred to obtain wild type, first generation (f1) knockout and heterozygote mice for the mTert and mTerc genotypes. The mTert-/- line was originally described by Chiang et al., (2004) [243] and mTerc-/- by Blasco et al., (1997) [244]. Moreover, mice tissues from a different mTert knock-out construct, originally described in Liu et al., (2000), were generously donated by Prof. Lea Harrington, University of Edinburgh, UK [245], and used as negative controls in mTert expression experiments. Animals used in all experiments were housed in the same room and were provided with sawdust as well as paper bedding. All animals had ad libitum access to water. Control animals in dietary restriction experiments had 24h access to standard rodent pelleted chow (Special Diets Services, Witham). Mice were accommodated at 20 ± 2 °C under a 12 h light/12 h dark photoperiod with lights on at 7 am. The project was approved by the Faculty of Medical Sciences Ethical Review Committee, Newcastle University. Experiments were conducted in accordance with UK Home office legislation under the Animals (Scientific Procedures) Act 1986.

2.2. Genotyping

DNA obtained from digestion of mouse ear notches was used for genotyping transgenic animals. Tissue in a buffer consisting of 25mM NaOH and 0.2mM EDTA was placed in a thermocycler (Hybaid) for 1 hour at 98°C and held afterwards at 15°C. Next, 40nM Tris-HCl (pH 5.5) was added and the samples were centrifuged at 4000 rpm for 3 minutes. Afterwards, 4µl of DNA rich supernatant was used in a PCR reaction where denaturation was carried out at 95°C for 3min. Then, 35 programmed cycles at 94°C for 30s, 68°C for 30s (annealing of mTert primers and 62°C for TERC) and 72°C for elongation. Primers for genotyping were purchased from
SIGMA (Table 1). PCR amplification products were visualised on a 3% agarose gel with SYBR® Safe DNA gel stain (Invitrogen) (Figure 2.1.).

<table>
<thead>
<tr>
<th>Primer name</th>
<th>sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTert</td>
<td></td>
</tr>
<tr>
<td>oIMR5359 FW</td>
<td>GGTCCTGGCTGTTTTCTAAG</td>
</tr>
<tr>
<td>oIMR5358 REV</td>
<td>CCCCAGGCGCCGCACAAAGG</td>
</tr>
<tr>
<td>oIMR5363 NEON2</td>
<td>CTGGATTCAATCGACTGTGGC</td>
</tr>
<tr>
<td>mTerc</td>
<td></td>
</tr>
<tr>
<td>oIMR1912 WT FW</td>
<td>CTCGGCACCTAACCCTGAT</td>
</tr>
<tr>
<td>oIMR1913 WT REV</td>
<td>CGCTGACGT TTGGTTTTTGAG</td>
</tr>
<tr>
<td>oIMR6916 KO FW</td>
<td>CTTGGGTGGAGAGGCTATTC</td>
</tr>
<tr>
<td>oIMR6917 KO REV</td>
<td>AGGTGAGATGAGACGCCGAGATC</td>
</tr>
</tbody>
</table>
Figure 2.1. Genotyping of *mTert* and *mTerc* mice. A. Representative visualisation of PCR products from *mTert* genotyping on a 3% agarose gel. The 420bp band represents the allele of *mTert* knockout and the 150bp band corresponds to the wild type allele. Two bands indicate heterozygotes. B. Visualisation of genotyping of *mTerc* animals. A band size of 280bp represents the knockout *mTerc* allele; the smaller 150bp band is a wild type and two bands indicate a heterozygote genotype. DNA for PCR was isolated from ear notch tissue.
2.3. Design of the dietary restriction experiments

Four independent DR experiments on male mice were performed in collaboration with Prof Thomas von Zglinicki and Dr Satomi Miwa. The control group of mice had free access to food (ad libitum). The restricted group (DR) was limited by 40% and food intake was calculated from the food intake of the AL group. The 60% chow amount was calculated based on the weight taken at the beginning of the experiment and remained unchanged over time. In experiment 1 (late onset DR), restriction started at 14 months of age and lasted for 3 months. Experiment 2 started with 3 months old animals and lasted for 6 months. The experiment 3 was started with 3 month old mice while dietary restriction was applied for another 3 months. Finally, the fourth experiment was performed on mTert+/+ and mTert-/- mice and started when animals were 6 months old and lasted for 16 months (Table 2). In this experiment, there were 12 mTert wild type animals and 11 mTert knock-outs in the control (AL) group. In the dietary restricted group there were 7 mTert wild type and 10 mTert knock-out animals.

Table 2. Design of dietary restriction experiments

<table>
<thead>
<tr>
<th>Exp.</th>
<th>% of DR</th>
<th>beginning of DR</th>
<th>end of DR</th>
<th>duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40%</td>
<td>14 months old</td>
<td>17 months old</td>
<td>3 months</td>
</tr>
<tr>
<td>2</td>
<td>40%</td>
<td>3 months old</td>
<td>9 months old</td>
<td>6 months</td>
</tr>
<tr>
<td>3</td>
<td>40%</td>
<td>3 months old</td>
<td>6-7 months old</td>
<td>3 months</td>
</tr>
<tr>
<td>4</td>
<td>40%</td>
<td>6 months old</td>
<td>22 months old</td>
<td>16 months</td>
</tr>
</tbody>
</table>

2.4. Rapamycin supplemented diet experiment in vivo

In total 38 male mice were used in the rapamycin diet experiment. The control group contained 7 mTert wild type animals and 9 mTert knock-outs. The rapamycin fed cohort contained 6 mTert wild type and 9 mTert knock-outs. Mice were 12 months old at the onset of the experiment and rapamycin supplementation lasted for 4 months. The dose was 0.067mg of rapamycin per day/per mouse. The experimental chow was a gift from John Strong, Texas [246, 247].

2.5. ROS generation in female mice brain mitochondria

To test if generation of hydrogen peroxide production in mouse brain is sex specific, female mice mTert+/+ and mTert-/- were used. In total 12 animals were tested.
Three young animals (aged 3-6 months) per genotype, and three old mice per genotype (aged 22 months) were tested.

2.6. Mouse and human cell lines
Commercially available 3T3 J2, MRC5 (human embryonic lung fibroblast, bought from ECACC), MCF7 (human breast adenocarcinoma) and HeLa (human cervix adenocarcinoma) cell lines were used (both from ATCC). In addition, as a positive control in a range of experiments MRC5/TERT overexpressing cells, constructed by retroviral transfection of MRC5 fibroblasts with the plasmid pLCP-hTERT (Clontech), were used [61]. Cells were cultured in high glucose (4.5g/L) DMEM (PAA Laboratories) medium supplemented with 10% Foetal Bovine Serum (FBS), 2mM L-glutamine, 50U/mL penicillin with 10mg/mL streptomycin (all from PAA Laboratories). Incubation was carried out under normoxic condition with 5% CO₂ at 37°C in a humidified water jacketed incubator, (Series II incubator, Forma Scientific).

2.7. Isolation of primary mouse neurons from embryos (E16)
Pregnant female mice were sacrificed by cervical dislocation at day 16 after fertilisation. Embryos were removed from the uterus, culled by cervical dislocation and brains were removed. Afterwards brains were cut into 3-4 pieces and placed in 1mL HBSS buffer at 37°C. Then, tissue was incubated with 0.025% trypsin at 37°C for 30 min. Next, the digested tissue was centrifuged at 1500rpm for 3 min. and resuspended in plating medium (Gibco Neurobasal®, Life technologies), containing 5% FBS (PAA Laboratories), 5% horse serum (SIGMA), 2mM L-glutamine (PAA Laboratories), 100 U/mL penicillin and 10mg/mL streptomycin (PAA Laboratories). Isolated cells were seeded on a 6 well plate coated with Poly-D-Lysine (SIGMA) at a concentration of 0.5x10⁶ cells/mL and cultured in 5% O₂ at 37°C. Growth of astrocytes was inhibited by 1µM AraC® (SIGMA) in order to avoid over-growth of neurons. After 5 days of AraC® treatment, the medium was gradually substituted (50% every 3 days) for Gibco Neurobasal® media supplemented with: Gibco B27® (Life technologies), 2mM L-glutamine, 50 U/mL penicillin and 10mg/mL streptomycin (all from PAA Laboratories).
2.8. Isolation and cultivation of epidermal neural crest stem cells

Epidermal Neural Crest Stem Cells (EPI-NCSC) were isolated from bulges of mouse whisker hair follicles. Whisker pads were dissected from mice culled as above and submerged in buffer HBSS containing 5mM HEPES. Excess of skin and fat tissue was removed and whisker follicles were dissected with microsurgery tools. Follicles were cut longitudinally, and afterwards, below and above of the bulge area. Next, bulges were removed from the collagen capsule and transferred from HBSS to a 12 well tissue culture plate coated with CELLstart® (Invitrogen) according to the manufacturer’s instructions (solution 1:50 in DPBS). Bulges were incubated for 1 hour at 37°C in 3% O₂ without any medium or buffer to allow attachment to the surface. For proliferation of EPI-NCSC, alpha modified MEM (PAA Laboratories) media was gently added to the bulges which contained the following supplements: 5% chicken embryo extract (Sera Laboratories), 10% FBS (Sigma) treated with Chelex-100 (Bio-Rad), 50U/mL penicillin and 10mg/mL streptomycin (PAA Laboratories), 2mM L-glutamine (PAA Laboratories) and 2.5µg/mL amphotericin B (Sigma). Emigrating EPI-NCSC were observed after 3 to 8 days after bulge isolation. Approximately 60% of isolated bulges released EPI-NCSC, similar to a report by Sieber-Blum et al. [248, 249]. Staining with Sox10 antibody was used as a marker of EPI-NCSC.

2.9. Isolation and cultivation of mouse ear fibroblasts

Mouse ear notches or whole ears were collected and stored in cold Serum Free Media (SFM) until processed, Gibco® Advanced DMEM/F12 (Life Technologies). Firstly, tissue was chopped and incubated in SFM with 1mg/mL of collagenase A (Roche) for 1.5h at 37°C in 5% O₂. After digestion, the tissue was passed through an 18G syringe needle and then centrifuged for 10 minutes at 1000 rpm. Next, pellets were resuspended in Gibco Advanced DMEM/F12 medium enriched by 10% FBS (PAA Laboratories), 2mM L-glutamine (PAA Laboratories) and a mix of 50U/mL penicillin and 10mg/mL streptomycin (PAA Laboratories). Isolated cells were incubated at 37°C in 3% O₂. The first fibroblasts to attach to the surface were observed at 2-3 days after isolation.
2.10. Generation of mouse tissue homogenates and isolation of mitochondria

2.10.1. Generation of mouse brain homogenate and isolation of mitochondria

Mice were culled by cervical dislocation, then dissected with the brain being removed from the skull and placed into isolation buffer (composition: 250mM sucrose, 10mM Trizma base, 0.5mM EDTA and pH 7.4 at 4°C). Next, brains were chopped and washed to remove blood. Afterwards, brains were homogenised in a glass Dounce homogeniser (type TKW-120-070J, Fisher). The obtained homogenates were sampled and the remaining material was used for the isolation of mitochondria [250].

Briefly, homogenates were centrifuged for 3 min. at 2000 x g at 4°C. Pellets were discarded and supernatants rich in mitochondria were further centrifuged at 12500 x g for 10 min. at 4°C. Then, pellets were separated from the remaining blood and resuspended in 3% Ficoll® (SIGMA). The obtained solution was suspended on top of 6% Ficoll®, in a centrifuge tube to obtain a visible phase between layers and centrifuged for 15 min. at 11500 x g at 4°C. Next, supernatants were sharply decanted and pellets were resuspended in the isolation medium and centrifuged for 10 min. at 11500 x g. The resulting pellets were used for functional experiments immediately or stored at -80°C for protein assays.

2.10.2 Generation of mouse liver homogenate and isolation of mitochondria

Livers from culled mice were chopped and homogenised in STE buffer (composition: 250mM Sucrose, 5mM Trizma base, 2mM EGTA and pH 7.5 at 4°C). Homogenates were aliquoted and the remaining material underwent an initial centrifugation for 3 min. at 1047 x g at 4°C. Supernatants with mitochondria underwent further centrifugation for 10 min. at 11630 x g at 4°C. Afterwards, supernatants were discarded; pellets rich in mitochondria were separated from the remaining blood with a glass rod and resuspended in STE buffer. This solution underwent additional centrifugation at 11630 x g for 10 min. at 4°C. Then, pellets were resuspended in STE, layered on top of 80%, 52% and 26% Percoll® gradient (SIGMA) and centrifuged at 41100 x g for 10 min. at 4°C. The mitochondrial layer was aspirated from the 26% - 52% Percoll® interface. Next, the mitochondria solution was centrifuged 11630 x g for 10 min. at 4°C to obtain pure mitochondria.
pellets. Purified mitochondria were used immediately in functional assays or stored at -80°C.

2.11. Mitochondrial function

2.11.1. Hydrogen peroxide generation

Mitochondrial superoxide generation linked to complex I, II and III was measured using AmplexRED® technique (Invitrogen) which is a highly sensitive, fluorogenic substrate for horseradish peroxidase. In total, 0.4mg of protein from freshly isolated mitochondria were used per reaction containing: 0.2mg/mL oligomycin, 50μM AmplexRED®, 2U/mL horse radish peroxidase, 1U/μL and KHE/BSA buffer (composition: 115mM KCl, 10mM KH₂PO₄, 2mM MgCl₂, 3mM HEPES, 1mM EGTA, 0.2% fatty acid free BSA; all from SIGMA). Measurements of fluorescence was conducted in real time at an excitation of A₅₄₄nm and an emission of A₅₉₀nm using a Fluostar Omega® plate reader (BMG Labtech). Gain was set at 5% for male and 10% for female brain mitochondria. Gain was established experimentally higher for females due to lower ROS production in female brain mitochondria. After establishing the basal H₂O₂ production level, 5mM pyruvate/malate (substrate for complex I) or 4mM succinate (substrate linked to complex II) were added to each reaction. Next, production of superoxide was inhibited with 2.5μM of rotenone (blocker of electron transport in complex I). Then, 2.5μM of antimycin A (SIGMA) was added to block complex III which results in maximal superoxide production. The final step was to measure the fluorescence increase after addition of the standard of 1pmole H₂O₂ to calibrate the fluorescence units and calculate the pmoles of H₂O₂ release in the samples per minute. Each sample was measured in triplicate.

2.11.2. Oxygen consumption

Oxygen consumption rate in isolated mitochondria and intact cells was analysed by an Extracellular Flux Analyser - XF24® (Seahorse Bioscience). This instrument measures O₂ and H⁺ levels with solid state probes which are submerged in solution above a monolayer of intact cells or isolated mitochondria [251]. The probe cartridge is also equipped with 4 injection channels to deliver reagents altering ETC.

Mitochondrial oxygen consumption was measured according to the following protocol. First, 5μg of freshly isolated liver or brain mitochondria (see paragraph 2.8
for isolation and purification method) was plated in *Seahorse* 24 well plates and centrifuged at 2500g for 10min. at 4°C. Afterwards, substrates for complex I (10mM pyruvate) diluted in KHE/BSA buffer were added to the supernatant above mitochondrial pellets and the plate was incubated in 37°C for 8 min. Next, the plate with mitochondria was assembled with a calibrated cartridge and measurements were conducted in the XF24® instrument at 37°C after delivery of the following reagents added through cartridge channels: 4mM ADP, 2.5µg/mL oligomycin, 4µM FCCP (carbonyl cyanide p-[trifluoromethoxy]-phenyl-hydrazone) and 4µM Antimycin A (all reagents and substrates from *SIGMA*). Readout of the assay is shown as Oxygen Consumption Rate (OCR) in pmoles O₂ / min.

A similar protocol was applied for the analysis of oxygen consumption of cells in culture. Briefly, 25000 mouse ear fibroblasts or neural crest stem cells were seeded per well of a *Seahorse*® plate and incubated overnight at 37°C in 5% O₂ and 5% CO₂ atmosphere of an incubator. Afterwards, cells were incubated with XF assay medium for 1h in atmospheric oxygen. The medium for the oxygen consumption experiment contained DMEM (*SIGMA*), 1xGlutaMax-1 (*Gibco*), 5mM glucose (*SIGMA*), 3.7g/L NaCl (*SIGMA*), 30mg/L phenol-red (*SIGMA*) and 10mM of pyruvate (*SIGMA*). After incubation, measurements were conducted in a XF24 analyser with the following reagents added through the cartridge channels: 1µg/mL oligomycin, 2µM FCCP, 0.5µM FCCP and 2.5µM antimycin A (all from *SIGMA*). Results are presented as OCR in pmoles O₂ / min /1x10⁵ cells. Results were normalised to cell number (counted after DAPI staining).

### 2.11.3. Flow cytometry analysis

Levels of ROS production in mitochondria of wild type and knock-out mTert mouse cells were measured using flow cytometry based on MitoSOX® (*Invitrogen*) and dihydrorhodamine 123 – DHR (*Molecular Probes*) staining. Prior to measurements, the flow cytometry apparatus PAS® (*Partec*) was calibrated using fluorescence stained 3µM yellow calibration beads (*Partec*) - in order to adjust the laser optics and stream flow. Cell populations were analysed using forward (FSC) and sideways scatter (SSC), and fluorescence was detected in channels FL1 (green) and FL3 (red). Autofluorescence was measured on unstained control cells and the value was subtracted from the FL3 fluorescence of measured samples.
Briefly, cells were trypsinised, washed with PBS and stained with 5µM of MitoSOX® in SFM for 15 min. at 37°C. After incubation, cells were centrifuged at 1600rpm for 2 min. and pellets resuspended in fresh SFM. Next, cells were gated in FSC/SSC and peak of red (FL3) fluorescence intensity was used to estimate superoxide production for at least 1x10⁴ cells. Measurements were conducted in triplicate. Similarly, cellular peroxide production was measured by staining with DHR. Cells were incubated with 30µM DHR for 30 min. at 37°C and centrifuged at 800rpm for 5 min. Next, the pellet was resuspended in SFM and measurement for at least 1x10⁴ cells in triplicate was conducted using the flow cytometry instrument. The gated FL1 and FL3 fluorescence peak was used to estimate cellular peroxide production.

2.12. Human post mortem brain tissue

Paraffin embedded and frozen human brain sections and tissue were provided by Newcastle Brain Tissue Resource (NBTR), Institute of Ageing and Health, Newcastle University, Newcastle, UK. Frozen brain samples were ground in liquid nitrogen using a mortar and pestle ceramic grinder. The obtained powder was used for RNA and protein isolation.

2.13. TERT ELISA

TERT protein abundance in cells, tissues and mitochondrial fractions was assessed by TERT ELISA Assay (GenWay). Cell pellets, homogenised or powdered tissue and mitochondria pellets were lysed in CHAPS (Roche) buffer (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate), for 30 min. on ice. Protein content was measured by Bradford assay (Bio-Rad). A total of 100µg protein was used per reaction. Samples were usually tested in duplicates or triplicates. During the first reaction at room temperature for 1h the TERT protein from tested samples and standard calibrators (recombinant TERT protein supplied with the kit) were bound to TERT antibody-precoated 96 microwell plate. Standard curve was prepared with following concentrations of recombinant TERT protein: 50ng/mL, 25ng/mL, 12.5ng/mL, 6.25ng/mL, 3.12ng/mL, 1.56ng/mL and 0.78ng/mL. After washing 3 times for 30s with washing buffer supplied with the kit, a biotin conjugated anti-TERT antibody was added at room temperature and incubated for 1h on a horizontal shaker (100rpm). Next, the wells were washed 5 times for 30s and streptavidin (HRP
– horseradish peroxidise labelled) was added. After further washing the substrate (tetramethylbenzidine – TMB) was added which turned the solution blue. To stop the reaction 100µl of sulphuric acid provided with the kit was added. After 10min. absorbance was measured (A₄₅₀nm) by Multiskan Ascent® plate reader (Thermo Labsystems). TERT protein concentration in the samples was calculated from absorbance of final product compared to absorbance of the standard curve made from provided recombinant TERT protein of a known concentration. Principles of the TERT ELISA technique are shown in Figure 2.2.
Figure 2.2. Principles of the TERT ELISA technique. A. An antibody to TERT is precoated to the plastic microwell plate. B. During the incubation TERT protein from sample binds to an antibody. Subsequently, unbound proteins are washed three times for 30 seconds with the washing buffer supplied with the kit. C. During second incubation an anti-TERT antibody bids to protein (“sandwich”) and wells are washed afterwards. D. Incubation with secondary antibody conjugated with streptavidin-HRP. E. Substrate TMB is added to the reaction what results with blue colour solution. Next, sulphuric acid solution is added to the wells what stops reaction and turns the solution yellow. TERT protein levels are quantified by measuring the absorbance of solution at 450 nm.

Telomerase activity was assessed by TeloTAGGG Telomerase PCR ELISA kit – TRAP assay (Roche) which measures the potential enzymatic activity of telomerase in sample of tissue or in cell lysate. In principle, the TRAP assay quantifies the extension of a telomere - like primer (TS – primer) by telomerase from the particular lysate. Cell pellets, homogenised or powdered tissue and mitochondrial pellets were lysed in CHAPS buffer for 30min. on ice. A total of 100ng protein per sample was used in the reaction. The first step – the elongation of the TS – primer (labelled with biotin) with telomeric repeats by telomerase from the sample was carried at 25°C for 30min. Then, after telomerase inactivation at 94°C for 5min. the product was amplified in the following steps: denaturation at 94°C for 30s, annealing at 50°C for 30s and polymerization at 72°C for 30s (30 cycles). First, 5µl of the generated amplicons were denatured for 10min. with a denaturation buffer (20µl) supplied with the kit at room temperature. Then 225µl hybridisation mixture that contains a digoxigenin labelled probe against the telomeric repeats were added. Afterwards, the PCR products were detected by ELISA. 100µl of each sample were incubated on 96 well microplate precoated with streptavidin (provided with the kit) for 2h at 37°C on a horizontal shaker (300rpm). In this step the hybridisation product was immobilised by the biotin labelled primer to the streptavidin. Afterwards, wells were washed 4 times with washing buffer and product incubated with an anti-digoxigenin antibody conjugated to horseradish peroxidase. In the final step, the probe was visualised by the reaction of peroxidase and substrate (TMB) which resulted in a blue colour product. Finally, the reaction was stopped with sulphuric acid and the intensity of resulting yellow colour was measured on a microplate reader - Multiskan Ascent® (Thermo Labsystems). The absorbance readings at 450nm were considered negative when the value was lower than 0.2 A.U. As a positive control MRC/TERT and 3T3J2 cells were used. MRC5 cells were used as negative controls. Principles of TRAP assay are shown in Figure 2.3.
Figure 2.3. Principles of the TRAP assay technique. In the 1st step telomerase from the samples adds telomeric repeats to the P1-TS primers and TTAGGG – telomeric like elongates are amplified by PCR. During 2nd step amplification product is denatured and hybridise with telomere specific probe conjugated with digoxigenin. In the 3rd step (ELISA) product is immobilised to streptavidin precoated well. In the final 4th step product is detected by anti-DIG-POD antibody and substrate TMB is transformed into blue colour product which turns yellow after reaction with sulphuric acid.
2.15. Western blotting

Cell pellets, tissue homogenates and mitochondria were lysed using CHAPS buffer \((Roche)\) as described in previous subchapter. A total of 60-100µg protein per sample was loaded. After preparing samples by mixing them with Laemmli\textsuperscript{®} \((SIGMA)\) buffer, lysates were denaturised on a heating block at 95°C for 5 min. and shocked on ice afterwards. Proteins from samples were separated on an 8 – 12% SDS – polyacrylamide gel at 35mA and blotted to nitrocellulose-ECL membrane \((GE Healthcare)\) at 100V for 90 min. at 4°C. The following step was blocking the membrane for 1h at room temperature with 5% low fat milk or with 5% BSA (when phosphorylated proteins were detected) diluted in TBST (composition: 10mM Tris, 50mM NaCl, 0.1% Tween 20, pH 7.5, all from \textit{SIGMA}). Next, the membrane was incubated with the primary antibody diluted in blocking solution over night at 4°C and washed 3 times for 10min. with TBST. Then, membranes were subjected to incubation with a peroxidase labelled secondary antibody for 1h at room temperature. For membrane development the Amersham Western Blotting detection kit \((GE Healthcare)\) was used. Images were taken with a chemiluminescence camera LAS 3000\textsuperscript{®} \((Fujifilm)\). Afterwards, stripping buffer \((Thermo Scientific)\) was used to draw off bound antibodies from the membrane for re-incubation with further antibodies and loading controls. Images were quantified by densitometry measurements and normalised to expression of loading controls \((\beta\text{-tubulin})\) by AIDA\textsuperscript{®} software \((Raytest)\).

2.16. Polymerase Chain Reaction - PCR

Gene expression was evaluated by the standard Polymerase Chain Reaction (PCR) and Quantitative Real-Time PCR (qPCR). Firstly, frozen tissue was powdered using mortar and pestle on liquid nitrogen and total RNA was isolated from \(\sim\)30mg of material using the RNeasy\textsuperscript{®} kit \((Qiagen)\) according to the manufacturer’s protocol. Similarly, mouse and human cell pellets \((\sim\)1x10\textsuperscript{6}) were processed using a RNeasy\textsuperscript{®} kit. Next, cDNA was obtained by Reverse Transcription PCR (RT-PCR). Briefly, 1µg of total RNA measured on a NanoDrop\textsuperscript{®} ND-1000 spectrophotometer \((Nanodrop technologies, Inc.)\) was mixed with 2 µl of random primers \((Promega)\), denatured for 7 min. at 75°C and chilled on ice. Then, a mix of reverse transcriptase, RNase inhibitor, 10mM dNTP, 0.1M DTT and first strand buffer was added (all from Invitrogen). Next, reverse transcription was carried out in a thermocycler at 42°C for
90 min. afterwards the enzyme was inactivated at 95°C for 5 min. and hold at 4°C. For PCR, 1µl of cDNA was mixed with a solution of ddH₂O (double distilled H₂O), forward and reverse primers (0.5µM each) and Taq PCR Master Mix (Qiagen). PCR was conducted in a thermocyclcer (Hybaid) programmed for denaturation at 94°C for 2 min. followed by 24 cycles for GAPDH or 40 cycles for TERT/TERC: denaturation at 94°C for 2 min., annealing at 55°C-68°C for 1 min. (temperature depends on primers, see Table. 3) and elongation at 72°C for 1 min. When finished, the reaction was hold at 4°C. Products obtained by standard PCR were visualised by electrophoresis on 2% agarose gel with SYBR® Safe DNA gel stain (Invitrogen) and detected with a UV camera - GelDoc 2000® (Bio-Rad).

Quantitative PCR (Real-Time PCR) was performed in a Step One Plus® instrument (Applied Biosystems). 1 µl of cDNA was mixed with ddH₂O (Qiagen), an internal reference dye (SIGMA), SYBR® Green Jump Start Taq Ready mix (SIGMA) and sets of primers (50nM of each forward and reverse primer). The instrument was programmed for denaturation at 95°C for 10min. followed by 40 cycles consisting of the following steps: denaturation at 95°C for 30s and annealing at 55°C-68°C for 1min. Afterwards, the quality of the PCR products was analysed by a melting curve stage performance with the following steps: 95°C for 15s, 60°C for 1min. and 95°C for 15s. The expression of the genes of interest was normalised to the expression of GAPDH and mRNA quantity was related to a reference sample (known lack of gene expression) according to a comparative threshold cycle method (ΔΔCₜ). Sequence and annealing temperatures of all used primers are detailed in Table 3.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>sequence 5’-3’</th>
<th>annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTert fw exons 2-3-4</td>
<td>GGATTGCCACTGGCTCCG</td>
<td>68°C</td>
</tr>
<tr>
<td>mTert rev</td>
<td>TGCCTGACCTCTCTCTTGTGAC</td>
<td></td>
</tr>
<tr>
<td>mTert fw exon 2</td>
<td>TCGCGTCTGATACTGGCAGAT</td>
<td>55°C</td>
</tr>
<tr>
<td>mTert rev</td>
<td>CTGTCTCTCATGTCTCCGA</td>
<td></td>
</tr>
<tr>
<td>mTert fw exons 11-12-13</td>
<td>GCAAAAAACCTTCTCAGCACC</td>
<td>58°C</td>
</tr>
<tr>
<td>mTert rev</td>
<td>ACTTCAACCGCAAGACGAC</td>
<td></td>
</tr>
<tr>
<td>mTert fw exons 6-7</td>
<td>TGGTGGAGGTGTGTCCACAAAA</td>
<td>58°C</td>
</tr>
<tr>
<td>mTert rev</td>
<td>ACTGGCATCTGAATCCTGC</td>
<td></td>
</tr>
<tr>
<td>mTerc fw</td>
<td>TCATTAGCTGTGGTTCTTGGT</td>
<td>60°C</td>
</tr>
<tr>
<td>mTerc rev</td>
<td>TGGAGCTCTCCTGCTGACGTG</td>
<td></td>
</tr>
<tr>
<td>mGapdh fw</td>
<td>GAACGGGAAGCTCACTGACC</td>
<td>62°C</td>
</tr>
<tr>
<td>mGapdh rev</td>
<td>GACAACCTGGCTCTCACTG</td>
<td></td>
</tr>
<tr>
<td>hTERT fw</td>
<td>TATGGCTGCTGGTGGTGAACCTTG</td>
<td>55°C</td>
</tr>
<tr>
<td>hTERT rev</td>
<td>CATAGCTGGAGTAGTGCGTCTCT</td>
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<td>hTERC fw</td>
<td>GCCCTCCACCGTTCATTCTAA</td>
<td>58°C</td>
</tr>
<tr>
<td>hTERC rev</td>
<td>CCTGAAAGGCGCTGAAACCTC</td>
<td></td>
</tr>
<tr>
<td>hGAPDH fw</td>
<td>AAACATCAACCACATCTCC</td>
<td>60°C</td>
</tr>
<tr>
<td>hGAPDH rev</td>
<td>GACTCCACGACGTACTGAC</td>
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</tr>
</tbody>
</table>
2.17. Immunofluorescence on cells

Proteins in cultured cells were localised using immunofluorescence. The following staining protocol was applied: cultivated cells grown on glass coverslips (VWR) in 12 well plates were washed with PBS and fixed with 4% paraformaldehyde (PFA), (SIGMA) in PBS for 10 min. Following permeabilisation with PBG (composition: 1xPBS, 0.5% BSA, 0.2% fish skin gelatine, and 0.2% Triton-X 100, all from SIGMA) for 45 min. at room temperature cells were incubated with the primary antibody over night at 4°C on a slow shaker. After three washes for 5 min. with PBS cells were incubated with a specific fluorescently labelled secondary antibody. A list of all antibodies used and their concentrations are shown in Table 4. Next, the coverslips were washed with PBS three times for 5 min. and incubated for 10 min. at room temperature with DAPI (Partec) for nuclear staining. Images were acquired using fluorescent microscopes: Leica DM 5500B, Axio Imager Z1 (Zeiss) and Axio Vision with filters for green and red fluorescence (Zeiss). Images were analysed with ImageJ® (free access software developed by Wayne Rasband - NIH) and Axio Vision® (Zeiss).

2.18. Immunofluorescence on tissues

Freshly dissected mouse brain tissue was fixed in 4% PFA for at least 24 hours in room temperature and afterwards embedded into paraffin (Zeiss). Next, 4µm sections were cut and attached to glass slide coated with 4% 3-Aminopropyltriethoxysilane, APES (SIGMA) and stored at room temperature. Paraffin embedded human brain sections were obtained from NBTR, Institute of Ageing and Health, Newcastle University.

To rehydrate tissue, slides were placed in decreasing concentrations of methanol (90% for 5 min. and 70% for 5min.) and submerged in water two times for 5 minutes. Then, the tissue was subjected to antigen retrieval by boiling them in 10mM citrate buffer with 0.05% Tween 20 (SIGMA) for 15min. Cooled sections were incubated with 70% formic acid for 10min. and blocked against unspecific antibody binding for 30min. in room temperature with blocking solution (10% goat serum and 0.1% BSA in PBS, all from SIGMA). Afterwards, the sections were incubated with the primary antibody in a humidified chamber over night at 4°C. Slides were then washed 4 times with PBS for 5 min. and incubated with a specific fluorescently labelled secondary antibody (Table 4.) for 90min. at room temperature.
in a dark humidified chamber. Once incubation was finished the glass slides with tissues were washed three times for 5 minutes with PBS and incubated with DAPI for 15min. After further washing the tissue sections were subjected to a reaction with Sudan Black III (SIGMA) for 30 min. in room temperature to reduce background autofluorescence. Following a short wash with PBS sections were mounted with Vectashield® medium (Vector Laboratories). Images were acquired with the same microscopes as described above for immunofluorescence of cells.

Table 4. List of primary and secondary antibody used in Western blotting (WB), immunofluorescence on cells (IFC) and in immunofluorescence on tissue (IFT)

<table>
<thead>
<tr>
<th>Antigen</th>
<th>conc./technique</th>
<th>manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary antibody</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hTERT</td>
<td>1:500 WB; 1:1000 IFC; 1:100 IFT</td>
<td>Rockland</td>
</tr>
<tr>
<td>βtubulin</td>
<td>1:1000 WB</td>
<td>Abcam</td>
</tr>
<tr>
<td>p-mTOR (Ser2448)</td>
<td>1:500 WB</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>mTOR</td>
<td>1:500 WB</td>
<td>Abcam</td>
</tr>
<tr>
<td>Sox10</td>
<td>1:500 IFC</td>
<td>Abcam</td>
</tr>
<tr>
<td>Tuj1</td>
<td>1:500 IFC; 1:200 IFT</td>
<td>Neuromics</td>
</tr>
<tr>
<td>COX II</td>
<td>1:200 WB 1:500 ITC; 1:100 IFT</td>
<td>Santa Cruz Bio.</td>
</tr>
<tr>
<td>FOX3</td>
<td>1:200 IFT</td>
<td>Abcam</td>
</tr>
<tr>
<td>HDAC</td>
<td>1:800 WB</td>
<td>Abcam</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary antibody</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP labelled goat anti rabbit</td>
<td>1:2000 WB</td>
<td>Abcam</td>
</tr>
<tr>
<td>HRP labelled donkey anti rabbit</td>
<td>1:2000 WB</td>
<td>Abcam</td>
</tr>
<tr>
<td>Alexa Fluor® 594 goat anti-rabbit</td>
<td>1:2000 IFC 1:1000 IFT</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Alexa Fluor® 488 goat anti-rabbit</td>
<td>1:2000 IFC 1:1000 IFT</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Alexa Fluor® 488 goat anti-chicken</td>
<td>1:2000 IFC 1:1000 IFT</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>
2.19. Neuromuscular coordination test

Neuromuscular coordination of mice of different ages, genotypes and diets was assayed using a tightrope test. The equipment consisted of a 60cm long and 2mm in diameter rope which was tied up 40cm high between two rods (chemical stands) above bedding cushioned with polystyrene foam and paper shredding (Figure 2. 4.). Mice were suspended above the tightrope for 20 seconds at the distance of their whiskers. Afterwards, the mouse was placed with its front paws in the middle of the rope and time between grabbing the rope and ability to pulling up the hind legs was recorded. There was 60 seconds period to complete the task and three attempts.
Figure 2.4. Principles of the tightrope test. A. Equipment used in tests. B. Mouse suspended above rope for 20 seconds. C. Mouse grabs the rope and trying to lift hind legs. D. Successful animal is moving to the rod and using four limbs.
2.20. Insulin ELISA

Blood samples were collected during mouse dissection or from the tail vein. Tubes with blood were centrifuged for 10 min. at 4°C and 2800 rpm to obtain serum. To determine the insulin level an ELISA method was used according to the manufacturer’s instructions (Ultra-Sensitive Mouse Insulin ELISA kit, Crystal Chem Inc.).

The kit used for the determination of insulin level is a classic sandwich ELISA and requires only 5µl of blood serum per sample. During the first reaction insulin is bound to an anti-insulin guinea pig antibody coated on the 96 well plates. After washing with the supplied washing buffer, a horse radish peroxidase (POD)-conjugated antibody bound to an immobilised complex of insulin and the guinea pig antibody. Following washing steps, TMB substrate solution was added to detect bound POD conjugates what resulting in a colour reaction. Absorbance was measured using a Fluostar Omega® plate reader (BMG Labtech). Readouts for $A_{630nm}$ were subtracted from $A_{450nm}$ values. Concentration of the insulin in serum was calculated in ng/ml from a standard curve evaluated by readout of the absorbance of the known concentration of mouse insulin standard supplied with the kit.

2.21. Glucose tolerance test

Determination of blood glucose clearance in mice under different dietary condition and in different genotypes was performed using a Glucose Tolerance Test (GTT) according to a protocol described in Selman et al., (2008) [107]. Glucose was delivered by abdominal injection (20% solution of d-glucose, SIGMA). Blood was obtained from a punctured tail vein and the glucose concentration was measured by a glucose monitor Accu-Chek®, Aviva Nano, (Roche).

Glucose clearance from the blood was measured at 4 time points. The first measurement was performed after an overnight fast and just before glucose abdominal injection (2mg/g of body mass). Afterwards, glucose clearance was assessed after 15, 30, 60 and 120 minutes. Animals were fed immediately after the last measurement.
2.22. Statistical analysis

All data presented in this thesis are results from duplicate or triplicate measurements and with at least three mice per group. To examine statistical significance of the parametric data a One Way Anova and Two Way Anova tests were performed using Sigmaplot® software. Non-parametric data sets where tested by One Way Anova on ranks or Mann-Whitney Rank sum test. All other calculations were completed by Excel Office® 2003 and 2010 (Microsoft). Error bars on the graphs represent standard error of the mean (±SEM) if not stated differently. Results were considered as statistically significant for two groups when the p value was <0.05.
Chapter 3 – Mouse Tert protein localises in neurons and accumulates in mitochondria after dietary restriction

3.1. Introduction

Telomerase shows high expression of its protein component (TERT), and has elevated activity in the brain during embryonic development in mammals [223, 224]. In the postnatal brain the activity of the enzyme decreases sharply, although TERT mRNA and TERT protein are still detected in different brain areas in adult mice - mostly in neuronal progenitors, stem cells, pyramidal neurons of the hippocampus and Purkinje neurons of the cerebellum [223, 225, 226]. Interestingly, it was shown recently by Eitan et al., (2012) that mTert protein is expressed in the cytoplasm of Purkinje neurons in mouse brain cerebellum. However, the exact localisation of mTert was not tested in this research [226]. So far there is very little data published showing mitochondrial localisation of mTert protein in mouse cells. For example, Haendeler et al., (2003) initially showed that TERT localised in the cytoplasm of HEK293 cells after treatment with H₂O₂ [207]. The cytoplasmic as well as mitochondrial localisation of hTERT was later confirmed independently by other groups in human fibroblasts and cancer cells [61, 209, 210].

It has been shown previously that improvement of mitochondrial function and reduction of ROS production is a major causal mechanism for the beneficial effects of DR. Furthermore, TERT was shown to protect mitochondrial function and reduce ROS production. Hence, the aim of this part of my research was to analyse: 1) whether telomerase is localised in mitochondria of mouse neurons in vitro, and in the mouse and human brain in vivo; 2) whether DR influences expression of telomerase, telomerase activity and mTert protein status in vivo in mice.

3.2. TERT co-localises with mitochondria in mouse and human brain

Since there are several publications showing extra-nuclear localisation of hTERT in vitro, the question emerged whether mouse cells and tissues also show a similar cytoplasmic or mitochondrial localisation. To obtain evidence for the hypothesis that mTert co-localises with mitochondria in mice similarly to in vitro human cells a series of fluorescence stainings with a specific antibody for the telomerase enzymatic component TERT was performed.
Firstly, the specificity of a commercially available antibody (Rockland) was confirmed on mouse cells. Surprisingly, mouse ear fibroblasts did not show a specific staining, although these cells show specific telomerase activity in the TRAP assay as will be shown later (Figure 3.5.). In contrast, in vitro cultivated neurons isolated from E16 mouse embryos expressed sufficient amounts of mTert protein and the specificity was confirmed by immunofluorescence on neurons isolated from wild type animals and mTert knock-out animals (Figure 3.1). Interestingly, the strongest signal of mTert expression was observed in the cytoplasm of neurons with negligible staining in the nuclear area. Furthermore, staining with an antibody against the mitochondrial marker coxII confirmed that mTert co-localises with these organelles (Figure 3.2). Remarkably, immunofluorescent staining with a TERT antibody in mouse and human brain paraffin sections exhibited the same expression pattern as isolated neurons. Human and mouse neurons identified by the neuronal marker Tuj1, showed a strong expression of telomerase protein component mostly in the cytoplasm of neurons in the frontal cortex and cerebellum (Figure 3.3).

Interestingly, telomerase was localised in mouse and human neuronal mitochondria in basal conditions without applying external oxidative stress in contrast to previous studies on human cell lines [61]. This may suggest a new function of the protein in this cell type.
Figure 3.1. TERT antibody (Rockland) is specific for the detection of the protein in cultivated mouse embryonic neurons. A. Neurons isolated from mTert wild type mice. B. Neurons isolated from first generation mTert knockout mice. The red signal shows expression of mTert protein. Green fluorescence shows expression of Tuj1 – a neuronal marker. Nuclei are stained with DAPI (blue).
Figure 3.2. Mouse and human TERT protein co-localise with mitochondria. A. Cultured mouse embryonic neurons immunolabelled with TERT (red) and mitochondrial COXII antibody (green). B. TERT staining (red) in human brain cortex and mitochondrial marker CoxII (green). Blue – DAPI staining for nuclei. Yellow colour = co-localisation of red and green signal.
Figure 3.3. TERT protein is expressed in the neurons of mouse brain. A. Immunofluorescence staining on cerebellum - Tert (red), Tuj1 (green). Arrows show Purkinje neurons stained with TERT and Tuj1 antibodies. B. Mouse frontal cortex shows expression of Tert protein in neurons. Tert protein (red) is localised mostly in the cytoplasm. Neurons were stained with the neuron specific marker FOX3 (green).
3.3. TERT ELISA - validation of the method

Until recently, the quantitative measurement of TERT protein was based on estimates from western blotting or immunofluorescence. However, these estimations are technically laborious and not very precise. A newly developed TERT ELISA (GenWay) measures the amount of TERT protein by using a recombinant TERT protein as a standard in an ELISA format. Quantification is performed using a standard curve from consecutive dilutions of recombinant TERT protein of a known concentration as described in the Material and Methods chapter. Before using the TERT ELISA kit, which was a very new kit on the market it had to be verified whether it indeed was applicable for the purposes of this study. Several mouse and human cell lines as well as tissues were tested (Figure 3. 4 A). A total protein extract of 100µg from either cells, whole organs/tissue homogenate and from mitochondria were assayed. Moreover, the standard curve was modified. Originally, the highest concentration of recombinant TERT protein was 500ng/ml. It was established experimentally, that our samples were in a range from 0 to 50ng/mL (Figure 3. 4 B). Therefore, the standard curve was adapted for this range in order to optimise the accuracy for the required concentration range. However, one has to be aware that the main disadvantage of this ELISA is the lack of a loading control. In general, the highest abundance of TERT protein was found in human MRC/TERT cells (high overexpression of TERT shown by Ahmed et al., 2008 [61]) and 3T3 mouse fibroblasts. As expected, no TERT protein was found in human MRC-5 fibroblasts and in fibroblasts from mTert knockout mice. Furthermore, established positive and negative controls were confirmed with Western blotting (Figure 3. 4 C). Thus, the TERT ELISA kit was confirmed to be reliable for this study.
Figure 3.4. Verification of the TERT ELISA technique. A. The TERT ELISA is applicable for human cells and mouse cells as well as tissues. B. A standard curve obtained from recombinant TERT protein was adapted and used for calculation of TERT amounts in the sample. C. The amounts of TERT protein in control samples obtained by ELISA antibody (GenWay) were confirmed by Western blotting with an anti TERT antibody (Rockland).
3.4. Comparison of telomerase activity and mTert protein in mouse cells, tissues and isolated mitochondria

Telomerase contains two main components – the catalytic subunit TERT that acts as a reverse transcriptase during telomere elongation, and the RNA part TERC that contains the template for telomeric repeats. Both subunits are necessary for telomerase activity (TA) and it was shown that TA is not always correlated with TERT or TERC gene expression levels due to additional regulatory mechanisms of telomerase activity [243, 245]. Hence, it was tested here whether the amount of mTert protein (by ELISA) was correlated with TA measured by TRAP assay in mouse tissues and mitochondria as well as in primary ear fibroblasts derived from mTert+/+ and mTert-/- animals.

Primary mouse fibroblasts showed high telomerase activity. Thus, to test the correlation between TA and mTert protein abundance, I used MEaF’s generated from mTert wild type and knock-out mice, both by TERT ELISA and TRAP assays. As expected, I found that there was no telomerase activity in the MEaF’s cells lysates from mTert knock-out and mTerc knock-out mice. Heterozygotes for the mTerc gene showed ~50% reduced telomerase activity when compared to fibroblasts from wild type mice. However, heterozygotes for the mTert gene did not show a significant decrease in TA. Moreover, ELISA results showed that the lack of enzymatic activity of telomerase is not evidential of deficiency or the presence of mTert protein. Lack of mTert was observed, as expected, only in fibroblasts from mTert-/- mice (Figure 3.5). The same samples were tested in parallel in the TRAP assay and TERT ELISA and Pearson coefficient test between TERT protein and telomerase activity showed a positive correlation between protein abundance and enzymatic activity (r = 0.68, Figure 3.5 C.).

There was none or only a background level (below the cut-off threshold of 0.2450nm A.U.) of telomerase activity (measured by TRAP assay) in mitochondria isolated from mouse brain and liver (Figure 3.6 A.). The whole tissue lysates from liver showed a rather high TA compared to brain (Figure 3.6 A.). Interestingly, the finding of measurable amounts of TA in brain lysates from 6 months old mice contradicts my observation in brain tissue lysates from young (18 days) and adult (11 months) mouse brain lysates prepared from tissue disrupted with a mortar and pestle.
in liquid nitrogen (Figure 3.6 C.). However, these differences might depend on the method used for sample preparation.

To clarify, brain samples were prepared on a Dounce homogeniser from freshly dissected mouse brain tissue (Figure 3.6 A. B.). Thus, this finding might suggest that samples prepared by mortar grinding in liquid nitrogen may lose some TA during preparation. The ground tissue stored previously in -80°C from adult mouse brains (6-11 months old mice) showed no TA. In contrast, brain lysates from one day old and eighteen days old mice pups prepared with the same (powdered) method confirmed, as previously reported, a tendency for the gradual decrease of TA in a postnatal mouse brain [223]. In the TA experiment on brains from young animals, as negative controls I used age matched mTert-/- animals and as expected there was no telomerase activity. However, there were no 11 month old, age-matched wild type animals available at the time of the experiment (Figure 3.6 C.).

Interestingly, there was more mTert protein in mitochondria isolated from brain and liver than in the respective homogenates generated using Dounce homogeniser (Figure 3.6 B.). This finding suggests that only the protein part (mTert) of telomerase shuttles to mitochondria, corresponding to data from Sharma et al., (2012) [219]. Furthermore, these results confirm findings from immunofluorescence staining, where mTert localised in mitochondria under basal condition without applying oxidative stress (Figure 3.3).
Figure 3.5. Comparison of telomerase activity and mTert protein abundance in mouse ear fibroblasts from telomerase wild type, \textit{mTert-/-} and \textit{mTerc-/-} as well as heterozygous mice. A. TRAP assay on MEaF’s cell lysates isolated from \textit{mTert} and \textit{mTerc} knock-out, wild type and heterozygote animals. B. TERT protein expression in MEaF’s from a different genotype of \textit{mTert} and \textit{mTerc}. 3T3 cells were used as positive controls. 2 animals per genotype were analysed in duplicates. Error bars represent standard deviation. C. Pearson correlation coefficient between TERT protein and telomerase activity, $r = 0.68$. 
Figure 3.6. Comparison of telomerase activity and telomerase protein abundance in mouse liver and brain. A. Telomerase activity measured by TRAP assay in mitochondrial fractions and whole tissue homogenates from liver and brain of 6 months old animals per 100ng of total protein (mito – mitochondrial fraction; homo – whole tissue homogenate). 3 animals were used for homogenates and mitochondria preparation, measured in duplicates. B. TERT protein concentration measured by ELISA in 100µg of total protein in the same samples as used in TRAP assay. C. TA decreases with age in postnatal mouse brain (sample prepared by grinding on liquid nitrogen). 1 animal used per genotype in duplicate. Error bars represent standard deviation.
3.5. Mouse Tert protein abundance, telomerase enzymatic activity and mRNA in mouse brain and liver after dietary restriction

Dietary restriction (DR) is a non-genetic intervention known to result in lower oxidative stress and improved mitochondrial bioenergetics [32, 78, 81]. Thus, I wanted to investigate the role of telomerase in brain during DR and analyse the localisation changes of the protein component – mTert.

To investigate potential changes in mTert protein abundance under DR in brain, I analysed the mitochondrial fractions and whole organ lysates. Furthermore, I analysed whole organ homogenates and mitochondria fractions from liver to test whether a potential DR effect on mitochondrial mTert is tissue-specific. In this analysis samples from three different dietary restriction experiments were used (Table 2). Animals were separated into an ad libitum (AL) and dietary restricted (DR) group. Three to seven animals per group and experiment were used.

Interestingly, results from TERT ELISA experiments showed a significant increase of mTert protein abundance in brain mitochondria under DR in all three independent experiments (Figure 3.7). Remarkably, there was no difference between AL and DR in whole organ homogenates in the tested samples. The oldest animals (17 months old) showed the highest difference in mTert protein level in mitochondria between AL and DR (Figure 3.7 A.). The younger animals (3 and 6 months old groups from experiment 2 and 3) showed a similar ~50% increase of mitochondrial mTert (Figure 3.7 B. and C.).

Since abundance of mTert protein does not always correlates with activity of the enzyme, brain samples from the DR experiment were analysed by TRAP assay to determine whether there was a change in telomerase activity in whole tissue. Therefore, TA was measured in the homogenates of whole mouse brain. The same samples as used in the ELISA experiments were tested. According to the manufacturer’s protocol, absorbance below 0.2450nm A.U. was considered negative for the TRAP assay.

Similar to the data shown in the previous subchapter, there was a low but detectable activity of telomerase in whole brain homogenates prepared by Dounce homogeniser from fresh tissue (the influence of lysate preparation on TA was shown in the previous subchapter). Interestingly, in the TRAP experiments the TA decreased significantly only in experiment 3 where 3 months old animals were
restricted for 3 months (Figure 3.8 C.). This could be due to the fact that the largest number of animals were tested in this experiment (5 per group and only 3 per group in experiments 1 and 2), or because they were the youngest animals among the three experiments. In contrast, in homogenates from experiment 1 and 2 the decrease in TA after dietary restriction was not significant due to large variation between animals from both groups (Figure 3.8).

To establish potential changes in mTert and mTerc gene expression levels under dietary restriction, mRNA levels in mouse brain were assayed. Tissues ground in liquid nitrogen from snap-frozen samples were used for total RNA isolation. Then RT-PCR (reverse transcription) was performed in order to obtain cDNA followed by conventional PCR with mTert and mTerc primers. There were only tissues from experiment 1 (17 months old animals) available. Altogether, there were eleven brain samples tested. Five were from control animals and six from dietary restricted mice. Products from the conventional PCR visualised on agarose gels showed no visible difference neither in mTert or mTerc mRNA abundance between the AL and DR groups (Figure 3.9 A.).

In order to analyse changes in mTert and mTerc mRNA in a more quantitative manner, a qPCR method was used (Real-Time PCR). The same samples were used as in the conventional PCR. I found that mTert gene expression in mouse brain decreased significantly after adult-onset of dietary restriction (experiment 1), which is in agreement with the results from the TRAP assay (both were from whole tissue, although the samples for the TRAP assay were processed using a Dounce homogeniser and for PCR on liquid nitrogen) (Figure 3.9 B.). However, the decrease in mTert transcript did not correspond to the findings from the TERT ELISA, where there was no change in mTert protein abundance in whole tissue homogenates. Interestingly, mTerc transcription level did not change significantly after DR (Figure 3.9 C.). This means that the decrease in TA correlates with a decrease in mTert mRNA.

To test whether the mTert response to DR was tissue specific, I tested the potential changes in the protein abundance and its activity in mouse liver homogenates and mitochondria fractions from three independent dietary restriction experiments (Figure 3.10).
Interestingly, similar to the brain, DR tended to enhance mTert protein concentrations in liver mitochondria in all three separate experiments, although the differences were not significant (Figure 3.10). Surprisingly, there were no consistent effects of DR on whole tissue mTert amounts. Remarkably, there was a significant increase of mTert in whole liver from experiment 1 (Figure 3.10 A.). In experiment 2 and 3 there were no changes in the mTert amount observed (Figure 3.10 B. and C.). Interestingly, telomerase activity in liver lysates after DR showed a similar pattern to those observed in brain homogenates. There were no changes in TA after DR in experiment 1 and 2, however, activity of telomerase declined significantly after DR in experiment 3 (Figure 3.11). Hence, it is probable, that accumulation of mTert in mitochondria may be differently regulated in brain and liver, while activity of telomerase during dietary restriction may share a similar mechanism in both organs. However, further investigation would be required to elucidate this question.
Figure 3.7. Mouse Tert protein in brain after short term dietary restriction. DR significantly increased accumulation of mTert in brain mitochondria. The average of mTert protein in *ad libitum* samples (AL) was set as 100%, and values from DR were compared per animal to the mean of the controls. A. Experiment 1 (late onset: 17 months old animals); mitochondria AL – mitochondria fraction from the control – *ad libitum* group; mitochondria DR – mitochondria from the restricted group; homogenates – whole tissue homogenates; 3 animals per group. B. Experiment 2 (3 months old animals restricted for 6 months); 4 animals per group were used. C. Experiment 3 (3 months old mice restricted for 3 months); 4 animals from AL and 7 animals from DR group were tested. *Statistical significance was tested by One Way Anova test. Error bars show S.E.M
Figure 3.8. Telomerase activity in mouse brain homogenates after dietary restriction. A. TA in mouse brain after DR in experiment 1, 3 animals from the AL group, 3 animals form DR group were used. B. Experiment 2, 3 animals per group were used. C. Experiment 3, 5 animals per group were used. *Statistical significance was tested by One Way Anova test. Error bars represent S.E.M.
Figure 3.9. Telomerase gene expression in mouse brain after dietary restriction. Brain samples shown here are from experiment 1. A. Expression of mTert in mouse brain after DR by conventional PCR. L: 100bp ladder; N/C: negative control; 1AL-5AL: control brain samples, 1DR-6DR: brain samples from 6 dietary restricted mice, mGapdh - reference; B. Expression of mTert measured by qPCR. 3T3 mouse fibroblasts and MEaF’s form a mTert+/+ mouse were used as positive controls. Brain samples and MEaF’s form mTert−/− mice were used as negative controls. C. Expression of mTerc gene in mouse brain after dietary restriction. MEaF’s and a brain from an mTerc−/− mouse were used as negative controls. Results in B. and C. are presented as log10 of gene expression relative to the negative control evaluated by the ΔΔC_T method, C_T values were normalised to mGapdh expression. Five animals per group in triplicate were used. *Statistical significance was tested on the means of 5 samples per group by One Way Anova test. Error bars represent S.E.M.
Figure 3.10. Mouse Tert protein levels in liver after dietary restriction. The average of mTert protein in ad libitum samples (AL) was set as 100% and values from DR were compared per animal to the mean of the controls. A. Experiment 1, mitochondria AL – mitochondria from control – ad libitum group; mitochondria DR – mitochondria from dietary restricted group; homogenates – whole tissue homogenates; 4 animals per group. B. Experiment 2, 4 animals per group were used. C. Experiment 3, 7 animals per group of mitochondria and 3 animals per group of homogenates were tested. *Statistical significance was tested by One Way Anova test. Error bars shows S.E.M.
Figure 3.11. Telomerase activity in mouse liver homogenates after dietary restriction. A. Experiment 1, 4 animals per group were used. B. Experiment 2, 3 animals per group were used. C. Experiment 3, 5 animals per group were used. *Statistical significance was tested by One Way Anova test. Error bars represent S.E.M.
3.5.1. Purity confirmation of brain mitochondria

To establish whether the analysed brain mitochondrial fractions used in the described experiments were free from nuclear and cytoplasmic contamination, a series of western blotting experiments were performed. The aim of this analysis was to confirm that the detected mTert protein was indeed originating from the mitochondrial fraction only. A histone deacetylase II (HDACII) antibody was used to analyse whether the mitochondrial fractions were not contaminated with nuclear proteins. Mitochondrial proteins were detected by a specific marker – an antibody for cytochrome c oxidase II (coxII). In order to test for the presence of cytoplasmic proteins in mitochondrial fractions a β-tubulin antibody was used.

As shown in Figure 3.12 there were no cytoplasmic proteins in the brain mitochondrial fraction and only a trace of nuclear protein was observed. Thus, the detected mTert protein was indeed of mitochondrial origin. The purity of liver mitochondria was not tested in this study.
Figure 3.12. Purity of brain mitochondria fraction. There were no cytoplasmic proteins in brain mitochondria and only a trace of nuclear contamination; Cox II – (cytochrome c oxidase II) was used as mitochondrial protein marker; HDAC II – (histone deacetylase II) was used as nuclear protein marker; β-tubulin – cytosolic protein; mito – mitochondrial fraction lysate; homo – whole brain homogenate.
3.6. TERT protein and enzymatic activity in different mouse and human brain areas

It was reported that telomerase is expressed in embryonic and adult mouse brain, as well as in human brain [141, 223, 230]. TERT protein and telomerase activity in the central nervous system (CNS) is mostly observed in neural stem cells of the dentate gyrus in the hippocampus and the subventricular zone of the lateral ventricle [229, 252]. Furthermore, mTert protein was localised by Eitan et al., (2012) in differentiated Purkinje neurons in adult and old mouse brain cerebellum in vivo using immunofluorescence [226]. Moreover, previous reports were focused on telomerase in whole brain lysates or gene expression tested by in situ hybridisation [232, 253]. However, quantitative analysis of TERT protein abundance and TA in different brain areas has not been comprehensively reported so far.

To test telomerase protein expression in different mouse brain areas, TERT ELISA experiments were performed and compared with human samples from relevant brain regions. Mouse frontal cortex showed a high mTert protein concentration and it was greater than cerebellum and hippocampus in brains from three different mice. Results of the experiment on human brain tissue showed a similar abundance of hTERT in corresponding areas (Figure 3.13 A). The fact that the human brain samples came from an 89 year old donor suggests that hTERT is expressed even in old human brain. Human hippocampus samples were not available at the time of performing this experiment.

In order to analyse any potential changes with age in mTert abundance in mouse brain I tested cerebellum and cortex of young (6 months) and old (17 months) mice. Three animals per group were used. Results from TERT ELISA experiments showed that in both areas the amount of mTert was reduced significantly with age (Figure 3.13 B).

Further experiments on brain were focused on TA. In mouse cerebellum, hippocampus and frontal cortex from 6 months old animals the enzymatic activity was tested by TRAP assay. There was negligible TA detected only in mouse cerebellum and that readout was consistent in two separate measurements in duplicates in brains from three different animals (Figure 3.13 C.). Only absorbance above 0.2_{450nm} A.U. is considered positive according to the manufacturers’ instructions. Low telomerase activity in cerebellum and no activity in mouse cortex
and hippocampus might be explained by the respective tissue processing method. As explained in Chapter 3.4, preparation of brain tissue on liquid nitrogen may result with reduced or diminished telomerase activity in TRAP assay. Furthermore, there was no telomerase activity detected in cortex and cerebellum of human brain areas prepared by frozen tissue grinding (data not shown). However, human tissue was available from only one donor. Thus, these findings must be confirmed with a larger sample number.
Figure 3.13. Telomerase expression in different areas of mouse and human brain. A. Expression of TERT protein in mouse and human brain, cbll – cerebellum, hippo - hippocampus B. Expression of mTert in young (6 months) and old (17 months) mouse brain, 3 animals per group were tested, * Statistical significance was calculated by One Way Anova test. C. Telomerase activity in mouse brain areas, only the cerebellum showed activity above 0.2450 A.U. Three mouse brains were tested. Error bars represent in panel A. show standard deviation and in panel B. and C. show S.E.M.
3.7. Critical appraisal of the mTert knock-out design used in the project

In order to establish reliable controls for experiments on telomerase in my project, two types of mTert knock-out mice were tested. The main mTert/- animals used in most of the experiments in this study were designed and described by Chiang et al., (2004) (Figure 3.14 A.) [243]. In this knock-out design the green fluorescent protein gene (GFP) and a neomycin resistance gene (neo) were inserted into the full length mTert gene into exon 1 on 5’site of the ATG starting codon [243]. Thus, these animals are rather a knock-in of the gene. In the paper Chiang and co-authors showed the absence of the wild type allele and the presence of a new allele (called knock-out) by southern blotting of material from mouse tails by using a specific probe; however there was no evidence in the publication for deletion of any part of the mTert gene. Moreover in the same publication, no mTert mRNA was detected in activated spleen lymphocytes from mTert/- animals by conventional PCR [243].

Surprisingly, in my analysis five brains from Chiang’s mTert/- animals were tested for mTert mRNA expression and all showed to be positive in conventional PCR (Figure 3.14 B.), as well as in qPCR using primers matching different regions of the gene (Figure 3.14 D.). To confirm these findings liver tissue and MEaF’s cells from matching animals were used. Interestingly, all tested livers from mTert/- animals with several sets of different primers were negative for mTert gene expression. Fibroblasts isolated from Chiang’s knockouts showed fewer transcripts than wild type in the PCR but were not completely negative. Moreover, results for MEaF’s were not consistent and depended on the primer sets used. Furthermore, two different animals showed different expression of the mTert gene in MEaF’s (Figure 3.14 B.).

Since Chiang’s construct seemed to have a “leaky” mTert expression in brain and in MEaF’s, we looked for an alternative negative control for mTert expression analysis. Thus, I used another mTert knock-out, generously provided by Professor Lea Harrington (University of Edinburgh, UK) and originally described by Liu et al., (2000) [245]. There is no difference in phenotype between mTert+/- and first generation of mTert/- reported in neither the Chiang nor Liu publications. The design of Liu knock-out was significantly different from Chiang’s mTert-/. Namely, exons two to six were replaced by a neomycin resistance gene (neo), which makes it a genuine knock-out (Figure 3.14 A.). The difference between the two
described knock-outs can be seen in the gene expression analysis (Figure 3.14 C. and D.). Interestingly, liver in both knock-out designs was negative for mTert (Figure 3.14 B. and C.). In contrast to Chiang’s mTert-/-, brains from the knock-out designed by Liu et al., (2000) showed no mTert expression in the same experiments with the same sets of primers (Figure 3.14 D.). Hence these samples were used as accurate negative controls in gene expression experiments (Figure 3.9, Chapter 3.4). All other experiments described in this thesis were conducted with Chiang’s mTert -/- design due to fact that all samples obtained from these animals were negative for telomerase activity and showed no mTert protein expression in neurons and brain.
Figure 3. 14. Expression of mTert gene in two different mTert knock-outs constructs. A. Comparison of wild type mTert mRNA and two mTert knock-outs. Wild type mTert+/+ contains 16 exons. The knock-out described by Chiang et al., (2004) contains a GFP and a neomycin resistance gene (neo) inserted into exon 1[243]. The knock-out described by Liu et al., (2000) has 5 exons replaced by a neo gene [245]. B. Expression of mTert in brain, liver and MEaF’s form Chiang mTert-/- design. There is an expression of mTert in brain from knock-out mice. Liver from mTert-/- mice showed no expression of the gene. MEaF’s showed low or no expression of the gene, dependent of the sample and primer set. C. Expression of mTert in brain and liver from the knock-out described by Liu. As expected mTert-/- show no expression of the gene. MEaF’s showed low or no expression of the gene, dependent of the sample and primer set. D. Expression of mTert in mouse brain measured by qPCR; mTert+/+ (L)-brain from mTert-/- described by Liu; mTert+/+ (C)-brain form mTert-/- described by Chiang using primers complementary to mGapdh expression. Data are presented as log10 of gene expression relative to the negative control, C_0 values were normalised to mGapdh expression. Error bars show standard deviation.
3.8. Discussion

Telomerase has been shown to localise in mitochondria of hTERT over-expressing human fibroblasts, HEK cells and human cancer cells. Furthermore, it was shown that this localisation has a protective effect against various internal and external stress factors [61, 206, 218]. The aim of this part of my research was to test whether the mTert protein localises in mitochondria from mouse tissue in vivo. Furthermore, my goal was to test whether there were any potential changes in the levels of mTert protein and telomerase activity after dietary restriction in vivo.

I have shown here by immunofluorescence that TERT protein is expressed in adult mouse brain as well as in human brain tissue. Furthermore, I have shown that telomerase protein in both species mainly accumulates in the mitochondria of neurons in the cerebellum and cortex. Similar results to those from brain were observed in isolated embryonic neurons as well as in adult mouse brain where mTert protein co-localises with the mitochondrial marker cox II. Data for hTERT accumulation in human brain mitochondria has not been published so far and findings in mouse brain and neurons are in agreement with data from a recent publication by Eitan et al., (2012), who showed by immunofluorescence that mTert protein localises in Purkinje neurons cytoplasm [226]. Furthermore, the same authors showed by Western blotting that mTert was expressed in mouse forebrain in the nuclear and cytoplasmic fractions. However, mitochondrial co-localisation was not reported [198, 226]. Surprisingly, in my Western blotting experiments mTert protein was not detected in mouse brain by using the same antibody as Eitan et al., (2012) as well as by an antibody from other manufacturers (i.e. anti mTert from Abcam and LSBio; data not shown) [198, 226].

Interestingly, 6 to 17 months old mouse brain samples showed some telomerase activity in my analysis. Moreover, there was no telomerase activity in isolated mitochondria in brain and liver which suggests that only mTert protein without mTerc shuttles into mitochondria. However, Sharma et al., (2012) showed that hTERT protein in mitochondria acts as a reverse transcriptase by associating with mitochondrial RNA’s in human cancer cells, and these complexes showed reverse transcriptase activity - although independently of the hTERC template. Furthermore, the same authors showed by qPCR on RNA isolated from highly
purified mitochondria that hTERC was not detected in these organelles, suggesting that the RNA part of telomerase is not imported into mitochondria [219].

To obtain a fast and reliable method for the evaluation of TERT protein abundance, a new commercially available TERT ELISA kit (GenWay) was validated and applied in this study. Since there was no publication of data available with this new kit I had first to evaluate its potential use for mouse and human samples. Application of the TERT ELISA for both species was confirmed by testing the same samples with two alternative methods – Western blotting and immunofluorescence. Reliable controls for mouse and human material were successfully established.

To test differences between telomerase protein expression and telomerase activity in a quantitative manner I conducted TERT ELISA and TRAP experiments on the same samples of mouse brain and liver. Interestingly, TA was high in whole tissue homogenates and negligible in mitochondrial extracts, while mTert protein tested by ELISA was significantly higher in mitochondria than in homogenates of liver and brain (Figure 3. 5). My findings for liver are in agreement with previous findings of Martin-Rivera et al., (1998) [141]. However, localisation of mTert protein in adult mouse brain was shown only recently by Eitan et al., (2012) in Purkinje neurons of the cerebellum [226]. Interestingly, mitochondrial mTert localisation in our study was observed in isolated neurons and brain tissue without any external stress, in contrast to what had been shown previously in human fibroblasts, endothelial cells and various cancer cell lines by our group and others [61, 206, 218]. This might suggest that mTert, in addition to its protective function during stress, may be an important regulator of the mitochondrial processes already under basal conditions, at least in mice.

The beneficial effects of dietary restriction were discussed in Chapter 1. 3. For example, several studies have revealed that the generation of new neurons can be stimulated by DR. Moreover, it has been shown on mouse models that restriction of food was efficient in restoration of neuronal plasticity and improved cognitive abilities of ageing animals [93]. According to the results from this part of the project, telomerase activity decreased in mouse brain under DR in whole brain lysates. Although significant data was obtained only from experiment 3, results in experiments 1 and 2 also showed a trend for TA reduction. These findings may be explained by the reduced proliferation events in tissues under DR. It has been
reported, that reduced cell proliferation is correlated with reduced telomerase activity in \textit{f4 mTert} knock-out mouse model by Lee \textit{et al.}, (1998) [192]. However, further research would be required to elucidate what is the influence on proliferation of cells in brain during DR, and more importantly what type of cell divide during this intervention.

It was shown previously \textit{in vitro} that the hTERT protein shuttles into mitochondria under oxidative stress [61, 207, 218]. Thus, since it is known that DR reduces oxidative stress we expected that restriction of food \textit{in vivo} might reduce the abundance of TERT protein in mitochondria. This hypothesis might be also supported by findings of Haendeler \textit{et al.}, (2004) who had shown that incubation of endothelial cells with antioxidants inhibited the exclusion of hTERT protein from the nucleus [254]. Interestingly, in my study in three independent DR experiments, \textit{mTert} protein significantly increased in mitochondria of mouse brain. Remarkably, there was no change in \textit{mTert} abundance in whole tissue homogenates in all 3 experiments. These findings might suggest a new important role of telomerase in mouse brain mitochondria. Furthermore, \textit{mTert} gene expression in mouse brain was significantly reduced after dietary restriction in adult mice (experiment 1), and \textit{mTerc} expression showed no changes in the same samples (Figure 3.9). This outcome, together with reduced TA, might suggest that \textit{mTert} may play an important role in brain mitochondria, independently from telomerase activity during dietary restriction. It also suggests that protein localisation was not dependent on \textit{mTert} transcription or increase in total \textit{mTert} protein in lysates.

A final step in this part of my research was to establish the distribution of telomerase in different mouse and human brain areas. Preliminary results from TERT ELISA tests on human and mouse samples showed that both species seem to have a similar abundance of TERT protein in cortex and cerebellum. These results might suggest a conserved function of the protein in mouse and human brain.

Interestingly, I showed here that the amount of \textit{mTert} protein in lysates of mouse frontal cortex and cerebellum was significantly reduced in old animals (17 months) when compared to young mice (6 months). These data are in agreement with new findings by Eitan \textit{et al.}, (2012), who showed that telomerase protein is significantly reduced in Purkinje cells, brain stem and frontal cortex from 24 months
old animals when compared to 3 months old mice. However, no biological effect of the observed changes was reported [226].

In conclusion, in this study telomerase was expressed in mouse and human brain and the highest protein abundance was observed in cerebellum and in frontal cortex. Furthermore, accumulation of the mTert component in brain mitochondria after dietary restriction and its reduction in cortex and cerebellum with age suggest a new possible link between telomerase, brain mitochondria function and ageing. The potential functional role of mitochondrial localisation of mTert in mouse brain will be analysed and discussed in the forthcoming chapter.
Chapter 4 – Mouse Tert status and dietary restriction influences mitochondrial function, metabolism and neuromuscular coordination

4.1. Introduction

To date there are few publications about the function of TERT in the mitochondria of eukaryotic cells. For example, Ahmed et al., (2008) and Haendeler et al., (2009) reported a protective function of hTERT in mitochondria against mtDNA damage [61, 206]. It was reported that in MRC5 hTERT-over-expressing cells, the amount of mtDNA damage was reduced after H2O2 treatment and under hyperoxia when compared with telomerase negative MRC5 cells. In addition, hTERT over-expressing cells showed better resistance to apoptosis when compared to normal MRC5 cells. Moreover, hTERT-over-expressing cells showed a decrease of ROS production and increased mitochondrial membrane potential [61]. Similarly, Haendelers’ group showed that localisation of telomerase to mitochondria not only reduced ROS production but also improved respiration in HEK cells. Furthermore, the same study showed that silencing of endogenous TERT via shRNA increases mitochondrial ROS formation. Moreover, the same group showed in a model of mTert+/+ and mTert-/- mouse that mTert improves respiration of heart mitochondria and protect against UV-induced degradation of mtDNA in isolated primary lung fibroblasts. The same group also suggested that the catalytic subunit of telomerase binds to mtDNA at regions coding for polypeptides of ETC complex I (ND1 and ND2). Subsequently, proposing that the function of TERT is vital in the protection and/or repair of mammalian mtDNA [206]. Recent studies from Santos’ group brought further evidence for a protective function of TERT in mitochondria. This group reported that cells with mutated NES (nuclear export signal for TERT) were unable to shuttle TERT protein from the nucleus while maintaining telomerase activity. Mutation in NES in this study resulted in increased mitochondrial ROS production, increased oxygen consumption and increased mtDNA damage. [215]. This is additional evidence for the protective function of telomerase shuttling into mitochondria.

To test directly whether localisation of TERT in mitochondria reduces oxidative stress our group used organelle specific vectors to express TERT protein in different subcellular compartments. In this method TERT was delivered on a plasmid
construct directly into mitochondria or the nucleus using the multiple NLS (nuclear localisation signal) or MLS (mitochondrial localisation signal) [218]. In a recent study, Singhapol and colleagues showed in cancer cells that only mitochondrial TERT but not nuclear was capable to reduce oxidative stress induced by ionising irradiation and H₂O₂, bringing direct evidence for the protective function of TERT in mitochondria [218].

The aim of this part of the project was to analyse oxygen consumption and reactive oxygen species generation in mice primary cell lines derived from animals of different mTert status. Furthermore, respiration and ROS production by brain mitochondria after dietary restriction was analysed in different mTert genotypes of mice. In addition, an aim was to address whether the mTert status influence physiological parameters known to be altered during ageing like glucose tolerance, insulin sensitivity and neuromuscular coordination under the condition of dietary restriction.

4.2. Reactive oxygen species production and oxygen consumption in EPI-NCSC and MEaF’s isolated from mTert wild type and fl knock-out mice

To test the hypothesis that mTert might be involved in the regulation of oxygen consumption and have the potential to reduce ROS production in mouse cells, I used isolated EPI-NCSC (epidermal neural crest cells) and MEaF cells (mouse ear fibroblasts). To compare the potential difference in the mitochondrial phenotype between mTert wild type and fl knock-out mice ROS production was measured using MitoSOX® staining (mitochondrial superoxide) and DHR staining (intracellular peroxides) of intact cells. Fluorescence intensity was measured by flow cytometry as described in subchapter 2.10.3.

Interestingly, there was no difference in ROS production in both primary cell cultures (stem cells and ear fibroblasts) isolated form mTert wild type and mTert knock-out animals (Figure 4.1). To complete this research further experiments are required. For instance, it would be beneficial to test ROS production in MEaF and EPI-NCSC isolated from different age groups, genders and after application of oxidative stress i.e. incubation for 5 – 7 days in 20% or 40% O₂ environment. To test respiration levels in both cell lines, oxygen consumption rates were tested in a Seahorse XF24 extracellular flux analyser. The level of basal respiration in cells was
established by measurements of oxygen consumption without any chemical blockers of respiration and after delivery of oligomycin to the cell culture (which blocks complex V of ETC and completely abolishes ATP production). The difference between O\textsubscript{2} consumption before and after oligomycin delivery reports a basal respiration level dependent on ATP production. Next, mouse cells were tested for maximal respiratory capacity of mitochondria by adding 2\(\mu\)M (final concentration) of the uncoupler FCCP that artificially enhances proton leakage into the inner mitochondrial membrane. To test whether maximal respiration capacity was reached, additional FCCP was added (to reach a final concentration of 2,5\(\mu\)M). Finally, O\textsubscript{2} consumption was completely inhibited with antimycin A (complex III blocker).

Both mTert genotypes showed similar oxygen consumption in stem cells and in mouse ear fibroblasts under all conditions (Figure 4.2). However, oxygen consumption experiments on MEaF’s and EPI-NCSC’s were conducted on cultures isolated from only two male mice (6 months old) per genotype. Thus, further studies are required in order to draw any final conclusion.

Interestingly, during cultivation of EPI-NCSC I found a reduced cell isolation efficiency and proliferation of cells derived from the f1 mTert knock-out mice (Figure 4.3). To confirm stemness and purity of EPI-NCSC cultures staining with sox10 – a neural crest stem cell marker – was conducted (Figure 4.3 B.). Isolation efficiency and proliferation of mouse ear fibroblast from different mTert genotypes was not quantified due to the difference in the amount of primary material used.
Figure 4.1. Reactive oxygen species levels in primary cultures from mTert wild type and f1 knock-out mice. A. Mitochondrial superoxide levels in EPI-NCSC’s measured by flow cytometry using MitoSOX, FL3 – quantification of red fluorescence in channel 3 of flow cytometer (Partec), n=2 animals per genotype; B. Intracellular peroxide levels in EPI-NCSC’s by DHR staining, FL1 – quantification of green fluorescence in channel 1, FL3 – quantification of red fluorescence in channel 3, n=2 animals per genotype; C. Mitochondrial superoxide levels measured by MitoSOX in MEaF cells, n=5 animals per genotype; D. Intracellular peroxide levels in MEaF’s evaluated by DHR staining, n=5 animals per genotype. Statistical significance was tested by One Way Anova test. In panel A. and B. error bars show standard deviation. In panels C. and D. error bars represent S.E.M.
Figure 4.2. Oxygen consumption in mouse primary cell culture. A. O₂ consumption of EPI-NCSC from mTert +/+ and mTert -/- male mice, two animals per group shown separately, oxygen consumption measured after oligomycin (OLIGO), FCCP – uncoupler (maximal respiratory capacity), Antimycin A (AA) – complex III blocker. B. O₂ consumption of MEaF from mTert +/+ and mTert -/- male mice, two animals per genotype were used. OCR – Oxygen consumption rate measured in pmoles/min. Data presented as OCR – oxygen consumption rate in pmoles of O₂ consumed per minute, normalised to basal respiration calculated per 1x10⁵ cells. Each primary culture is shown separately, named 1 and 2 of each genotype. Error bars show standard deviation of the average from measurements in quintuplicates.
Figure 4.3. Isolation and proliferation of EPI-NCSC form mTert +/+ and mTert -/- mouse whisker bulges. A. Whisker hair follicle, expression of neural crest stem cell marker sox10 (red) in the EPI-NCSC niche (white arrows) in a whisker bulge, staining performed by Talveen Purba [255]; B. Stemness and purity of EPI-NCSC colony was confirmed by sox10 staining. Red fluorescence shows expression of sox10; C. Stem cells release from whisker bulges after 6 days from dissection, representative bulges from mTert+/+ and mTert-/- adult male mice (6-9 months); D. Proliferation of EPI-NCSC form mTert wild type and knock-out mice (two weeks after isolation); E. EPI-NCSC number after 20 days from whisker bulges isolation. Graph bars show average cell number derived from 5 animals per genotype. Error bars represent S.E.M. *Statistical significance was tested by One Way Anova test.
4.3. Oxygen consumption and hydrogen peroxide production in mouse brain mitochondria from mTert wild type and f1 knock-out mice after 16 months of dietary restriction

I have shown in the previous chapter that dietary restriction increases mTert protein content in brain mitochondria in mice. Thus, the question emerged what could be the role of this mitochondrial localisation of telomerase? The first hypothesis was that mTert might reduce oxidative stress and improve mitochondrial respiration. To test this hypothesis a dietary restriction experiment was performed on mTert wild type and f1 knock-out animals. Restriction started when the animals were 6-7 months old and lasted for 16 months. Only male animals were engaged in the experiment. By the end of the experiment four body weight matching animals per group and genotype, were chosen for dissection, mitochondria isolation and functional evaluation.

The bioenergetics experiments were conducted in the presence of pyruvate:malate (complex I substrate), and separately with succinate (complex II substrate). Consumption of O2 by isolated mitochondria during active state 3 respiration (ATP being produced) was measured after adding ADP (substrate for ATP) to the mitochondria. Next, state 4 respiration (resting state) was measured after delivery of oligomycin to mitochondria, which blocks complex V of ETC and completely inhibits ATP production. Afterwards, the uncoupler FCCP was added to mitochondria and maximal capacity for oxygen consumption was measured. Finally, antimycin A was delivered to mitochondria to block complex III and fully inhibit mitochondria respiration. The readout from the XF24® Seahorse extracellular flux analyser is OCR (Oxygen Consumption Rate) shown as pmoles O₂/min/5µg of mitochondrial proteins. Moreover, the Respiratory Control Ratio (RCR) for mitochondria was calculated. RCR characterises the state3/state4 respiration ratio and its value corresponds to the coupling of mitochondria. The greater the RCR value the more coupled the mitochondria are. Mitochondria are considered coupled when all protons H⁺ pumped into the inner membrane space are used by complex V (ATP synthase) for production of ATP. Uncoupled mitochondria, on the other hand, have a leakage of protons bypassing complex V, which causes a reduction of membrane potential (ΔΨm). Another effect of uncoupling is oxygen consumption of mitochondria due to increased demand of O₂ for faster electron flow. In other words: the more coupled mitochondria are, the more efficient respiration is.
Isolation of functional mitochondria from several animals is a laborious technique. Furthermore, freshly isolated mitochondria need to be used in two independent experiments immediately after isolation to keep their functional capacity intact (H₂O₂ generation and respiration). Thus, to obtain high quality data measurements, these experiments were conducted in collaboration with Dr Satomi Miwa and PhD student – Amy Johnson.

In our experiments dietary restriction significantly increased oxygen consumption of brain mitochondria under basal condition (state 2 respiration) in the presence of pyruvate:malate (complex I substrate) (Figure 4.4 A.). Furthermore, consumption of O₂ was increased in mTert+/+ mice after DR during state 4 mitochondrial respiration (resting state after oligomycin delivery) in the presence of pyruvate:malate. Interestingly, this difference was not observed in mTert-/- animals. Moreover, DR caused an increase in O₂ consumption after delivery of antimycin A. The addition of ADP (state 3 respiration) and the uncoupler (FCCP) showed no changes in the consumption of oxygen between genotypes or dietary groups. Surprisingly, the delivery of antimycin A also revealed the highest consumption of oxygen in mitochondria from the mTert+/- dietary restricted group (Figure 4.4 A.).

Oxygen consumption rates measured in the presence of succinate (complex II substrate) showed no significant difference between mTert wild type and knock-out in all respiration states. There was also no significant difference in oxygen consumption between the ad libitum group and dietary restricted animals. Furthermore, there was no significant difference in RCR values in brain mitochondria when the measurements were performed with pyruvate:malate or succinate, this means there was no significant change in the coupling of mitochondria after DR or dependent on genotype (Figure 4.5). Moreover, comparable RCR values means that mitochondria preparations from all tested groups were conducted consistently.

In addition to oxygen consumption, isolated brain mitochondria were analysed for H₂O₂ generation by AmplexRED®, a method described earlier. This technique measures H₂O₂ production, which is derived from O₂⁻ via interaction with SOD which results in a fluorescent product during reaction with the AmplexRED® reagent. Readout from the measurement of fluorescence was used for the calculation of H₂O₂ in pmoles/min/mg of mitochondrial proteins. Initial measurements were
conducted during state 2 mitochondrial respiration in the presence of specific substrates for ETC complexes \textit{i.e.} pyruvate:malate for complex I and succinate for complex II. Moreover, a specific inhibitor – rotenone – was used for the inhibition of complex I which resulted in maximal superoxide $O_2^-$ production in the presence of pyruvate:malate due to the accumulation of electrons in the NAD$^+$ reduction site. Furthermore, rotenone was added to mitochondria in the presence of succinate which allowed the calculation of the residual superoxide ($O_2^-$) production separate from complex I. Finally, in the reaction with succinate – antimycin A (blocker of complex III) was added allowing the calculation of maximal hydrogen peroxide generation linked to complex III.

Surprisingly, there was no significant difference in hydrogen peroxide generation in mouse brain mitochondria between dietary groups or genotypes in all respiratory states as well as in the presence of substrates for complex I and complex II (Figure 4.6)
Figure 4.4. Oxygen consumption by brain mitochondria after dietary restriction from mTert wild type and knock-out mice. A. Oxygen consumption in brain mitochondria from mTert +/+ and mTert −/− animals after 16 months dietary restriction in the presence of complex I substrate – pyruvate:malate; B. Oxygen consumption in brain mitochondria in the presence of complex II substrate – succinate. Data are presented as OCR (oxygen consumption rate) measured in pmoles O₂ /minute/5µg proteins. OCR was measured during basal condition, after ADP delivery, after oligomycin (OLIGO), after FCCP and after antimycin A (AA). AL – ad libitum (control group), DR – dietary restricted group. Graph bars show average OCR measured in quintuplicates from 4 animals per dietary group and genotype. *Statistical significance was calculated by One Way Anova and Two Way Anova with treatment and genotype as factors. Error bars show S.E.M.
Figure 4.5. Respiratory control ratio (RCR) in mouse brain mitochondria after dietary restriction. A. RCR calculated for mitochondria respiration in the presence of complex I substrate – pyruvate:malate, the difference between mTert wild type and knock-out is not statistically significant; B. RCR calculated for mitochondria respiration in the presence of complex II substrate – succinate. **AL** – *ad libitum* (control group), **DR** – dietary restricted group. Graph bars show average RCR measured in quintuplicates from 4 animals per dietary group and genotype. Statistical significance was calculated by One Way Anova to compare two groups and Two Way Anova test with treatment and genotype as factors. Error bars show S.E.M.
Figure 4.6. Hydrogen peroxide production by brain mitochondria from mTert wild type and knock-out mice after DR (measurement and analysis performed by Amy Johnson). A. H$_2$O$_2$ production in mitochondria in the presence of complex I substrate (pyruvate:malate - PM) and rotenone (ROT); B. H$_2$O$_2$ production in mitochondria in the presence of complex II substrate (succinate - SUCC), after delivery of rotenone and antimycin A (AA), C. ROS production during reverse electron flow. Data are shown as pmoles of H$_2$O$_2$ per minute, per mg of mitochondria proteins. AL – ad libitum (control group), DR – dietary restricted group. Graph bars show average pmoles/H$_2$O$_2$/min/mg proteins measured in triplicates from 4 animals per dietary group and genotype. Statistical significance was calculated by One Way Anova test. Error bars shows S.E.M.
4.4. Hydrogen peroxide production by brain mitochondria in female mTert wild type and f1 knock-out mice

Experiments described in the previous subchapter were performed only on male animals. Thus the question emerged: what would be effect of mTert status on hydrogen peroxide production in female brain mitochondria?

To address this question, brain mitochondria were isolated from different age groups/genotypes of female mice and tested by AmplexRED® in the same conditions as described in the previous subchapter. Three animals were tested per group (age/genotype). Young mTert wild type female animals at the age of 3 to 6 months and old mice aged 22 months were matched with their knock-out littermates.

Interestingly, analysis of freshly isolated mitochondria from female brains revealed different results than males. For complex I there was a higher H$_2$O$_2$ generation in brain mitochondria from young mTert knock-out female animals compared to wild type in the presence of pyruvate:malate and oligomycin (complex I substrate, state 4 respiration). Under the same condition this difference was not observed in old female mice (Figure 4. 7 A.). However, 22 month old knock-out females showed a higher H$_2$O$_2$ release than wild type females, when maximal ROS production capacity was measured in the presence of rotenone. Strikingly, hydrogen peroxide release, linked to complex I by reverse electron flow, showed no difference between genotypes in young females. However, brain mitochondria from knock-out old females showed significantly higher free radical production by reverse electron flow than age-matching wild type female littermates (Figure 4. 7 C.). Interestingly, H$_2$O$_2$ release in the presence of succinate (complex II substrate) showed no changes in brain mitochondria of young and old females. Maximal capacity of ROS production linked to complex III measured after antimycin A inhibition also showed no difference between mTert genotypes in both age groups of females (Figure 4. 8).

Altogether, data from hydrogen peroxide production in female brain mitochondria support the hypothesis of a protective function of mTert against age related increase of ROS production in vivo in brain. Furthermore, these results might suggest that telomerase may have a different role in the regulation of mitochondrial ROS production in male and female brains.
Figure 4.7. Hydrogen peroxide generation in mouse female brain mitochondria in the presence of pyruvate:malate. **A.** $\text{H}_2\text{O}_2$ generation in brain mitochondria from young animals (3-6 months), PM – measurement after delivery of complex I substrate (pyruvate:malate) during state 2 respiration, PM+ROT – measurement after delivery of rotenone (complex I blocked); **B.** $\text{H}_2\text{O}_2$ generation in the presence of pyruvate:malate in brain mitochondria from old animals (22 months); 3 animals per age group/genotype were used for each experiment. *Statistical significance was calculated by Anova test. Error bars show S.E.M.
Figure 4.8. H2O2 generation in female brain mitochondria in the presence of succinate. A. Hydrogen production linked to complex I and II in female brain mitochondria from young animals (3-6 months), SUCC – measurement after delivery of complex II substrate (succinate), ROT – measurement after delivery of rotenone (complex I blocker), +AA – measurement after delivery of antimycin A; B. Hydrogen peroxide production in female brain mitochondria form old females (22 months) in the presence of succinate; C. H2O2 release in brain mitochondria linked to complex I, during reverse electron flow. 3 animals per age group/genotype were used for each experiment. Statistical significance was measured by Anova test. Error bars shows S.E.M.
4.5. Physiological parameters in mTert wild type and f1 knock-out mice after dietary restriction

Glucose tolerance and insulin resistance has often been associated with an age dependent physiological decline, and along with body weight as well as food intake, are suitable features for assessing general health of animal models [242, 256]. Hence, I asked whether the mTert gene status might influence basic physiological parameters in our dietary restriction model.

Male animals at the beginning of the DR experiment had similar body weight. For instance, in the AL group mTert+/+ animals were on average 32.23g ± 3.6; mTert-/- were 32.58g ± 3.6. In the DR group mTert+/+ animals were on average 33.31g ± 2.3; mTert-/- had 33.76g ± 3.2 of body weight. Food intake was calculated per animal (3.14g ± 0.24 of chow) thus 40% restricted animals had access to 1.9g of food per individual per 24 hours. However, food intake was not adjusted during the experiment, thus during some periods food intake was restricted from 30 to 50%. Within the first 60 days of the experiment DR mice lost an average of 13 grams of body weight and it remained steady for the rest of the experiment in both genotypes. Ad libitum mice were gaining weight steadily until 250 days when it stabilised (Figure 4.9 A.). Food intake was fluctuating during all 16 months of the experiment from 3 to 4g/animal/24h (Figure 4.9). Interestingly, the body weight of AL mTert-/- animals started to decrease in the last few months of the experiment (although not significantly), while the weight of AL mTert+/+ mice was more stable (Figure 4.9 A.) Thus, the general observation was that the mTert gene status did not influence weight or food intake significantly during dietary restriction.

To test what impact dietary restriction might have on glucose metabolism of mTert wild type and knock-out mice I conducted a Glucose Tolerance Test (GTT). The GTT measures a fasting organism’s ability to clear injected glucose in the blood. Moreover, it is known that this ability is reduced with age and in diabetes. Furthermore, it was shown in humans and rodents that the ability for blood glucose clearance is increased by dietary restriction and reduced with age [75, 257]. Since impaired glucose clearance from the blood is an effect of increased insulin resistance, because this hormone negatively regulates glucose levels, I have investigated the additional question - what is the potential change in the insulin level after dietary restrictions in different mTert genotypes?
32 animals were tested in total at the end of 16 months of dietary restriction as described in the materials and methods - subchapter 2.3. Specifically, there were 10 mTert wild type and 11 fI knock-out mice in the ad libitum group. The DR group contained 4 wild type and 7 knock-out mice. Only male animals were used in the experiment.

As expected, the obtained results showed that DR accelerates glucose clearance from the blood. Moreover, both genotypes in the DR group had a significantly lower basal fasting blood glucose concentration measured after 24h of fasting. However, glucose level in AL animals was on average 6.1mmol/L, which is usual for healthy mammals. Interestingly, there was no difference between genotypes in the AL group at all time points measured after the glucose injection: 15min., 30min, 1h and 2h (Figure 4.10 A.). Surprisingly, there was a significant difference observed at the 1h and 2h time point in the DR group, where mTert/- animals had significantly lower average blood glucose (Figure 4.10 B.).

Similarly to glucose tolerance, insulin resistance becomes impaired with age, and DR is thought to diminish the age related increase of insulin levels in plasma. To test whether mTert plays a role in this phenomenon, the same animals as in the GTT were used in the analysis of insulin levels using an ELISA kit after dissection of the mice. As expected, after 16 months of dietary restriction the insulin levels in mTert+/+ animals was reduced significantly (Figure 4.10 C.). Interestingly, among the mTert/- mice no difference was observed after DR. This may suggest an involvement of mTert in the regulation of insulin levels.
Figure 4.9. Body weight and food intake of mTert +/- and mTert-/- mice during the DR experiment. A. Average body weight of mice during 16 months of dietary restriction, AL – ad libitum fed animals (control group) consisted of 10 mTert+/- and 11 mTert-/- animals, DR – dietary restricted group consisted of 4 mTert+/- and 7 mTert-/- animals, n/s – not significant, statistical significance was analysed using Anova; B. Average food intake per day per animal during DR experiment, food intake in the restricted group was limited to 1.9g per animal per day. Error bars show S.E.M.
Figure 4.10. Blood glucose clearance and insulin levels after dietary restriction in mTert+/+ and mTert-/- mice. A. Glucose tolerance test (GTT) in ad libitum fed animals after 12h fasting, the group consisted of 10 mTert+/+ and 11 mTert-/- animals; B. GTT in mTert +/+ mice, AL (n=10) vs. DR (n=4); C. GTT on dietary restricted animals, the DR group consisted of 4 mTert+/+ and 7 mTert-/- animals; D. GTT in mTert-/- mice, AL (n=11) vs. DR (n=7); E. Quantitation of area under the curve of glucose tolerance test (GTT) F. Fasting insulin levels in plasma of mTert+/+ and mTert-/- mice after 16 months dietary restriction, 4 mice per group/per genotype were analysed in duplicate. *Statistical significance was tested by One Way Anova on Ranks. Error bars show S.E.M.
4.6. The influence of mTert status and DR on neuromuscular coordination

A beneficial effect of DR on brain function has been described previously [258]. To determine a potential effect of dietary restriction and mTert genotype on neuromuscular coordination a series of tightrope tests was performed on mice at the beginning of the DR and at the end of the DR experiment. The technique is a widely used method for assessing sensorimotor performance, strength and vigour of rodents. Particularly, the tightrope test is used in ageing and oxidative stress research focused on the function of the nervous system and muscles, particularly in their coordination [259-262].

The equipment for the tightrope test was described in chapter 2.18. It consists of a rope, which is tightly tied up between two chemical stands above cushioned bedding. Mice were suspended above the tightrope for 20 seconds at the distance of their whiskers. Afterwards, the mouse was placed with its front paws in the middle of the rope and the time between grabbing the rope and pulling up its hind legs was recorded. The experiment ran for 60 seconds and three trials were performed. The same male mice were tested at two time points i.e. at 8 months old – after 2 months of the DR and at 21 months old. The number of unsuccessful attempts (when the mouse fell down or did not lift its hind legs up) was averaged and analysed.

Interestingly, young males (8 months old) at the beginning of the DR experiment (2 months DR which started at 6 month of age) performed similarly in all groups, with less than one unsuccessful attempt on average to lift hind legs up in the test. There was no significant difference between dietary groups or between the mTert genotypes (Figure 4.11 A.). After 14 months of DR experiment all groups apart from the mTert+/+ under DR had decreased their performance compared to the 8 month time point (Figure 4.11 B.). Both genotypes in the AL group showed a two fold increase in the number of unsuccessful attempts (Figure 4.11 B.). However, in the DR group ability to perform the test was reduced only in mTert-/- animals, while wild type animals performed similarly as at the beginning of DR. Thus, these results suggest that: i) DR delays age-related neuromuscular coordination decline seen in the mTert wild type AL group and ii) mTert might play a possible role in neuromuscular coordination during ageing and dietary restrictions.

To test whether the effect of mTert status on neuromuscular coordination is sex specific, young (5-6 months) female mice were also tested for their tightrope
performance. In total, 22 \textit{mTert}+/+ and 14 \textit{mTert}-/- female mice were tested. Interestingly, in females the effect of \textit{mTert} status can be observed already in young animals. Knock-out female mice were significantly less successful in the experiment than their wild type littermates; however the number of mice was much higher than for males (Figure 4.11 C.). Moreover, there were no dietary restricted females available.
Figure 4.11. Neuromuscular coordination of mTert+/+ and mTert-/- mice during ageing and after dietary restriction. A. Unsuccessful attempts to lift up hind legs in young male mice (8 months) after 2 months of dietary restriction, AL – ad libitum, the group consisted of 10 mTert+/+ and 11 mTert-/- animals, DR – dietary restricted, the group consisted of 4 mTert+/+ and 7 mTert-/- animals; B. Unsuccessful attempts to lift hind legs up in old male mice (20 months) after 14 months of dietary restriction, the same animals had been analysed as at the beginning of the experiment, statistical significance between age groups was performed by Mann-Whitney rank-sum test: #P=0.019, †P=0.050, ‡P=0.0573, ɨP=0.005; C. Unsuccessful attempts to lift hind legs up in young female mice (5-6 months), the female cohort consisted of 22 mTert+/+ and 14 mTert-/- animals. *Statistical significance was measured by Mann-Whitney rank-sum test. Error bars show S.E.M.
4.7. Discussion

The protective role of mitochondrial telomerase against oxidative stress was previously reported by our group and others. Human telomerase reverse transcriptase was shown to reduce apoptosis and generation of reactive oxygen species in a number of \textit{in vitro} studies [61, 206, 218]. However, the role of mitochondrial TERT had not been sufficiently demonstrated to date \textit{in vivo} except for data published by Haendeler \textit{et al.}, (2009), where it was shown that \textit{mTert} deletion resulted in reduced respiration in mouse heart mitochondria but not in liver mitochondria [206]. Thus, we performed various relevant mitochondrial function experiments on cells and mitochondria isolated from brain of the same \textit{mTert} wild type and \textit{mTert} knock-out animals as Haendeler \textit{et al.}, (2009). Moreover, restriction of nutrients was used for elucidating a potential function of mTert in the bioenergetics of brain mitochondria under DR conditions.

The status of \textit{mTert} in this project was shown to have no impact on ROS production in MEaF and EPI-NCSC cells isolated from different \textit{mTert} genotype mice (Figure 4.1). This contradicts previous \textit{in vitro} findings published by Ahmed \textit{et al.}, (2008) and Haendeler \textit{et al.}, (2009), where overexpression of \textit{hTERT} resulted in reduced oxidative stress in human fibroblasts and HEK cells respectively [61, 206]. Similarly, in our study oxygen consumption in MEaF and EPI-NCSC cells was at the same level in both \textit{mTert} genotypes (Figure 4.2). However, the respiration data is based on a small number of animals thus further experiments are required in order to draw any final conclusion.

Interestingly, tests performed on cultivated EPI-NCSC from mouse whisker pads showed that the \textit{mTert} status was crucial for the isolation efficiency and proliferation ability of those cells. This effect of mTert was shown here for the first time in (\textit{f1}) \textit{mTert} knock-out animals (Figure 4.3). Sahin \textit{et al.}, (2011) showed a similar reduction in proliferation of hematopoietic stem cells on fourth generation (\textit{f4}) \textit{mTert} knock-out animals, which was a telomere length dependent phenomenon [193]. Furthermore, Nitta \textit{et al.}, (2011) showed a decrease of proliferation in stem cells isolated from mouse hair follicle as a result of \textit{mTert} deficiency and increased oxidative stress independently of telomere length [204]. Interestingly, Flores \textit{et al.}, (2008) showed that stem cells in mice have longer telomeres than cells in the surrounding tissue, however, there is no data on telomere length in (\textit{f1}) \textit{mTert} knock-
out mice [24]. Thus our data suggests that perhaps stem cells depend more on telomerase not only due to its telomere length function, but also due to its function as regulator of the transcription of development genes (i.e. WNT/β-catenin) or due to protective role of the enzyme. Further systematic experiments, especially on stem cells are required to elucidate the role of mTert in oxidative stress (i.e. hyperoxia, irradiation) and proliferation in different generations of mTert knock-outs, age groups and genders. Moreover, a critical and accurate measurement of telomere length would help to better evaluate whether the diminished growth of mTert-/ stem cells could be attributed to telomere-dependent or independent functions of telomerase.

The next question we wanted to address was about the role of mTert in brain mitochondria function. Interestingly, published data on mitochondrial respiration in organs, other than the brain, after dietary restriction are often contradicting. For example, it was shown that DR reduces oxygen consumption (state 4 mitochondrial respiration) in mice brain, heart and kidney as well as in the skeletal muscle of rat [263, 264]. However, other groups showed antagonistic results. For instance, Nisoli et al., (2005) showed that DR increases significantly oxygen consumption in brain and liver mitochondria in mice [265]. Moreover, Hempenstall and co-workers showed recently that dietary restriction increases oxygen consumption in mouse skeletal muscle mitochondria [266]. In our dietary restriction experiment, oxygen consumption increased significantly in brain mitochondria from restricted animals in the presence of complex I substrate. This rise in respiration was only observed in mTert+/+ animals, which would suggest a possible role of mTert in mouse brain mitochondria function (Figure 4. 4). However, this DR effect in the mTert+/+ mice was observed only during resting state of mitochondrial respiration. After delivery of ADP no changes in oxygen consumption were observed. Surprisingly, O2 consumption was not different between mTert+/+ and mTert/- male mice in the presence of pyruvate:malate – complex I substrate (Figure 4. 4). Interestingly, Haendeler et al., (2009) showed that mouse heart mitochondria from mTert knock-out animals had reduced respiration in the presence of complex I substrates (i.e. malate and glutamate), however in liver mitochondria there was no genotype effect on respiration. Probably one reason could be that the heart utilises high amounts of energy compared to liver and therefore mTert status effect could be more pronounced
[206]. Thus, the role of mTert on respiration may be tissue specific. However, research of Sahin et al. (2011) showed a significantly lower oxygen consumption in mitochondria from heart and liver in (f4) mTert/- than in the wild type mice as an effect of telomere shortening [193].

The next functional parameter we tested for brain mitochondria was hydrogen peroxide generation. It was shown by various groups in a number of animal models and tissues (i.e. mouse liver, intestine) that dietary restriction potentially attenuates ageing by reduction of oxidative stress [32, 79, 80]. A telomere dependent role in this process was also reported, showing that shortening of telomeres in late generations of mTert knock-out animals caused mitochondrial dysfunction, increased ROS production and accelerated ageing [193, 194]. However, the role of mitochondrial mTert was not analysed in vivo yet. In our experiments performed in collaboration with Dr Satomi Miwa and Amy Johnson, we found that dietary restriction did not reduce H2O2 production by mitochondrial complex I, II and III in brain (Figure 4. 6). That is in contrast to published data that DR significantly reduces ROS production in mouse and rat brain in different age groups [263, 267]. Moreover, in our study there was also no difference in brain mitochondria ROS production between different mTert genotypes in male mice. However, it would be interesting to test the dietary restriction effect on telomerase in female brain since it was shown that there are gender specific differences in mitochondria function after DR. For example, Colom and co-workers showed that in dietary restricted rat females there was reduction in ROS production in cardiac mitochondria to significantly higher extend than males when compared to the corresponding ad libitum controls [268]. Most importantly, mitochondria from rat female brain, liver and skeletal muscles showed lower ROS production than from their male counterparts [269-271]. An explanation of this phenomenon might be fact that estrogen was shown to reduce ROS production in female rat brain and in males supplemented with estrogen in vivo [272].

Since, female mice were not used in our dietary restriction study, we could only analyse the potential influence of mTert on genotype and age in this gender group. Interestingly, H2O2 generation linked with complex I substrate was significantly higher in brain mitochondria from female mTert/- animals than in wild type female littermates what was not observed in males (Figure 4.7). Remarkably, ROS production linked to complex II substrate showed no difference in both young
and old females. This data seems to suggest that mTert might be involved in mitochondrial complex I ROS production. Furthermore, free radical generation by reverse electron flow was similar in both genotypes in young animals but significantly increased in mTert-/- brain mitochondria from old females (Figure 4, 7). There was no influence of mTert status on hydrogen peroxide generation linked to complex III (blocked by antimycin A). Altogether these results suggest that: i) mTert seems to protect the female brain against oxidative stress; ii) mTert function in ROS generation seems to be differently regulated in male and female brain. Thus, it would be useful to conduct further research on the influence of telomerase on gender and sex steroids for the function of brain mitochondria. Interestingly, there are data published that might support this hypothesis. For example, it was described that estrogen is positive regulator of telomerase in human by two mechanisms: 1) estrogen is mediating expression of hTERT by estrogen response element on hTERT promoter and 2) estrogen is known for stimulation of c-Myc, a potent positive transcriptional hTERT regulator [273, 274]. Furthermore, withdrawal of testosterone was shown to be potent to elevate telomerase activity in healthy rat and monkey prostate [275, 276]. Taking all this points together, we propose that mTert function in brain mitochondria might be regulated by sex hormones.

Another question we addressed was the influence of telomerase on metabolic parameters in mice during dietary restriction. It was shown previously that dietary restriction decreased glucose and insulin levels in mammalian blood/serum, however the role of TERT in this effect was not tested to date [277, 278]. In this project, fasting insulin level was significantly decreased under dietary restriction as expected. Interestingly, the reduction of insulin concentration in fasting blood plasma was significantly reduced after DR only in mTert+/+ animals while mTert-/- mice showed no changes. This result might suggest that telomerase is a possible player in the regulation of insulin changes during DR. However, there were no significant changes in insulin level between mTert+/+ and mTert-/- under normal (AL) conditions that suggests that telomerase is rather indirectly involved in insulin regulation. On the other hand, while the glucose clearance was more efficient in the dietary restricted animals, mTert knock-out animals under DR showed a faster clearance of blood glucose in the first and second hour after glucose injection. Interestingly, this effect was not observed in the ad libitum group. These metabolic data confirm beneficial
effects of dietary restriction on mice. However, our glucose clearance data suggests that male $mTert-/ -$ mice might be more efficient in GTT after DR than $mTert+/+$ animals. No effect of first generation $mTert$ knock-out on glucose metabolism and insulin level was reported so far. However, an influence of telomerase length on glucose tolerance and insulin level was reported recently by Kuhlow and co-workers [279]. Authors of this publication showed that telomerase deficient mouse ($mTert-/-(f4)$) had significantly reduced glucose tolerance as well as diminished insulin secretion, all correlated with significantly reduced telomere length in pancreatic islets [279].

Mouse $Tert$ status also revealed interesting results on neuromuscular coordination. In my experiments it was tested whether telomerase and dietary restriction can protect mice against age related decrease in neuromuscular performance. Guyerbas et al., (2005) suggested that age related decrease in coordination can be restored by antioxidant supplementation, suggesting ROS levels as an important cause for the decline of the function of the nervous system and muscles [262]. Furthermore, it was shown that sensorimotor performance in a tightrope test was inversely proportional to the levels of oxidative stress markers in mouse brain like Cu/Zn-SOD and Mn-SOD [280]. Interestingly, my results from the tightrope experiments seem to show that $mTert$ status has a significant influence on neuromuscular performance of mice. However, taking together the data from ROS production in male and female brains mitochondria it is surprising that $mTert-/-$ in both genders have higher number of unsuccessful attempts in the test. It would be expected that female $mTert$ knock-out with higher ROS production in the brain should perform worse in neuromuscular coordination test. Why then old male $mTert-/-$ with no changes in ROS production in brain also performed worse? The question could be addressed by analysing ROS generation in muscle mitochondria from both genotypes and genders, since tightrope test is directed to assess central nervous system and muscle.
Chapter 5 - Is mTert in mitochondria regulated via mTOR pathway?

5.1. Introduction

Life span extension mechanisms induced by DR are often explained mechanistically by down-regulation of the mTOR pathway. mTOR is a kinase that regulates signals for cellular growth control in response to nutrients. Furthermore, the mTOR pathway is widely studied due to its key role in apoptosis and cancer development. It was shown that elevated activation of mTOR signalling reduces apoptosis and increases proliferative capacity of cancer cells [108]. Furthermore, it was shown that mTOR can be a link between nutrients and energy signalling pathways [109]. Recently, the kinase was also described as a regulator of nutritional and hormonal signals in the brain [110]. There are reports suggesting that inhibition of the mTOR pathway extends the life span of yeast, worms, flies and mice [111-113]. Furthermore, it was shown that mTOR downstream targets are engaged in the reduction of oxidative stress, as well as the response for DNA damage and induction of apoptosis. For example, Schieke et al., (2006) reports that inhibition of mTOR with rapamycin resulted in disruption of the mTOR-raptor complex, which resulted in lower mitochondrial membrane potential, oxygen consumption and decreased ATP synthesis [117, 118]. Moreover, Wilkinson and colleagues showed that rapamycin supplementation decreases tumour incidence, and reduces degenerative age related changes in liver and heart [120]. Remarkably, a recent publication by Fok et al., (2012) suggests that rapamycin and dietary restriction might have a similar effect on mice [121]. In these experiments, mice were dietary restricted or supplemented with rapamycin, the authors reported similar inhibition of S6K1 – mTOR pathway downstream targets as well as a similar increase in autophagy in liver after both treatments. Interestingly, animals from the DR group and the rapamycin treated group showed opposite effects on blood glucose clearance and different insulin tolerance, showing the benefits of dietary restriction and the potential deteriorative effect of rapamycin [121].

Since mTert shows a response in mouse brain mitochondria to dietary restriction, the question emerged whether a decreased mTOR activity might be involved in this phenomenon? To address this question two separate approaches were
considered: 1) to analyse the phosphorylation level of mTOR protein in mouse brain after dietary restriction, 2) to inhibit mTOR activity by rapamycin dietary supplementation in vivo and investigate whether the response of mTert is similar to DR under this treatment and 3) to test if brain mitochondria from mTert+/+ and mTert−/− mouse treated with rapamycin shows similar bioenergetics to those from dietary restriction experiments.

5.2. Rapamycin supplementation effect on mTert wild type and knock-out mice

5.2.1. Inhibition of mTOR pathway in mouse brain after rapamycin and DR

Rapamycin was shown to be a potent mTOR inhibitor. Thus, a short term supplementation experiment was conducted in mice. There were a total of 31 male mice in the experiment. In the control group, there were 7 mTert+/+ and 9 mTert−/− mice, while in the rapamycin fed cohort there were 6 mTert+/+ and 9 mTert−/− animals. All the mice had a similar weight (40.7g ± 7.5g) and were twelve months old when the supplementation started (Figure 5.1 C.). Rapamycin supplementation lasted for 4 months. Body weight during the rapamycin study did not change in either group. Furthermore, food intake, although it fluctuated, was similar in all tested groups.

Mouse brain tissue from experiment 1 (late onset short term DR) were tested by Western blotting to detect potential changes in mTOR protein phosphorylation in mouse brain. The ratio of phosphorylated to non-phosphorylated mTOR was quantified by densitometry analysis of protein visualisation on a Western blotting membrane. The phosphorylation of mTOR was significantly reduced after dietary restrictions in mouse brain from experiment 1 (Figure 5.1 A.). Furthermore, activation of mTOR was tested in mouse brain tissue after rapamycin supplementation. Western blotting experiments revealed that rapamycin treatment was efficient to significantly reduce mTOR phosphorylation in brain, with a similar effect to the one observed in brains after dietary restriction (Figure 5.1 B.). Thus, these results support the hypothesis that rapamycin supplementation might mimic the effects of dietary restrictions on mTOR in brain.
Figure 5.1. Phosphorylation of mTOR in mouse brain and body weight of mice after rapamycin supplementation. A. A decrease of mTOR activity after dietary restriction in mouse brain after 3 month of dietary restriction (exp. 1), upper panel: immune-blot with phosphor-mTOR and total mTOR antibodies, lower panel: densitometric quantification, the graph shows the average ratio of phosphorylated mTOR to non-phosphorylated mTOR, AL1-AL4 – ad libitum fed animals, DR1-DR4 – dietary restricted mouse; B. A decrease of mTOR activity after rapamycin supplementation for 4 months in mouse brain, C1-C4 – mTOR in control animals, R1-R4 – animals fed with rapamycin, C. Body weight of mice before and after 4 months of rapamycin treatment. *Statistical significance was calculated by One Way Anova. Error bars show S.E.M.
5.2.2. The influence of rapamycin supplementation on mTert protein expression in mouse brain

To analyse the effect of rapamycin on mouse brain and potential ability to mimic dietary restriction response in mouse brain, mitochondrial mTert protein levels were measured after 4 months of rapamycin treatment. In addition, insulin levels in plasma of mice from the rapamycin experiment were evaluated by ELISA.

It was shown earlier in this study that dietary restriction increased the abundance of mitochondrial mTert in brain - in three independent experiments. However, this effect of DR on mTert in liver mitochondria varied between experiments. Thus, a TERT ELISA was performed on mouse brain mitochondria and whole brain tissue homogenates from rapamycin fed mice compared to controls. Moreover, mitochondrial fractions from livers were also tested for mTert protein amount under this condition.

Strikingly, the effect of rapamycin on mTert in mitochondria was the same as observed in dietary restriction. In brain, mTert abundance was higher for 38% in brain mitochondria from rapamycin treated animals compared to controls, very similar to all three DR experiments. Furthermore, the total amount of mTert in whole brain homogenates remained unchanged (Figure 5.2 A.). Interestingly, the results from liver mitochondria in the rapamycin experiment were also similar to those obtained from DR experiments where no difference in mTert protein amount was observed (Figure 5.2 B.). Liver homogenates from rapamycin treated mice were not tested. Altogether, this data strongly supports the hypothesis that the dietary restriction effect on an increase of mTert in brain mitochondria can be mimicked by rapamycin dietary supplementation. Therefore, the shuttling of mTert into brain mitochondria might be regulated by the mTOR pathway.
Figure 5.2. The influence of rapamycin on mTert protein abundance in brain and liver mitochondria, brain homogenates and plasma insulin level. A. Telomerase concentration in mouse brain mitochondria and whole organ homogenates after 4 months of rapamycin supplementation, 5 animals were used per group in the experiment; B. Telomerase concentration in mouse liver mitochondria after rapamycin supplementation, 5 animals were used per group; *Statistical significance was tested by Anova. Error bars show S.E.M.
5.2.3. The influence of rapamycin on insulin level in mouse blood

To test what effect rapamycin supplementation has on mouse metabolism I tested insulin levels in plasma of mTert+/+ and mTert/- animals. Blood samples were collected from all animals during dissection (after feeding) and after blood serum isolation insulin levels were quantified by ELISA. Interestingly, in this experiment insulin levels after 4 months of rapamycin supplementation were significantly increased in mTert+/+ animals, which is in contrast to our findings from DR. However, this increase was not observed in mTert/- animals. Interestingly, insulin levels in mTert/- animals were significantly increased in the control and rapamycin treated group when compared to wild type control animals (Figure 5.3). This observation might suggest that mTert is involved in the metabolism of insulin. However, these results also show that rapamycin potentially can have deteriorative effects on liver, thus a lowered dosage of rapamycin is recommended in future experiments. Therefore, liver should be tested for functional markers to confirm a potentially toxic effect of rapamycin.
Figure 5.3. Insulin concentration in mouse serum after rapamycin supplementation. Graph bars show an average insulin concentration from 4 animals per group/genotype measured in duplicates. *Statistical significance was tested by One Way Anova. Error bars show S.E.M.
5.3. Oxygen consumption and reactive oxygen species generation in brain mitochondria after rapamycin supplemented diet

To determine a potential influence of rapamycin dietary supplementation for the function of brain and liver mitochondria we measured ROS generation and oxygen consumption levels. Experimental settings were identical to those used to analyse mitochondria from animals after dietary experiments. Due to the complex nature of functional parameters the experiment on freshly isolated mitochondria was conducted in cooperation with Dr Satomi Miwa and another PhD student – Amy Johnson.

Oxygen consumption was tested in the presence of pyruvate:malate as a substrate for complex I and independently with succinate a complex II substrate. Respiration of isolated brain mitochondria was measured during active respiration state 3 by adding ADP to mitochondria. Moreover, respiration state 4 was analysed after introduction of oligomycin to mitochondria to block complex V. Furthermore, as earlier described, the uncoupler FCCP was added to mitochondria to measure maximal capacity of oxygen consumption. Finally, antimycin A was added to block complex III and stop oxygen consumption. Moreover, the level of mitochondria coupling was quantified by calculation of the RCR value.

In our experiments rapamycin had no effect on O2 consumption in both genotypes in all states of respiration as well as with inhibitors and the uncoupler (Figure 5.3). Furthermore, RCR during respiration of brain mitochondria also remained unchanged after 4 months of rapamycin supplementation in both mTert genotypes, which means that rapamycin have no influence on coupling of mitochondria. Moreover, comparable RCR values indicate consistent mitochondria preparation (Figure 5.4).

Production of ROS by brain mitochondria after rapamycin treatment was measured by AmplexRED® under the same conditions as during measurements on mitochondria from the DR study. Briefly, hydrogen peroxide release was analysed during mitochondrial respiration state 4 (with oligomycin) in the presence of specific substrates for complex I and II. Furthermore, rotenone was used for the inhibition of complex I (maximal capacity of superoxide O2− production in the presence of pyruvate:malate). In the presence of succinate, rotenone was added to block complex
I and calculate $O_2^-$ generation during reverse flow, associated with complex I. Antimycin A was added to calculate the maximal H$_2$O$_2$ release linked to complex III.

Results from ROS production experiment revealed that rapamycin was able to significantly reduce maximal (in presence of rotenone) superoxide release linked to complex I in $m$Tert+/- animals (Figure 5.5 A.). Interestingly, brain mitochondria isolated from $m$Tert-/- showed no difference in generation of H$_2$O$_2$ after rapamycin supplementation. Furthermore, there was no difference in H$_2$O$_2$ production after rapamycin when only substrate for complex I (pyruvate:malate) was present in all genotypes (Figure 5.5 A.). Thus, this data strongly suggests that regulation of superoxide production in mouse brain mitochondria during inhibition of mTOR pathway depends on $m$Tert status. Moreover, ROS generation in the presence of succinate (complex II substrate) also seemed to be strongly influenced by rapamycin. In the presence of succinate only brain mitochondria from $m$Tert+/- rapamycin treated mice showed significantly reduced H$_2$O$_2$ production when compared to control samples (Figure 5.5 B.). In contrast, there was no difference observed among mitochondria from $m$Tert-/- mice. After addition of rotenone there was no difference in ROS production in all groups and genotypes.

Hydrogen peroxide production during reverse electron flow, linked to complex I can be calculated, as described earlier, from values obtained during measurements of ROS in presence of succinate and rotenone. Interestingly, rapamycin strongly reduced H$_2$O$_2$ production in brain mitochondria during reverse electron flow (Figure 5.5 C.). This reduction was only observed in $m$Tert+/- mice, while mitochondria from $m$Tert-/- animals shows no difference between control and rapamycin supplemented group what suggests that mTert seems to be crucial for the response to rapamycin.

Surprisingly, data from AmplexRED experiments showed that brain mitochondria from $m$Tert-/- animals produced less H$_2$O$_2$ (P<0.001) than mitochondria from $m$Tert+/- male animals when subjected to complex I substrate (pyruvate:malate). However, this effect was not observed in control wild type and knock-out in experiments on animals from dietary restricted group. Although similar animal number was tested, the age of animals in both experiments was different. Mice from dietary restriction analysis were 22-23 months old and animals from rapamycin study were 16 months old.
Figure 5.4. Oxygen consumption in mouse brain mitochondria after rapamycin supplementation. 

A. Oxygen consumption in the presence of complex I substrate – pyruvate:malate, brain mitochondria form mTert +/+ and mTert -/- animals after 4 months of rapamycin supplementation, 5 animals per group/per genotype were analysed; 

B. Oxygen consumption in brain mitochondria in the presence of complex II substrate – succinate. Data presented as OCR (oxygen consumption rate) measured in pmoles of O₂ per minute, per 5µg of proteins. OCR was measured during basal condition, after ADP delivery, after oligomycin (OLIGO), after FCCP and after antimycin A (AA). Statistical significance was calculated by Anova. Error bars show S.E.M.
Figure 5.5. Respiratory control ratio (RCR) in mouse brain mitochondria after rapamycin treatment. A. RCR calculated for mitochondria respiration in the presence of complex I substrate – pyruvate:malate; B. RCR calculated for mitochondria respiration in the presence of complex II substrate – succinate. Statistical significance was calculated by Anova. Error bars show S.E.M.
Figure 5.6. H$_2$O$_2$ generation of brain mitochondria from mTert$^+/+$ and mTert$^−/−$ mice after rapamycin treatment (measurements performed by Amy Johnson). A. H$_2$O$_2$ generation in mitochondria in the presence of pyruvate:malate - PM (complex I substrate) and rotenone (ROT); B. H$_2$O$_2$ generation in the presence of succinate - SUCC (complex II substrate), after delivery of rotenone and antimycin A (AA). C. ROS production during reverse electron flow; 4 animals per group and genotype were analysed in triplicate. *Statistical significance was calculated by Anova. Error bars shows S.E.M.
5.4. Discussion

Mammalian target of rapamycin (mTOR) and its downstream targets (*i.e.* S6K1) were shown to play a crucial role in regulation of lifespan [113]. It was shown by genetic manipulation as well as by treatment with rapamycin that inhibition of mTOR pathway extends lifespan of many animal models [113, 281-283]. Importantly, mTOR signalling was recognised to coordinate nutrient sensing in dietary restriction response and be responsible for a lifespan increase effect when food was limited [109]. Furthermore, inhibition of mTOR pathway by rapamycin was shown to protect the mouse brain against cognitive decline in normal aged C57BL/6J mice as well as in Alzheimer’s disease model (PDAPP mice) [284, 285].

The role of telomerase in ageing due to its telomere length related function has been previously established [175, 286]. However, its potential interaction with mTOR is poorly understood. Interestingly, it was shown that rapamycin inhibited growth of cervical cancer cells, leukaemia and endometrial cancer cells *in vitro* through reduction of the *hTERT* mRNA expression [287-289]. Strikingly, there are also reports suggesting that *hTERT* protein interacts physically with mTOR forming *hTERT*-mTORC1-RAPTOR complex in prostate cancer cells and NK (natural killer) cells which was suggested to influence telomerase activity [290, 291].

To test whether the mTert effect of dietary restriction in brain mitochondria might be regulated by the mTOR pathway, the level of mTOR phosphorylation (*i.e.* its activity) in tissue was tested. Interestingly, both dietary restriction and rapamycin treatment decreased the phosphorylation of mTOR in mouse brain. Similar results were shown previously in mouse liver, where short term dietary restriction and rapamycin supplementation decreased phosphorylation of S6K1 to the same extent [121]. This suggests that telomerase shuttling to mitochondria during dietary restriction and rapamycin supplementation might be regulated via the mTOR pathway.

The body weight of animals remained unchanged during our rapamycin supplementation study. It was shown by Miller *et al.*, (2011) that rapamycin reduces the body weight of mice, however the changes were observed after 5 months of supplementation and our study lasted for only 4 months [292]. Strikingly, the insulin level in serum at the end of the rapamycin experiment was significantly increased in mTert wild type animals (Figure 5.3), while the DR intervention attenuated insulin
levels in mouse plasma. This data is in agreement with reports from similar experiments on mice published recently, suggesting that DR and rapamycin might influence insulin metabolism by distant mechanisms [121, 293]. Glucose tolerance was not tested in our rapamycin experiment. However, Fok et al., (2013) showed that DR improved clearance of glucose from mouse blood while rapamycin supplementation significantly increased glucose levels in GTT [121]. In addition, recent data of Lamming and co-workers showed that rapamycin, apart from inhibiting the mTORC1 complex, also decreased the activity of the mTORC2 complex in vivo what resulted in decreased hepatic gluconeogenesis as an effect of increased insulin level [294]. Altogether, data from this study and others’ suggests that rapamycin might result in impaired liver function (high glucose and insulin), however, surprisingly, without affecting longevity [121, 294]. Furthermore, a recent study by Lamming et al., (2013) suggests that rapamycin influence on glucose and insulin metabolism may be dependent on the genetic background of mice. The authors show that in contrast to the study on C57BL/6 mice, heterogeneous HET3 mice exhibit glucose intolerance after rapamycin treatment, but insulin levels as well as insulin tolerance remain unchanged in young and old animals [295]. Interestingly, my results showed that telomerase seems to be involved in the rapamycin effect since the elevation of insulin levels was not observed in mTert-/- mice. Remarkably, absolute values of insulin in all groups in the rapamycin study were higher than levels measured in blood from the dietary restriction experiment. This result could probably be due to the collection of blood from fed animals which is not recommended, whereas the dietary restricted animals were tested after overnight fasting, hence data on insulin in our experiments cannot be compared since blood in the dietary restriction study was collected from fasted animals and in the rapamycin experiment blood was obtained after feeding of the mice.

The rapamycin supplementation experiment was performed on first generation mTert knock-out mice to understand the potential role of mTert protein in brain mitochondria during mTOR inhibition on parameters such as hydrogen peroxide generation and oxygen consumption. Although rapamycin is able to cross the blood-brain barrier, the level of the substance was shown to be two fold lower in brain than in blood after supplementation of mice [296]. There is no data so far about the effect of rapamycin on ROS generation in brain mitochondria. However,
rapamycin intervention was shown to significantly reduce ROS production in dendritic cells [297], in human corneal endothelial cells [298] and in lung carcinoma cells [299]. Furthermore, it has been shown in vitro and in vivo on mouse models that increased ROS production might elevate the activity of mTOR and rapamycin was potent to reverse this process and reduce oxidative stress [300, 301].

Interestingly, in our study rapamycin treatment was sufficient to reduce H$_2$O$_2$ generation linked to complex I (only with rotenone) and II in mouse brain mitochondria. Surprisingly, this effect was not observed in mitochondria isolated from $m$Tert/-$. Thus, these results suggest that telomerase accumulating in mitochondria might be a novel and important participant in mediating the effects of the mTOR pathway and its influence on mitochondria function. As shown in Chapter 4, mitochondria isolated from mouse brain after dietary restriction showed increased oxygen consumption after DR in basal and resting respiration states in the presence of pyruvate:malate. Interestingly this effect was not observed in $m$Tert/- mice. In the rapamycin supplementation experiment these changes were not observed. Stimulation of ETC complexes by pyruvate:malate and succinate resulted in no changes in O$_2$ consumption in isolated mitochondria in all tested groups. Furthermore, coupling of mitochondria (represented quantitatively by RCR values) remained also unchanged after rapamycin supplementation in both $m$Tert genotypes as it was observed after DR.

Rapamycin supplementation was potent to reduce ROS production in our experiment in the presence of pyruvate:malate with rotenone in $m$Tert wild type animals. Furthermore, beneficial effect of rapamycin treatment on ROS production was also observed in the presence of succinate (complex II blocker). Interestingly, these changes were not observed in $m$Tert knock-out animals. The most surprising result, however, was that control $m$Tert/- animals showed less ROS production than their wild type littermates in the presence of complex II inhibitor – succinate. This effect was not observed in the dietary restriction experiments. To clarify, there was a similar animal number in all groups of tested males in both experiments (DR and rapamycin treatment). The explanation might be the difference in age of the analysed animals in DR and rapamycin experiments or a methodological error in measuring of hydrogen peroxide. To elucidate this phenomenon further experiment would be required to assess what is the influence of $m$Tert status on H$_2$O$_2$ production in male
and female mouse brain mitochondria from different age groups. ROS production linked to complex III remained unchanged after rapamycin similarly to the results obtained after dietary restriction.

Our data did not answer clearly the question whether mTert protects mouse brain mitochondria under rapamycin treatment. However, we showed that telomerase might be potentially important in regulation of the response for rapamycin on ROS production. Different results from our dietary restriction and rapamycin study suggest that we not fully understand how much similarity there is between both treatments. Although published to date results from oxygen consumption studies are ambiguous, ROS generation was shown consistently to decrease after dietary restriction and rapamycin treatment in many models [77, 117, 302, 303].

Altogether, data from this part of my project suggests that rapamycin was able to mimic dietary restriction on mTOR phosphorylation. However, mitochondrial function in brain showed distinctive effect on mTert status. Furthermore, the influence of rapamycin and DR on liver function was also diverse. This study showed that mTert might be an important new player in the mTOR signalling pathway and its influence on mitochondrial function. Further research is required in order to elucidate how mTOR and rapamycin might influence male and female brain mitochondria since this study was conducted only on male mice. Moreover, experiments are required to elucidate what is the exact regulatory mechanism of coordination between mTOR and telomerase since both are known for their crucial role in ageing and senescence processes.
Chapter 6 - General discussion

Telomerase is a well-recognised key player in the ageing and senescence processes, mainly due to its canonical function – telomere elongation [194, 200]. However, there is a growing number of publications supporting the fact that the protein part of telomerase – TERT – shuttles from the nucleus as a response to stress and localises in mitochondria. This mitochondrial localisation was shown by various groups to have beneficial and protective effects against oxidative stress [61, 207, 209]. In my project I was aiming to show that TERT also localises in mammalian brain/neurons and that mitochondrial localisation of the protein has a beneficial effect on the organelle in vivo.

Research on mitochondrial telomerase to date was conducted mostly on human fibroblasts and cancer cell lines. Saretzki’s group was one of the first to show that shuttling of telomerase to mitochondria in hTERT overexpressing human fibroblasts has a protective effect against oxidative stress and apoptosis induced by H2O2 and etoposide [61]. More recently the same group showed in Singhapol et al., (2012) that mitochondrial telomerase also protects human cancer cells against nuclear DNA damage and apoptosis induced by oxidative stress and irradiation [218]. Furthermore, Haendeler et al., (2009) reported that telomerase binds to the mtDNA regions coding complex I genes (i.e. ND1 and ND2) leading to an increase of respiration efficiency and complex I activity. Moreover, the same group showed that binding of hTERT to mtDNA protects the mitochondrial DNA against damage caused by UV and ethidium bromide. In addition, mitochondrial TERT was responsible for increase of respiration efficiency in mice heart mitochondria and reduction of ROS production in HEK cells [206]. However, there was still no evidence whether hTERT is shuttling into brain mitochondria in vivo.

The first comprehensive data on the expression and role of telomerase in mammalian brain was published by Mark Mattson in the beginning of the last decade [223, 231, 304]. For example, Fu et al., (2000) showed that telomerase is present in mouse and rat brain and in cultivated primary rodent’s neurons [230]. Furthermore, the group of Han-Woong Lee also showed by in situ hybridisation the expression of mTert and mTerc in rat and mouse brain [232, 233]. Both mentioned groups as well as Caporaso et al., (2003) reported a detectable telomerase activity in whole brain or its particular regions (i.e. cerebellum, subventricular zone) of neonatal and adult
rodents [229]. However, mitochondrial localisation of mTert in mammalian brain was not discussed so far. Only recently Eitan et al., (2012) showed mTert protein expression in the cytoplasm of Purkinje neurons in mouse cerebellum, however, without discussing its exact localisation [226]. Using a new TERT ELISA method I found that the enzymatic component of telomerase is localised in mitochondria of mouse brain and liver. Furthermore, this cytoplasmic localisation was observed in normal condition, without application of any external stressor. To establish what type of brain cells contained telomerase, I isolated mouse embryonic neurons. I showed here that mTert localises in neurons and co-localises with mitochondria using an immunofluorescence method. Furthermore, staining of brain sections of mouse and human brain also confirmed that telomerase is expressed in the CNS and it localises outside nucleus and mostly in mitochondria. Moreover, abundance of mTert was significantly reduced with age in frontal cortex and cerebellum in mouse. These findings are in accordance to a recent publication by Eitan et al., (2012), where it was shown that mouse telomerase localises mostly in the nucleus as well as cytoplasm (not specified what organelle) of Purkinje neurons and that the abundance of the protein decreases with age [226]. Thus, telomerase appears to be a new potential factor in the ageing of brain. Moreover, due to its cytosolic localisation this new unknown role of TERT is clearly not related to telomere maintenance function.

To investigate a potential functional role of telomerase in brain, a dietary restricted mouse model was used. DR was shown by many groups to have beneficial effects on health and longevity in number of species. The DR regimen was shown to reduce oxidative stress in brain and improve neuronal mitochondria function [258, 303]. Furthermore, Mattson et al., (1999) had previously shown that restriction of nutrients causes mild stress effects in rodent neurons with increased levels of HSP-70 and GRP-78 proteins, what resulted in an increased expression of the neuroprotective trophic factors such as NGF [305-307]. This findings are in accordance with the theory of hormesis which suggest that physiological amount of ROS are required for normal function of the organelle and causes an adaptive and/or protective reaction against oxidative stress beneficial for longevity [308]. Thus we asked what would be the role of mTert in mitochondria in vivo during dietary restriction.

Recent experiments published by our group showed that telomerase under stress protect mitochondria against damaging agents such as reactive oxygen species.
In this study we demonstrated that mitochondrial hTERT decreased oxidative stress in human cancer cells [218]. In my experiments in mice in vivo the enzymatic part of telomerase – mTert was significantly increased in brain mitochondria after short term dietary restriction. This finding was confirmed in three independent DR experiments, while there was no increase of mTert amount in whole tissue homogenates. However, telomerase in liver mitochondria was not increased in any DR experiment, what might suggest a specific function for mTert in brain (for example protection of neurons by reducing ROS production). Surprisingly, telomerase activity and mTert gene expression in brain were decreased after DR that suggests mTert shuttled from the nucleus to mitochondria without de novo transcription on the gene. These findings were surprising for us since dietary restriction is expected to reduce oxidative stress; hence mTert content should be reduced in brain mitochondria. In conclusion, it means that there seem to be additional triggers for the TERT shuttling process to oxidative stress. Increase of telomerase in brain mitochondria as a response for nutrient limitation is shown here for the first time. Next, we asked what effect this accumulation of mTert might have on the organelle function.

Haendeler et al., (2009) showed that respiration in heart mitochondria in mTert−/− mice was significantly lower than mTert+/+. However, there was no difference between genotypes in liver mitochondria suggesting a tissue specific role of mTert in this organelle [206]. Interestingly, in our experiments there was no difference between mTert genotypes in respiration of brain mitochondria. Comparable results were obtained from two experiments on control animals in DR experiment and rapamycin study (i.e. ad libitum fed mice from the DR experiment and the control mice form the rapamycin study). Interestingly, dietary restriction increased oxygen consumption in brain mitochondria from mTert+/+ animals during basal and resting respiration state in the presence of pyruvate:malate (complex I substrate) and in the presence of complex III blocker antimycin A. However, there was no difference during active state 3 respiration (delivery of ADP to mitochondria) and in the presence of succinate – complex II substrate. This result suggest that mTert might improve complex I function by protection of mtDNA accordingly to the results from Haendeler at al., (2009) that TERT binds to mtDNA sequence coding subunits of complex I, namely ND1 and ND2 [206].

The changes in oxygen uptake by mitochondria during dietary restriction are under ongoing debate [263-266]. However, our results on brain mitochondria and recent findings of Hempenstall et al., (2012) suggest that DR causes an increase in
mitochondrial oxygen consumption [266]. Furthermore, since mitochondrial respiration decline with age [309] and DR was shown to protect the organelle against age related dysfunction [77], it would be expected that dietary regimen would increase oxygen consumption. However, the effect of DR on mitochondrial respiration might be species-, age-, gender-, and tissue specific. Furthermore, in our DR experiments there was no difference in H$_2$O$_2$ generation in brain mitochondria, neither between mTert genotypes or dietary groups. Although it was shown by others that DR reduces ROS production and oxidative stress in different mammalian tissues, recent findings of Radak et al., (2013) showed there is no difference in ROS production in rat brain between ad libitum and restricted groups [310]. Thus further experiments are required in order to establish the influence of DR on brain ROS production. Interestingly, supplementation of mice with rapamycin which is believed to mimic DR effect showed a reduction of hydrogen peroxide production linked to complex II of mitochondrial ETC.

Rapamycin was shown to slow ageing and to be potent to mimic some beneficial effects of dietary restriction due to a common regulation via mTOR pathway [119, 121, 246, 311, 312]. However, it was shown that rapamycin might increase liver degeneration, cataract severity, testicular degradation in mice and increase glucose intolerance [120, 313]. In our experiments dietary restriction and rapamycin show distinct effect on insulin content in mouse serum. While DR significantly reduced insulin levels, rapamycin significantly increased the content of this hormone. However, in the rapamycin experiment blood was collected from fed animals. Thus, the results cannot be comprehensively compared. Nevertheless, it was suggested by others that rapamycin might have detrimental effects on liver function [294]. Interestingly, a recent study by Lamming et al., (2013) suggests that rapamycin influence on glucose and insulin metabolism might be dependent on the genetic background of mice. The authors show that in contrast to the study on C57BL/6 mice, heterogeneous HET3 mice exhibit glucose intolerance after rapamycin treatment but insulin level as well as insulin tolerance remain unchanged in young and old animals [295]. Furthermore, in our DR study glucose tolerance was improved and although we did not analyse glucose tolerance in the rapamycin experiment it was show by others that inhibition of mTOR reduced the ability to clear glucose from the blood [294].

To test the hypothesis that mTert shuttling in brain is regulated by the mTOR pathway experiments with rapamycin supplementation were conducted on mTert wild type and knock-out mice. I have shown in this thesis that indeed mTOR activation was decreased in mouse brain after dietary restriction and after rapamycin treatment to a
similar extend. Similar mTOR pathway inhibition after DR and rapamycin were shown on mice liver by Fok and co-workers [121]. In addition, rapamycin treatment in our experiments also increased mTert protein in brain mitochondria and remained unchanged in whole organ homogenates similarly to the dietary restriction study. Telomerase activity was not tested in our rapamycin experiment. Nonetheless, it was shown on a different model by Zhou et al., (2003) that rapamycin is potent to decrease TA by reduction of hTERT transcription in cancer cells [288]. Interestingly, in our study rapamycin was also capable to reduce hydrogen peroxide production linked to complex II of ETC in brain and this phenomenon was dependent on the mTert status. Brain mitochondria from mTert/- animals did not show any difference in the generation of H$_2$O$_2$ as a response for rapamycin. These findings suggest that telomerase might be a new player in the mTOR pathway and for ROS production in brain mitochondria. The oxygen consumption rates were unchanged in brain mitochondria from both mTert genotypes after the mTOR pathway inhibition by rapamycin in all tested respiration states. However, Schieke et al., (2006) showed that rapamycin disrupted the complex of mTOR and raptor in vitro in cancer cells what resulted with reduced oxygen consumption [117].Surprisingly, in the rapamycin experiment we found that brain mitochondria from mTert/- animals produced less ROS than mTert+/. These findings are in contrast to our results from the control group (AL) from dietary restriction experiment where no differences between mTert genotypes were observed. Moreover, Haendeler et al., (2009) showed that knock-down of mTert increased significantly ROS production in vitro [206].

Since only male animals were tested in both experiments (DR study and rapamycin treatment), I was interested in analysing mitochondria isolated from female mice brain. Interestingly, mitochondria from female brains generated less H$_2$O$_2$ in mTert wild type animals when compared to the knock-out littermates, supporting a protective role of telomerase in females but not in males. Furthermore, this protective effect was observed in young and old animals. Remarkably, the difference between male and female brain ageing is under extensive research [314]. It was suggested that sex hormones influence brain physiology and are important factors during stem cells differentiation into neurons [315]. For example it was shown by Diaz et al., (2007) that there is gradual increase in expression of estrogen and progesterone receptors genes during differentiation of stem cells into neurons [316]. Thus, it could be beneficial to analyse for example estrogen receptor $\alpha$ (ER$\alpha$) level in mouse brain from different mTert genotype after DR and test its correlation with ROS levels. Furthermore, there are data
showing gender dependent response to DR. For example, it was shown that dietary restriction reduces ROS production in cardiac mitochondria significantly higher in rat females than in males [268]. Finally, estrogen was shown to reduce ROS production and oxidative stress in brain of rat female as well as male supplemented with the hormone [272].

Protection against oxidative stress was shown to improve neuromuscular coordination in mouse brain [262]. Interestingly, in this project mTert was shown to be efficient in protection against age related decline in neuromuscular coordination. Dietary restricted animals from the mTert/- genotype were performing significantly worse that their wild type littermates while there was no significant difference between genotypes in the ad libitum group. These data strongly support the hypothesis that telomerase preserves neuromuscular coordination ability of ageing mice and the mTert protein might play a role in the dietary restriction response. However, brain function per se (i.e. spatial memory and learning) needs to be tested by more specific methods such as Barnes maze, which tests the ability of rodents to learn and remember the location of a target zone. Interestingly, the protective function of DR on neuromuscular coordination might be also dependent on the mTOR pathway. It was shown that supplementation of mice with rapamycin significantly improved cognitive function of the brain like learning and memory [284].

To further develop this project there are still areas which should be investigated. Further experiments on ROS generation and oxidative damage in mouse brain mitochondria from different genotypes of mTert male and female animals are necessary. Besides, it would be interesting to elucidate what is the exact pathway of telomerase shuttling into mitochondria in vivo. Is it mTOR as suggested in this thesis? It was proposed that research on the inactivation of mTOR by rapamycin is more robust when the phosphorylation of downstream targets like S6 kinase or 4EBP is probed instead of mTOR itself [121, 284]. Furthermore, a comprehensive analysis of DNA damage in telomeric regions in brains from mTert wild type and knock-out mouse would deliver evidence whether the observed results are independent from the telomere-dependent role of telomerase. Since our group and others showed that mitochondrial localisation of hTERT reduced mtDNA damage, the question is what level of mtDNA damage could be observed in brain when mTert is deleted and what effect it might have on brain function [206, 218, 219]. Finally, our results might be used to investigate a potential role of telomerase in neurodegenerative diseases.
example, it was shown recently by Eitan et al., (2012) that telomerase has a protective effect on brain in an amyotrophic lateral sclerosis mouse model. In this study chemical activator of telomerase (AGS-499) was shown to be effective in the delay of the disease onset and progression as well as survival. Furthermore, increased expression of mTert protected motor neurons against oxidative stress and apoptosis [198].

To conclude, telomerase was shown here to be an important new player in the response to the dietary restriction and mTOR inhibition. It was shown that TERT co-localises with mitochondria in mouse and human brain. Moreover, I showed here for the first time that mTert accumulates in brain mitochondria during short term dietary restriction. Interestingly, the same accumulation of mTert was observed after rapamycin supplementation that together with similar inhibition of mTOR in both treatments shows a close interaction between telomerase and the mTOR pathway (Figure 6.1). However, DR and rapamycin showed distant effect on brain mitochondria function. While restriction of nutrients increased oxygen consumption linked to complex I of ETC, rapamycin reduced generation of hydrogen peroxide linked to complex II. Moreover, whereas DR significantly reduced insulin level in mouse serum, rapamycin seemed to impair liver function what resulted in increased insulin level. Finally, mTert and DR showed beneficial, age dependent effect on brain function in neuromuscular coordination test what might suggest that mTert could be a possible new therapeutic target in neurodegenerative diseases like Alzheimer’s or Parkinson’s.
Figure 6.1. Summary of the results from dietary restriction and rapamycin experiment. Both interventions have no influence on total mTert protein in whole brain homogenates while result with significant increase of protein abundance in brain mitochondria. Mechanistically the observed effect might be correlated with the decrease in mTor phosphorylation. Dietary restriction resulted with increased oxygen consumption in isolated mitochondria and rapamycin decreased hydrogen peroxide production in these organelles.
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Appendix: Abstracts of publications

1. Article in *Impact Ageing* journal, September, 2010

**Article title:** Adult-onset, short-term dietary restriction reduces cell senescence in mice  

**Authors:** Chunfang Wang, Mandy Maddick, Satomi Miwa, Diana Jurk, Rafal Czapiewski, Gabriele Saretzki, Sabine A.S. Langie, Roger W.L. Godschalk, Kerry Cameron, Thomas von Zglinicki  

**Abstract:**
Dietary restriction (DR) extends the lifespan of a wide variety of species and reduces the incidence of major age related diseases. Cell senescence has been proposed as one causal mechanism for tissue and organism ageing. We show for the first time that adult-onset, short-term DR reduced frequencies of senescent cells in the small intestinal epithelium and liver of mice, which are tissues known to accumulate increased numbers of senescent cells with advancing age. This reduction was associated with improved telomere maintenance without increased telomerase activity. We also found a decrease in cumulative oxidative stress markers in the same compartments despite absence of significant changes in steady-state oxidative stress markers at the whole tissue level. The data suggest the possibility that reduction of cell senescence may be a primary consequence of DR, which in turn may explain known effects of DR such as improved mitochondrial function and reduced production of reactive oxygen species.

2. Article in *PlosONE* journal, January, 2013

**Article title:** Mitochondrial telomerase protects cancer cells from nuclear DNA damage and apoptosis  

**Authors:** Chatchawan Singhapol; Deepali Pal; Rafal Czapiewski; Mahendar Porika; Glyn Nelson; Gabriele Saretzki  

**Abstract:**
Most cancer cells express high levels of telomerase and proliferate indefinitely. In addition to its telomere maintenance function, telomerase also has a pro-survival function resulting in an increased resistance against DNA damage and decreased apoptosis induction. However, the molecular mechanisms for this protective function remain elusive and it is unclear whether it is connected to telomere maintenance or is
rather a non-telomeric function of the telomerase protein, TERT. It was shown recently that the protein subunit of telomerase can shuttle from the nucleus to the mitochondria upon oxidative stress where it protects mitochondrial function and decreases intracellular oxidative stress.

Here we show that endogenous telomerase (TERT protein) shuttles from the nucleus into mitochondria upon oxidative stress in cancer cells and analyzed the nuclear exclusion patterns of endogenous telomerase after treatment with hydrogen peroxide in different cell lines. Cell populations excluded TERT from the nucleus upon oxidative stress in a heterogeneous fashion. We found a significant correlation between nuclear localization of telomerase and high DNA damage, while cells which excluded telomerase from the nucleus displayed no or very low DNA damage. We modeled nuclear and mitochondrial telomerase using organelle specific localization vectors and confirmed that mitochondrial localization of telomerase protects the nucleus from inflicted DNA damage and apoptosis while, in contrast, nuclear localization of telomerase correlated with higher amounts of DNA damage and apoptosis.

It is known that nuclear DNA damage can be caused by mitochondrial generated reactive oxygen species (ROS). We demonstrate here that mitochondrial localization of telomerase specifically prevents nuclear DNA damage by decreasing levels of mitochondrial ROS. We suggest that this decrease of oxidative stress might be a possible cause for high stress resistance of cancer cells and could be especially important for cancer stem cells.

3. Oral communication on Neuroscience North-East Conference, Durham 2011

Talk title: Mitochondrial TERT protects neurons and increases in brain during dietary restriction via TOR pathway

Abstract:
The central nervous system is particularly exposed to oxidative stress due to high energy demand and high oxygen consumption rate. It was shown that reactive oxygen species (ROS) - products of respiration - play a crucial role in development and progress of neurodegenerative diseases in animal models as well as in human. Hence research for factors that protects brain and neurons in particular seems to be high priority.
It appears that one of important neurons protectors might be telomerase – an enzyme consisting two major components TERT (protein) and TERC (RNA) best known for its function in telomere maintaining. However, recently it was shown that TERT has telomere- and TERC-independent functions.

Our lab was one of the first to show that telomerase shuttles to mitochondria improving their function and decreasing cellular oxidative stress. Oxidative stress and mitochondrial dysfunction are well known to increase during ageing and have been implicated as a cause for age-related neurodegenerative diseases. We now demonstrate that using targeted localisation of TERT into mitochondria but not nuclei significantly decreases mitochondrial ROS levels under basal and stressed conditions. Although telomerase activity is negligible in brain we found considerable amounts of the telomerase protein TERT in mouse and human brain. Immunohistochemistry showed extra nuclear localisation of TERT in neurons from human cortex, hippocampus and cerebellum. Moreover, TERT was co-localised by fluorescence immunocytochemistry with the mitochondrial marker COXII in isolated neurons from E16.5 mouse as well as in human brain sections.

Dietary restriction (DR) has been shown to ameliorate hallmarks of neurodegenerative diseases such as Alzheimer's disease and cognitive decline including hyperphosphorylated tau protein deposits and amyloid. To test how DR influences TERT we conducted three independent short term DR experiments on mice. We found that TERT protein resides in mouse brain mitochondria already in control animals and increases in brain tissue from 3 independent DR experiments. Furthermore, we demonstrate that ROS generation is significantly decreased in brain mitochondria from DR mice. Decreased signalling through mTOR has been described as a major mechanism of DR. Accordingly; we found that mTOR phosphorylation was significantly down regulated in brains from DR animals. These findings were confirmed by rapamycin (TOR inhibitor) treatment of cells. Moreover, rapamycin increased the extra nuclear TERT amounts while decreasing intracellular oxidative stress. We conclude that down-regulation of mTOR is a possible mechanism to increase TERT protein levels within mitochondria under DR.
Finally, our preliminary data shows that neurons from human hippocampus and frontal cortex with hyperphosphorylated tau protein do not express TERT and vice versa. Together, our data suggest that mitochondrial TERT can protect neurons and improve mitochondrial function and delay or prevent age related neurodegenerative diseases.

4. Oral communication on BSRA meeting, Birmingham 2012
Talk title: Does mitochondrial TERT protect the brain?
Abstract:
Dietary restriction (DR) is the only manipulation proven to increase lifespan in wide range of animal models such as yeast, worms, flies and mammals. The exact mechanism for the delay of is not clear yet. It has been shown that DR significantly reduces the reactive oxygen species production, improves mitochondrial function and seems to delay cell senescence in mammals.

Telomerase is an enzyme well known for protecting and maintaining telomeres consisting of the catalytic subunit TERT and the RNA component TERC. Recently our group showed that telomerase protects mitochondria under oxidative stress. We hypothesise that mitochondrial TERT could play a role for the beneficial effects of DR in vivo in mice.

We did not find telomerase activity (using TRAP PCR) in isolated mitochondria but high levels in homogenates of whole organs. In contrast, the mitochondrial amount of TERT was significantly higher in the mitochondrial fraction than in tissue homogenates. These results suggest that only TERT but not TERC localised to mitochondria.

By using TERT ELISA assay (GenWay) we measured TERT protein level in mitochondrial fractions from liver, brain and muscle in three independent short term DR experiments. We found highly significant increase in TERT protein abundance in brain mitochondria under DR in all three experiments. There is also clear tendency of increase in TERT protein in mitochondria and homogenates in liver.

We suggest that catalytic component of telomerase might be involved in mitochondrial protection and that this effect is increased under conditions of dietary restriction and therefore contribute to the beneficial health effects of DR.