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Investigating the Protective Effects of Telomerase Reverse Transcriptase on Neuronal Metabolism and Resistance to Amyloid-beta

Olivia Singh, *The University of Western Ontario*

Supervisor: Dr. Robert C. Cumming, *The University of Western Ontario*

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Abstract

Maintenance of telomere length during cell division is dependent on the catalytic subunit telomerase reverse transcriptase (TERT), which adds TTAGGG repeats to the ends of chromosomes to prevent telomere shortening during DNA replication. However, non-telomeric roles of TERT have emerged under oxidative stress whereby TERT translocates from the nucleus to the mitochondria and protects against mitochondrial dysfunction through a poorly defined mechanism. A major pathological feature of Alzheimer's Disease (AD) is the progressive accumulation of amyloid-beta (Aβ) peptide within the cortex and hippocampus. Aβ can directly interfere with mitochondrial respiration and promote mitochondrial dysfunction, ROS production, and neuronal cell death. A shift in metabolism away from oxidative phosphorylation towards aerobic glycolysis has been shown to confer neuroprotection against Aβ toxicity. Hence, it was hypothesized that elevated TERT expression can protect neurons from mitochondrial dysfunction and Aβ toxicity via metabolic reprogramming. TERT overexpression in the neuronal cell line HT22 promoted a shift towards aerobic glycolysis by altering levels of glycolytic enzymes following exposure to H_2O_2 and Aβ. Furthermore, TERT overexpression decreased mitochondrial ROS levels and improved cell viability during oxidative stress. Following exposure to H_2O_2 and Aβ, TERT translocation to the cytoplasm and mitochondria was observed. The results from this study suggest that TERT may be a therapeutic target for potential treatment of AD.

Keywords

telomerase reverse transcriptase, aerobic glycolysis, Warburg effect, metabolism, mitochondria, reactive oxygen species, oxidative stress, amyloid-beta, Alzheimer's Disease

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List of Abbreviations

AAV	adeno associated virus vectors
ABAD	amyloid-beta binding alcohol dehydrogenase
acetyl-CoA	acetyl coenzyme A
ADP	adenosine diphosphate
ALS	amyotrophic lateral sclerosis
APP	amyloid precursor protein
ATP	adenosine triphosphate
Aβ	amyloid-beta
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
BDNF	brain derived neurotrophic factor
c-Abl	Abelson murine leukemia viral oncoprotein
CHIP	C terminus of HSC70-Interacting Protein
CRM1/XPO1	nuclear export receptor chromosomal maintenance 1/exportin 1
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
E-boxes	enhancer-boxes
embryonic day	E#
ETC	electron transport chain

FADH2	flavin adenine dinucleotide
FBS	fetal bovine serum
GCL	glutamate cysteine ligase
GPx	glutathione peroxidase
H_2O_2	hydrogen peroxide
hEST1A/hEST1B	human homologs of the yeast essential telomerase proteins (EST)
HIF-1α	hypoxia inducible factor 1 alpha
Hsp90	heat shock protein 90
IFN-α	interferon alpha
IMM	inner mitochondrial membrane
JNK	Jun NH_2-terminal kinase
LDHA	lactate dehydrogenase A
Mad	Max associated protein D
Max	c-Myc/Myc associated factor X
MKRN1	Makorin Ring Finger Protein 1
MOMP	mitochondrial outer membrane permeability
MPC	mitochondrial pyruvate carriers
mtDNA	mitochondrial DNA
mTOR	mechanistic target of rapamycin
MTS	mitochondrial targeting sequence

MZF-2	myeloid-specific zinc finger protein 2
NADH	nicotinamide adenine dinucleotide
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	N-methyl-D-aspartate
NT-3	neurotrophin-3
O_2^-	superoxide
OH^-	hydroxyl radical
OMM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation
P/S	penicillin/streptomycin
p66Shc	66-kilodalton isoform of the adaptor protein Src homology 2 domain
PBS	phosphate buffer saline
PDH	pyruvate dehydrogenase
PDK1	pyruvate dehydrogenase kinase 1
PEP	phosphoenolpyruvate
PET	positron emission tomography
PFK	phosphofructokinase
PGC-1α/PGC-1β	peroxisome proliferator-activated receptor gamma coactivator-1 alpha/beta
PK	pyruvate kinase
PKCα	alpha isoform of protein kinase C

postnatal day	P#
POT1	protection of telomeres protein 1
PPP	pentose phosphate pathway
pRB	retinoblastoma protein
Prx1	peroxiredoxin 1
PTP	permeability transition pore
Ran GTPase	GTP-binding nuclear protein Ras-related nuclear protein
RdRP	RNA-dependent RNA polymerase
RMRP	mitochondrial RNA processing endoribonulease
ROS	reactive oxygen species
RT	reverse transcriptase
SOD	superoxide dismutase
Sp1	specificity protein 1
TCA	citric acid cycle
TERC	telomerase RNA component
TERT	telomerase reverse transcriptase
TGF-β	transforming growth factor beta
TIM23	translocase of the inner membrane
TIN2	TRF1-interacting nuclear factor 2
TNFα	tumor necrosis factor alpha

TOM 20/40	translocases of the outer membrane 20/40
TPP1	human homolog of yeast RAP1
TRF1/2	telomeric repeat binding factor 1/2
TrkB	tropomyosin receptor kinase B
UCP2	uncoupling protein 2
WT1	Wilms' tumor 1

Chapter 1: Introduction and Literature Review

1.1 Telomerase and TERT

1.1.1 *Telomerase and associated proteins*

Telomeres are specialized structures located on the ends of chromosomes that are comprised of double stranded guanine (G)-rich hexanucleotide tandem repeats (TTAGGG) followed by a single stranded TTAGGG overhang. The primary function of telomeres is to prevent linear DNA from being recognized as a broken end, thereby preventing DNA end-to-end joining and DNA degradation[1]. However, DNA polymerases are unable to replicate the extreme ends of the 5' strand during DNA replication resulting in a progressive loss of telomeres from chromosomes after each cell division. This phenomenon is known as the end-replication problem[2]. Consequently, the shortening of telomeres to a critical length, termed the Hayflick limit, leads to a loss of proliferation and initiation of cellular senescence[3,4]. Telomere attrition has been used as a biomarker for aging and is associated with many age-related diseases such as compromised immune function, various cancers, cardiovascular diseases and cognitive disorders[4–6].

Fortunately, telomere length can be preserved by the eukaryotic protein telomerase. Telomerase is a dimeric protein comprised of two main subunits; telomerase RNA component (TERC) and telomerase reverse transcriptase (TERT)[7]. Telomerase recognizes the hydroxyl group at the telomeric 3' overhang and uses the TERC RNA template to catalytically add TTAGGG nucleotide repeats to telomeres[7]. In addition, the maintenance of telomeres relies on the shelterin complex which is required for the protection and stability of telomeres in a conserved T-loop configuration[8]. The shelterin complex is composed of six major subunits: telomeric repeat binding factor 1 and 2 (TRF1, TRF2), TRF1-interacting nuclear factor 2 (TIN2), protection of telomeres protein 1 (POT1), TPP1 and the human homolog of yeast RAP1 (RAP1)[8]. Telomere elongation is dependent upon the structure of the telomere itself. Longer telomeres attract more shelterin complexes which maintains the T-loop structure and enhances POT1 binding to

inhibit telomerase activity, while shorter telomeres surrounded by less shelterin are in a more open linear configuration that favours the recruitment of telomerase by TPP1[9].

TERT expression and activity is high in cells undergoing extensive proliferation such as germ line cells of the testis, embryonic stem cells or malignant cancer cells[10]. In contrast, TERT is downregulated in most post-mitotic somatic cells[11]. Expression of telomerase in adulthood is generally restricted to cells in highly regenerative tissues, such as lymphocytes in bone marrow and peripheral blood, epithelial cells in the skin, hair follicle, intestinal mucosa and endometrium, and adult stem cells[12–16].

1.1.2 *Telomerase reverse transcriptase (TERT)*

Since the reactivation of TERT expression in somatic cells is a key step in cancer cell transformation, TERT is the most extensively studied telomere-associated protein. Human TERT (hTERT) is a 127kDa protein encoded by 1132 amino acids and consists of three main domains: the ~400 amino acid N-terminal region, a central region containing reverse transcriptase (RT) motifs and the ~200 amino acid C-terminal region[17]. The N-terminal region contains the highly conserved GQ motif which is essential for telomere maintenance, followed by a non-conserved linker sequence which is proposed to be important for nucleic acid binding, and conserved CP, QFP and T motifs responsible for binding to TERC[18]. The RT domain consists of 7 conserved motifs that contain three critical aspartic acid residues needed for enzymatic activity, termed the catalytic triad, and regions needed for nuclease activity, nucleotide binding, rNTP/dNTP discrimination and nucleotide addition[18]. The C-terminal region is proposed to be involved in nucleotide addition and processivity[18].

1.1.3 *Transcriptional/post-translational regulation of TERT*

Transcriptional regulation of TERT is important for determining a proliferative or post-mitotic phenotype in cells. The ~14kb hTERT gene consists of 16 exons and 15 introns located on the small arm of chromosome 5 (5p15.33) as a single copy[19]. Although expression of full length hTERT is required for telomerase activity, TERT mRNA can be alternatively spliced to generate α-TERT which contains a deletion in exon 6 and β-

TERT which has a complete deletion of exon 7 and 8[20]. The hTERT promoter is GC-rich with a CpG island 330 bp upstream of the ATG start codon[21]. The hTERT promoter contains specific domains for transcription factors that promote hTERT expression, such as two enhancer-boxes (E-boxes) for c-Myc/Myc associated factor X (Max) binding, five GC-boxes for binding of specificity protein 1 (Sp1) which cooperates with c-Myc, and estrogen response elements located more than ~950 bp upstream of the ATG[22,23]. More importantly, transcriptional repression of hTERT can be regulated by the Max/Max associated protein D (Mad) complex which competitively binds to E-boxes[22]. Another transcriptional repressor of hTERT is the cell cycle arrest and tumor suppressor protein p53 which interacts with Sp1 to prevent binding to the hTERT promoter[24]. The transcription factor E2F-1 can bind to the promoter of hTERT to promote expression, however this effect can be abolished with the expression of retinoblastoma protein (pRB) which recruits histone deacetylases to promoter-bound E2F-1[25]. Other repressors of hTERT transcription is the tumor suppressor Wilms' tumor 1 (WT1) and myeloid-specific zinc finger protein 2 (MZF-2)[26,27]. In addition, proteins involved in cell cycle arrest and differentiation can also inhibit hTERT expression such as interferon alpha (IFN-α) and transforming growth factor beta (TGF-β). Moreover, certain naturally occurring or synthetic chemicals such as of vitamin D_3, retinoic acid and dimethyl sulfoxide (DMSO) can repress hTERT expression[28–31].

Post translational regulation of hTERT occurs through protein interactions, phosphorylation, and ubiquitination. The p23 foldasome chaperone binds to the N-terminal region of hTERT and recruits heat shock protein 90 (Hsp90), both of which are required for assembly of active telomerase with TERC and are the only proteins that remain associated with active telomerase after assembly[32]. In addition, human homologs of the yeast essential telomerase proteins (EST), hEST1A and hEST1B, can bind to hTERT and contribute to telomere maintenance and protection[33]. Other proteins shown to bind to hTERT to enhance telomerase activity are the DNA-PKC interacting protein KIP which can bind to amino acids 762-855, and Ku which links hTERT with TRF1 and TRF2[34,35]. A negative regulator of telomerase is the nucleolar TRF1- binding protein PinX1, which binds to and sequesters hTERT thereby inhibiting interaction with TERC and preventing activation[36]. While phosphorylation of hTERT at the N-terminal region

by proteins such as the alpha isoform of protein kinase C (PKCα) contributes to active telomerase, phosphorylation by Abelson murine leukemia viral oncoprotein (c-Abl), phosphatase 2A, and PKCζ proteins inhibit telomerase activity[37]. The ubiquitin ligase Makorin Ring Finger Protein 1 (MKRN1) has also been reported to associate with the C-terminal region of hTERT to promote degradation[38]. Other ubiquitin ligases that can negatively regulate TERT activity is C terminus of HSC70-Interacting Protein (CHIP) and Hdm2 (E3 Ligase)[39,40].

1.1.4 *TERT expression in the brain*

TERT activity is generally limited by its restricted expression and is generally downregulated in most post-mitotic cells. However, TERT has unusual expression patterns in the brain, irrespective of telomerase activity. Telomerase activity is high during embryonic and early postnatal development, but declines shortly thereafter and is undetectable in adult brains[41,42]. When mouse brain extracts corresponding to the cortex, hippocampus, and brain stem were examined, telomerase activity peaked in all regions at embryonic day 13 (E13), declined to low levels by E18, and was undetectable by postnatal day 10 (P10)[43]. In contrast, high TERT mRNA levels persist until P5 and low levels can be detected in the same regions of the adult brain[43]. By E18 the hippocampus contains fully differentiated post mitotic neurons and upon further analysis, it was found that TERT is expressed primarily in hippocampal pyramidal neurons and cortical neurons but not in astrocytes. In addition, experiments in which TERT expression was either increased or decreased demonstrated that the protein plays a pivotal role in suppressing apoptosis induced by trophic factor withdrawal well before mitochondrial dysfunction or caspase activation[44]. Since access to trophic factors determines neuronal survival during development, it has been proposed that TERT regulates synaptic pruning and neuronal differentiation during early postnatal brain development[43,44].

In the adult brain, telomerase activity can be detected in areas with high proliferation, such as the subventricular zone of the lateral ventricle, a region which contains neural stem cells[45]. In addition, TERT expression has been detected in the dentate gyrus of the hippocampus and the glomeruli, mitral/tufted cell layer, and the olfactory granule cell

layer of the olfactory bulb, independent of TERC expression[46]. Interestingly, TERT may be important for regulating normal brain functions since TERT knock out mice display altered anxiety-like behaviours and abnormal olfaction[46]. TERT has also been detected in the cerebellum, specifically in the nucleus and cytoplasm of Purkinje neurons in adult and old mice[47].

1.2 Non-canonical functions of TERT

1.2.1 *Non-canonical functions of TERT in the nucleus*

Several studies have suggested that TERT may have functions independent of telomere extension. TERT overexpression has been reported to promote tumorigenesis by influencing the expression of genes involved in cell growth and proliferation, independent of telomere elongation[48]. Furthermore, downregulating TERT expression dramatically increases apoptotic cell death well before detectable telomere shortening[49]. Studies in TERT-deficient mouse models have revealed that TERT can regulate survival, tumorigenesis, stem cell properties and gene regulation independent of changes in telomere length[50,51]. TERT may also play a critical role in maintaining healthy mitochondria, as restricting TERT localization exclusively to the nucleus increases telomeric and extra-telomeric DNA damage, reactive oxygen species (ROS) production and mitochondrial dysfunction[52].

Apart from TERT's canonical function as a subunit in telomerase, non-canonical functions of TERT within the nucleus have been reported. Nuclear import of TERT is dependent on a bipartite nuclear localization signal residing within amino acid residues 222-240 and by phosphorylation on serine 227 by the Akt serine/threonine kinase[53]. Another mechanism of nuclear import is through the interaction of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) with TERT in the cytosol, which is recognized by tumor necrosis factor alpha (TNFα) leading to nuclear translocation[54]. In the nucleus, NF-κB can induce TERT expression which can then act as a transcriptional modulator in the NF-κB signaling pathway by binding to p65 and p50 to influence transcription of NF-κB target genes regulating inflammation and cancer progression[55,56]. TERT is also known to bind to the SWI/SNF-related chromatin remodelling protein

BGR1 to regulate genes involved in proliferation and stem cell renewal via the Wnt/β-catenin signalling pathway[57]. In addition, TERT may be responsible for the maintenance of chromatin structure and plays a role in the response to DNA double strand breaks[58,59]. Moreover, TERT may participate in telomere capping to stabilize telomeres and increase lifespan, independent of telomere lengthening[60,61].

1.2.2 *Properties and functions of mitochondria*

Surprisingly, many non-canonical functions of TERT have been reported as a result of its mitochondrial localization. The mitochondria are cytoplasmic organelles that play a vital role in maintaining cellular homeostasis and energy production. The structure of this organelle consists of the mitochondrial matrix surrounded by the inner mitochondrial membrane (IMM) organized in the form of folds or cristae. The IMM is separated from the outer mitochondrial membrane (OMM) by the intermembrane space. The mitochondria has key functions in producing adenosine triphosphate (ATP) and ROS, initiating apoptosis by releasing proteins that activate caspases, and controlling intracellular calcium concentrations[62].

Also termed the “powerhouse” of the cell, the mitochondria are essential for producing ATP via oxidative phosphorylation (OXPHOS) to meet cellular energy demands. After glucose enters the cell via GLUT transporters, it is metabolized to pyruvate through a series of enzyme catalyzed reactions collectively terms glycolysis[63]. Pyruvate is actively shuttled to the mitochondria through mitochondrial pyruvate carriers (MPC), where it is converted to acetyl coenzyme A (acetyl-CoA) by the enzyme pyruvate dehydrogenase (PDH) to act as the first substrate within the citric acid cycle (TCA)[63]. The high-energy reduced products from the TCA, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2), are subsequently oxidized in the electron transport chain (ETC), consisting of five complexes named I-V that are located in the IMM[63]. The transfer of electrons through complexes I-V transports protons across the IMM to the intermembrane space, resulting in a membrane potential across the IMM. The electrochemical proton gradient across the IMM drives protons back into the matrix through complex V (ATP synthase), which uses that energy to convert adenosine

diphosphate (ADP) to ATP[63]. The entire process produces a net of 34 ATP molecules per oxidation of one glucose molecule compared to a net of 2 ATP molecules per glucose molecule produced during glycolysis[63]. However, complexes I-III can sometimes leak electrons resulting in the production of oxygen radicals such as superoxide (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2)[64]. Low levels of ROS may be beneficial to the cell, as ROS can be used as a signalling molecule that initiates signalling cascades important for the redox balance of a cell. Conversely, excessive ROS can lead to oxidative stress characterized by damage to cell structures, DNA, lipids and proteins[65]. Levels of intracellular ROS can be controlled by a variety of antioxidant enzymes such as catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPx)[66].

The mitochondria also play a key role in initiating cell death via the intrinsic apoptosis pathway. Apoptosis is regulated by the activation of a family of cytosolic caspase proteases, which cleave cellular contents to initiate programmed cell death[67]. The intrinsic pathway of apoptosis relies heavily on mitochondrial components and the release of cytochrome C, which is a part of the apoptosome complex and can initiate the activation of caspases[68]. The anti-apoptotic B-cell lymphoma 2 (Bcl-2) proteins reside in the OMM and maintain mitochondrial outer membrane permeability (MOMP) to block cytochrome C release[69]. However, pro-apoptotic proteins such as Bcl-2-associated X protein (Bax), which is a subset of proteins from the Bcl-2 family, can translocate from the cytosol to the mitochondria, undergo oligomerization thereby promoting changes in MOMP to release cytochrome C from the intermembrane space to the cytosol[69]. The translocation of Bax to the mitochondria is considered a key upstream initiator of intrinsic apoptosis[69].

Calcium regulation is another important function of the mitochondria. Calcium acts as a signalling molecule that has spatially and temporally complex functions, some of which include roles in metabolism, secretion, muscle contraction, gene expression, and proliferation[70]. Typically, the cytosol maintains a very low concentration of Ca^{2+} with the aid of Ca^{2+} pumps and transporters[70]. The mitochondria can act as a buffer and respond to Ca^{2+} influx to the cytosol by providing the rapid removal and storage by

uptake of excess Ca^{2+} [70]. In neurons, calcium signalling plays a vital role in signal transmission, excitability, and synaptic plasticity[70].

1.2.3 *TERT localizes to the mitochondria under oxidative stress*

Under normal conditions, approximately 10-20% of the total cellular TERT is localized to the mitochondria[71]. However, oxidative stress has been shown to trigger increased TERT accumulation in the mitochondria; an event linked to mitigation of mitochondrial dysfunction[72,73]. Nuclear exclusion occurs in as little as 45 mins after oxidative stress treatment, reaching a maximum exclusion level of 60% after 24 hours and can persist up to 5 days, depending on the cell type[74]. TERT phosphorylation on tyrosine 707 by the proto-oncogene Src kinase triggers nuclear export by promoting association with GTP-binding nuclear protein Ras-related nuclear protein (Ran GTPase) and the nuclear export receptor chromosomal maintenance 1/exportin 1 (CRM1/XPO1)[75]. Alternatively, nuclear export of TERT can be negatively regulated by the protein tyrosine phosphatase Shp-2, which is known to antagonize the activities of the Src kinase family[76,77]. Nuclear export can also be inhibited by the 14-3-3 signaling protein which interferes with CRM1/XPO1 binding to the nuclear export signal located at the C-terminus of TERT[78]. After successful nuclear export, cytosolic TERT is directed to the mitochondria via a mitochondrial targeting sequence (MTS), which is encoded within the first 20 amino acids[79]. TERT interacts with mitochondrial transport protein complexes translocases of the outer membrane 20/40 (TOM 20/40) and translocase of the inner membrane (TIM23) to cross the mitochondrial membranes and enter the mitochondrial matrix[80,81].

1.2.4 *TERT has catalytic activity in the mitochondria*

In the mitochondria, TERT can have reverse transcriptase activity even in the absence of TERC. TERT interacts with mitochondrial tRNAs to synthesize cDNA, suggesting a role in mitochondrial DNA (mtDNA) replication or repair[71]. Conversely, TERT has been shown to have a novel RNA-dependent RNA polymerase (RdRP) activity that is independent of its DNA reverse transcriptase function, and is the only RdRP identified in mammals[82]. TERT forms a complex with the mitochondrial RNA processing endoribonulease (RMRP) and is able to produce siRNAs from double-stranded RNAs,

which can influence transcriptional or post-transcriptional gene silencing of the RMRP gene[82].

1.2.5 *TERT prevents mitochondrial dysfunction under oxidative stress conditions*

Recent studies have demonstrated that mitochondrial localization of TERT contributes to the proliferative and survival properties of cancer cells[79,83]. Under oxidative stress conditions, TERT prevents mtDNA oxidative damage and degradation, decreases mitochondrial superoxide production and ROS levels and maintains a high mitochondrial membrane potential[73,74,77,80,81]. These effects on mitochondrial function may indirectly protect against nuclear DNA damage and apoptosis in response to oxidative stress[74,80]. A possible mechanism for TERT-mediated reduction in ROS production and suppression of apoptosis is by decreasing ROS-induced cytosolic acidification and blocking translocation of Bax to the mitochondria, which is critical for pro-apoptotic signalling[81].

TERT plays a central role in cell viability through regulation of intrinsic mitochondrial-induced apoptosis, which makes it an attractive target for cancer therapeutics[84]. While some studies have shown that TERT catalytic activity in conjunction with TERC expression may be essential for suppression of apoptosis, overexpression of an catalytically inactive form of TERT has been shown to inhibit Bcl-2 and p53 dependent apoptosis[85–87]. In addition, TERT inhibition via siRNA sensitizes cells to the activation of Bax and subsequent apoptosis[88]. Downregulation of TERT results in activation of the JNK/p38-MAPK pathway and increased cleavage of caspase 3, 8 and 9, which are involved in mitochondrial induced apoptosis[89,90]. Downregulation of TERT is also associated with an increase of cytosolic cytochrome C due to disrupted mitochondrial membrane potential[91].

TERT overexpression reduces basal ROS levels and overall ROS production during oxidative stress conditions[81]. This may be accomplished through improving respiratory chain activity or antioxidant activity. Mitochondrial TERT reduces succinate-linked respiration and decreases complex I activity, both of which contribute to mitochondrial ROS production[80]. TERT can bind to mtDNA at coding sequences for NADH:ubiquinone oxidoreductase (complex I) subunit 1 and 2 (ND1 and ND2), which is thought to increase

synthesis of these subunits and allow for replacement of subunits damaged by ROS production[80]. Furthermore, TERT overexpressing cells improve mitochondrial function by limiting levels of the uncoupling protein uncoupling protein 2 (UCP2), which is transcriptionally upregulated by high ROS levels[73]. TERT can also affect the production and availability of various antioxidants to reduce ROS production. TERT overexpressing cells have a higher reduced glutathione compared to oxidized glutathione, which is an important co-factor for GPx activity[81]. It was also observed that TERT overexpressing cells upregulate glutamate cysteine ligase (GCL), the enzyme needed for the production of glutathione and exhibit a more rapid recovery of peroxiredoxin isoforms from their hyperoxidized state[81]. Moreover, TERT overexpressing cells exhibit increased complex IV activity, which contributes to the electrochemical proton gradient utilized by ATP synthase (complex V) for ATP production[81].

1.2.6 *Mitochondrial localization of TERT is neuroprotective*

Mitochondrial localization of TERT may also benefit neurons due to its anti-apoptotic functions, implicating a vital role in aging and neurodegenerative disease. Specifically in brain tissue, dietary restriction or inhibitors of the mechanistic target of rapamycin (mTOR) pathway decreases ROS production, but this is dependent on an increase of TERT transcription and protein localization to the mitochondria[92]. In cerebellar Purkinje neurons, neuronal stressors such as glutamate excitotoxicity increases mitochondrial TERT localization[93].

Primary neuronal cultures derived from transgenic mice overexpressing TERT have increased neuronal survival following N-methyl-D-aspartate (NMDA) exposure compared to neuronal cultures from control mice[94–96]. This increase in cell survival may occur due to TERT mediated effects on mitochondrial membrane potential and mitochondrial uptake of Ca^{2+}, thereby decreasing the accumulation of cytosolic free calcium Ca^{2+} after NMDA receptor activation[94]. In addition, TERT was detected in the cytosol and nucleus of microglia up to 7 days after excessive stimulation of NMDA receptors by the seizure-inducing drug kainite[97].

TERT is also protective against initiators of apoptosis in neurons following exposure to various forms of stress. TERT inhibition in cultured neurons that are deprived of oxygen and glucose results in increased caspase 3 cleavage, decreased Bcl-2/Bax expression, enhanced ROS production, and reduced mitochondrial membrane potential[98]. In a transgenic mouse model of amyotrophic lateral sclerosis (ALS), increased TERT expression protected against spinal cord motor neuron death[96]. Neuroprotection is believed to be mediated, in part, by tropomyosin receptor kinase B (TrkB) induced signalling of brain derived neurotrophic factor (BDNF) which can upregulate transcription factors c-Myc, Sp1 and NF-κB, thereby increasing TERT expression and telomerase activity in spinal cord motor neurons undergoing injury[99]. This confers resistance to apoptosis mediated through Bcl-2, Bax, p53 and a maintenance of mitochondrial membrane potential[99]. Another study has shown that in hippocampal neurons, TERT localizes to cytosolic RNA granules[100]. Within the RNA granules, TERT colocalizes with p15INK4B mRNA, which is known to promote cell cycle arrest by inhibition of cyclin-dependent kinases CDK4 and CDK6[100]. Upon acute stress, TERT disassociates from the P15INK4B mRNA allowing for translation, suggesting this may be a mechanism for TERT's pro-survival effect in neurons[100].

There is also evidence that TERT may contribute to the protective roles in cell types other than neurons in the brain after tissue injury. After permanent ischemic damage, TERT mRNA is rapidly increased within 4 hours in the cytoplasm of surviving ipsilateral cortical neurons[94]. However, a separate study showed that TERT protein expression shifts from neurons to astrocytes after 3 days in a hypoxic-ischemic rat model[101]. Transient ischemic brain injury also induced increased TERT mRNA expression in astrocytes in the uninjured ipsilateral hemisphere[102]. TERT expression in astrocytes corresponds with an upregulation of the cell cycle regulator p15 to inhibit proliferation of reactive astrocytes and the increased expression of neural growth factor neurotrophin-3 (NT-3) to promote neuronal survival[101].

1.3 Alzheimer's Disease and TERT

1.3.1 *Alzheimer's Disease and oxidative stress*

Alzheimer's disease (AD) is the most prevalent form of dementia worldwide. It is characterized by the deposition of intracellular neurofibrillary tangles composed of hyper-phosphorylated tau protein and extracellular plaques composed of amyloid-beta (Aβ) peptides[103]. Early-onset AD occurs in 1%-6% of cases and is most commonly the result of a mutation in the amyloid precursor protein (APP) or the secretases (PSEN1, PSEN2) that shift the processing of the APP to the pathogenic 1-42 amino acid Aβ peptide ($A\beta_{1-42}$)[104]. This $A\beta_{1-42}$ peptide can spontaneously aggregate into soluble oligomers, and further accumulate to form insoluble beta-sheet fibrils, finally forming deposits of diffuse or compact plaques[105]. Over time, there is progressive degeneration of neurons and synapses in areas of the brain involved in memory and cognition such as the hippocampus, entorhinal cortex, and basal forebrain[106].

Mitochondrial dysfunction and oxidative stress are prominent features of many neurodegenerative diseases[107]. A neuron's high energy demand makes it reliant on mitochondrial oxidative phosphorylation to produce ATP, which is more efficient at ATP production than glycolysis[108]. However, due to the high number of neuronal mitochondria and the relatively low levels of antioxidant enzymes compared to other organs, neurons are especially vulnerable to ROS[109]. Aging can lead to increased mitochondrial DNA mutations and damaged complexes in the ETC, leading to elevated electron leakage and reduced ATP synthesis[63]. It has also been reported that patients with AD have altered mitochondrial morphology, reduced cytochrome c oxidase (complex IV) activity and reduced activity of enzymes within the TCA cycle[62,110]. In addition, glycolysis is perturbed as evidenced by lower levels of glucose transporters (GLUT1 and GLUT3) and enzymes involved in glycolysis (phosphofructokinase (PFK), glucose6-phosphate isomerase and lactate dehydrogenase in the brains of AD patients compared to age matched controls[111,112]. A decrease in glucose consumption and oxygen metabolic rates are observed during normal aging, but this effect is further exacerbated during neurodegenerative disease, such as AD[113].

In AD, oxidative damage occurs well before Aβ plaque accumulation and is believed to be a major contributor to pathogenesis[114]. Recent studies suggest that neuronal dysfunction and degeneration is due to the accumulation of intracellular Aβ, which

eventually causes lysis of the cell leading to extracellular Aβ plaque deposition[115,116]. The $A\beta_{1-42}$ oligomers have been shown to be toxic to neurons, synapses and the mitochondria by promoting ROS production, oxidative damage, and tau phosphorylation[117,118]. $A\beta_{1-42}$ oligomers directly interfere with mitochondrial function via Aβ binding alcohol dehydrogenase (ABAD) resulting in mitochondrial impairment, ROS production, and cell death[119]. In addition, transgenic mice that express mutant human APP reveal decreased basal levels of mitochondrial membrane potential at 3 months of age compared to littermate controls[109]. At this time, only moderately elevated intracellular Aβ load is detected, suggesting that Aβ production interferes with mitochondrial function well before Aβ plaque deposition[109]. Since current insights of AD pathology propose there is a cyclic relationship between oxidative damage and Aβ production, it may be useful to investigate targets that reduce the damaging effects of oxidative stress[62]. Recently, it has been proposed that lifestyle factors such as dietary restriction, intellectual activity and exercise may target intrinsic biological mechanisms to limit oxidative damage[120,121].

Surprisingly, amyloid-deposition is observed in about 25% of cognitively normal elderly individuals[122]. Investigation of Aβ resistant cells reveal an upregulation of antioxidant enzymes such as peroxiredoxin 1 (Prx1), GPx and catalase. In addition, Aβ resistant cells exhibit an increase in glucose uptake and glycolysis due to an activation of transcription factor hypoxia inducible factor 1 (HIF-1)[123–125]. Increased activation of the alpha subunit of HIF-1 (HIF-1α) is frequently observed in tumors resulting in an increase of glucose uptake and lactate production, even in the presence of oxygen[126]. This phenotype is termed the Warburg effect or aerobic glycolysis. *In vitro* studies of Aβ resistant cells demonstrate that elevated aerobic glycolysis is a neuroprotective mechanism that decreases mitochondrial ROS production and increases resistance to Aβ -induced apoptosis[127]. Furthermore, increased expression of rate-limiting enzymes in aerobic glycolysis, such as lactate dehydrogenase (LDHA) or pyruvate dehydrogenase kinase 1 (PDK1), can protect against Aβ toxicity[128]. Positron emission tomography (PET) imaging of AD patients demonstrated that aerobic glycolysis correlates spatially in individuals that have high Aβ plaque deposition but are cognitively normal[129]. Altogether, these results suggest that neurons may become resistant to the toxic effects of Aβ by altering

their metabolism, moving away from OXPHOS and towards aerobic glycolysis for their energy needs.

1.3.2 *TERT is protective against amyloid-beta*

Telomere shortening and a decrease in TERT expression occurs significantly more in hippocampal neurons of AD patients compared to aged matched controls[130]. Recent *in vitro* studies demonstrate that increasing TERT expression and activity may be beneficial against Aβ toxicity. One study showed that estrogen receptor activation is neuroprotective against Aβ toxicity in a manner dependent on upregulation of TERT activity[131]. A proposed mechanism is through activation of TERT via phosphorylation by Akt kinase, causing TERT to associate with NF-κB and translocate to the nucleus where telomerase activity can occur[131]. A separate study determined that treating primary murine cerebrovascular endothelial cells with calf serum was able to reverse Aβ-inhibition of HSP90 expression, a protein which binds to TERT, and facilitate an upregulation of telomerase activity to prevent apoptosis induced by Aβ[132]. Similarly, TERT may protect mouse hippocampal neurons from Aβ induced apoptosis by limiting mitochondrial ROS production and by maintaining mitochondrial membrane potential[133]. Interestingly, the vaccine peptide GV1001 derived from the TERT sequence blocks Aβ toxicity by localizing to the mitochondria of rat neural stem cells and reduces ROS levels, mitochondrial DNA damage, and alters expression levels of survival/death related proteins to prevent apoptosis[134]. More recently, *in vivo* studies in mice have confirmed that while TERT is expressed in both aged control and transgenic AD adult hippocampal neurons, the organelle localization of TERT differed between these two groups[132]. Mitochondrial localization of TERT was observed in transgenic AD brains and the presence of TERT was mutually exclusive from hyper-phosphorylated tau, suggesting a neuroprotective function against tau pathology[135].

1.4 Rationale

The current field of research has recognized many physiological functions of TERT apart from its canonical role in maintaining telomeres. Most importantly, it has been shown that mitochondrial localized TERT protects against oxidative stress and the toxic effects

of the Aβ, a peptide strongly implicated in AD pathogenesis. In addition, Aβ resistant cells have been known to switch their metabolism to aerobic glycolysis through a mechanism which is poorly defined. **I hypothesize that TERT will localize to the mitochondria and alters metabolism in a manner which renders neurons less susceptible to toxicity following exposure to Aβ or oxidants.** As a result of mitochondrial localization of TERT, mitochondrial ROS levels will lower and cell viability will increase in Aβ challenged cells. Since mitochondrial dysfunction is a prominent feature of AD, the discovery of mitochondrial associated functions of TERT may implicate TERT as a potential therapeutic target for AD and other neurodegenerative diseases.

Chapter 2: Materials and Methods

2.1 Plasmids

Human TERT (072-hTERT, referred to as TERT) and 071-FLAG-hTERT (referred to as FLAG-TERT) cDNA expression plasmids were generously provided by Dr. Lea Harrington (IRIC, Montreal, Quebec, Canada). The pcDNA 3.1 plasmid (referred to as pcDNA; Invitrogen) was used as a transfection control in all experiments. The pcDNA3-EGFP plasmid (referred to as GFP; Addgene) was used as a transfection control for MitoTracker CMXROS experiments. Transfection efficiency was between 40-50%, which was assessed following co-transfection of the TERT plasmid with GFP and visualization by fluorescence microscopy.

2.2 Cell Culture

The HT22 immortalized mouse hippocampal neuronal cell line was a gift from Dr. Dave Schubert (Salk Institute for Biological Sciences, California, USA). HT22 cell cultures were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza) supplemented with 10% Fetal Bovine Serum (FBS) (Corning) and 1% penicillin/streptomycin (P/S) (Gibco). Cells were cultured in an incubator at 37°C and 5% CO_2.

2.3 Aβ Preparation

$A\beta_{(25-35)}$ was purchased from California Peptide and dissolved in sterile ddH_2O to a concentration of 1 mM then left overnight at room temperature to allow fibril formation before storing at -20°C, as previously described[127].

2.4 Western Blot

HT22 cells were seeded at 150 000 cells per 60 mm cell culture dish and grown to 50%-70% confluency then transfected with 3 µg DNA (TERT, pcDNA) using 1 mL Opti-MEM (Gibco) and 5 µL Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Transfection media was replaced with DMEM + 10% FBS + 1% P/S media

after 5 hours and the cell cultures recovered overnight. The following day cell cultures were treated for 4 hours with 400 µM H_2O_2 (Sigma) or 25 µM $A\beta_{25-35}$ (California Peptide),then washed twice in Phosphate Buffer Saline (PBS; Lonza) and lysed in ice-cold RIPA buffer (10 mM Tris-Hcl pH 8, 1% Triton X-100 (Sigma), 0.1% Sodium deoxycholate, 0.5 mM EGTA, 0.1% SDS, 140 mM NaCl) containing a protease inhibitor cocktail (2 mM leupeptin (Sigma), 0.1 mM pepstatin A (Sigma)), phenolmethanesulfonyl fluoride (Sigma), and sodium orthovanadate (Sigma). Protein content from whole cell lysate was quantified using the DC protein assay (Bio-Rad). Protein samples were resolved by 10% SDS-PAGE, immunoblotted onto polyvinylidene fluoride membrane (Bio-Rad), and blocked in TBS-T buffer containing 3% BSA (VWR), 1% non-fat dry milk (Cell Signalling). Blots were hybridized with the following primary antibodies: anti-hTERT (600-401-252S; Rockland), anti-PDH (ab110334; abcam), anti-Actin (sc-47778; Santa Cruz), anti-pser^{232} PDH (AP1063; EMD Millipore), anti-LDHA (#2012; Cell Signalling), anti-PDK1 (ADI-KAP-PK112-F; Enzo Life Sciences), anti-PKM1 (#7067; Cell Signalling), and anti-PKM2 (#3198; Cell Signalling). Following incubation with HRP-conjugated secondary mouse (sc-2005; Santa Cruz) and rabbit (sc-2030; Santa Cruz) antibodies, blots were visualized by enhanced chemiluminescence using the Luminata Forte substrate (EMD Millipore) and imaged in a Chemidoc XRS System (Bio-Rad). Densitometric quantification of band intensity of three independent experiments was performed using Image Lab software (Bio-Rad).

2.5 MitoTracker Red CMXROS

To determine mitochondrial ROS levels of the cells, the fluorescent dye MitoTracker Red CMXRos (Life Technologies) was used. HT22 cells were seeded at 80, 000 cells per 35 mm cell culture dish and grown to 50%-70% confluency. The cell cultures were transfected with 1.2 µg DNA (TERT/pcDNA:GFP, 2:1 ratio) and 2 µL Lipofectamine 2000 in 800 µL Opti-MEM according to manufacturer's instructions. Transfection media was replaced with DMEM + 10% FBS + 1% P/S media after 5 hours. The following day, cell cultures were treated for 12 hours with 400 µM H_2O_2 or 25 µM $A\beta_{25-35}$. After treatment, the cell cultures were incubated at 37°C for 20 minutes with phenol-red free DMEM + 10% FBS + 1% P/S containing 200 nM MitoTracker Red CMXRos. Cells were

washed twice with PBS and incubated with 10 ug/mL Hoeschst (Life Technologies) in PBS for 1 minute at room temperature. Cells were rinsed with PBS, placed in phenol-red free DMEM + 10% FBS + 1% P/S and analyzed by fluorescence microscopy using a Leica DMI6000 B microscope equipped with an ORCA Hamamatsu digital camera. All digital images were captured at the same exposure time and analyzed using ImageJ software (National Institute of Health). The mean red fluorescence of three images per treatment was calculated for each of three independent experiments.

2.6 Cell Viability Assay

The MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay was used to assess cell viability. 150 000 HT22 cells were seeded in a 60 mm cell culture dish and grown to 50%-70% confluency. Cell cultures were transfected with 3 µg DNA (TERT, pcDNA) and 5 µL Lipofectamine 2000 in 1 mL Opti-MEM according to manufacturer's directions. Transfection media was replaced with DMEM + 10% FBS + 1% P/S media after 5 hours and the next morning, cells were trypsinized and 7 000 cells per well were seeded in a 96-well plate with DMEM + 10% FBS + 1% P/S media. After cell adherence (approx. 5 hours), the cells were treated for 24 hours with DMEM + 5% FBS + 1% P/S media containing 400 µM H_2O_2 or 25 µM $A\beta_{25-35}$. The concentration of Aβ was chosen based on previous studies[127] whereas the H_2O_2 concentration was determined following a dose response curve (see Appendix 5). Live cells were visualized and representative images were taken for each treatment using a Leica DMIL LED phase contract microscope. Then the media was replaced with DMEM + 5% FBS + 1% P/S media with MTT reagent (Sigma) added at a final concentration of 10%. Culture plates were incubated at 37°C for 3 hours. After incubation, culture medium containing MTT was aspirated, replaced with DMSO and the optical density was measured at 595 nm using a microplate reader (Bio-Rad Model 3550). All treatments were seeded in triplicates and the mean viability was calculated from three independent experiments.

2.7 Immunofluorescence

Cover slips were placed in a 6-well plate and treated with 50 µg/mL Poly-D lysine

(Sigma) overnight. After washing with sterile water, 60 000 HT22 cells per well were seeded and grown to 50% confluency. Cell cultures were transfected with 1.2 µg DNA (FLAG-TERT, pcDNA) 2 µL Lipofectamine 2000 (Invitrogen) in 800 µL Opti-MEM according to manufacturer's instructions. Transfection media was replaced with DMEM + 10% FBS + 1% P/S media after 5 hours and the next morning, cells were treated for 12 hours with 400 µM H_2O_2 or 25 µM $A\beta_{25-35}$. Cells that adhered onto cover slips were transferred to a 12-well cell culture plate, washed twice with PBS and fixed with ice cold 4% paraformaldehyde in PBS for 10 minutes at room temperature. Cells were washed once with PBS and then twice with PBS + 1% Triton X-100 before blocking with 3% goat serum (Invitrogen) in PBS + 1% Triton-X-100 for 1 hour at room temperature. Fixed cells were hybridized with the primary antibodies anti-FLAG (F3165; Sigma) and anti-TOM20 (sc-11415; Santa Cruz) diluted 1:200 in in 3% goat serum in PBS + 1% Triton-X. Following an overnight incubation at 4°C, coverslips were washed twice with PBS + 1% Triton-X followed by hybridization with anti-mouse IgG secondary antibody conjugated to Alexa Fluor 488 (Invitrogen) and goat anti-rabbit IgG secondary antibody, Alexa Fluor 594 (Invitrogen) diluted 1:200 in 3% goat serum in PBS + 1% Triton-X for 1 hour at room temperature. Cover slips were then washed twice with PBS + 1% Triton-X and then mounted on a glass slide using ProLong Gold Antifade Mountant with DAPI (Invitrogen). Clear nail polish was used to seal the edges. Slides were visualized by fluorescence microscopy using the Leica DMI6000 B equipped with an ORCA Hamamatsu digital camera. Images were captured at the same exposure time for all treatments.

2.8 Statistical Analysis

Data are presented as means ± SD resulting from three independent experiments. Data were analyzed statistically using a one-way ANOVA followed by a Tukey test (GraphPad software). Results were considered statistically significant at $p \leq 0.05$.

Chapter 3: Results

3.1 TERT partially localizes with mitochondria following H_2O_2 or Aβ exposure

Previous studies have shown that TERT localizes to mitochondria under oxidative conditions[136–138] . Due to low expression of endogenous TERT and the lack of anti-TERT antibodies suitable for immunofluorescence staining, HT22 cells were transfected with a FLAG-TERT plasmid to allow the use of an anti-FLAG antibody to detect overexpressed TERT. Cells were then treated with 400 μM H_2O_2 and 25 μM $A\beta_{25-35}$ for 12 hours and co-staining was performed using anti-FLAG and anti-TOM20 (mitochondrial marker) antibodies. DAPI was used as a nuclear marker. Under control conditions, TERT expression is primarily nuclear, as illustrated by colocalization of FLAG (green) with DAPI (blue) (Figure 1A). However, after 12 hours of oxidative stress induced by 400 μM H_2O_2 and 25 μM $A\beta_{25-35}$ exposure, there are two patterns of TERT localization that emerge (Figure 1B,C). The first is characterized by reduced localization of TERT from the nucleus and increased localization to mitochondria. The second pattern shows TERT is present in both the nucleus and the mitochondria. Both patterns illustrate that TERT may have non-canonical mitochondrial functions under oxidative stress conditions.

Figure 1

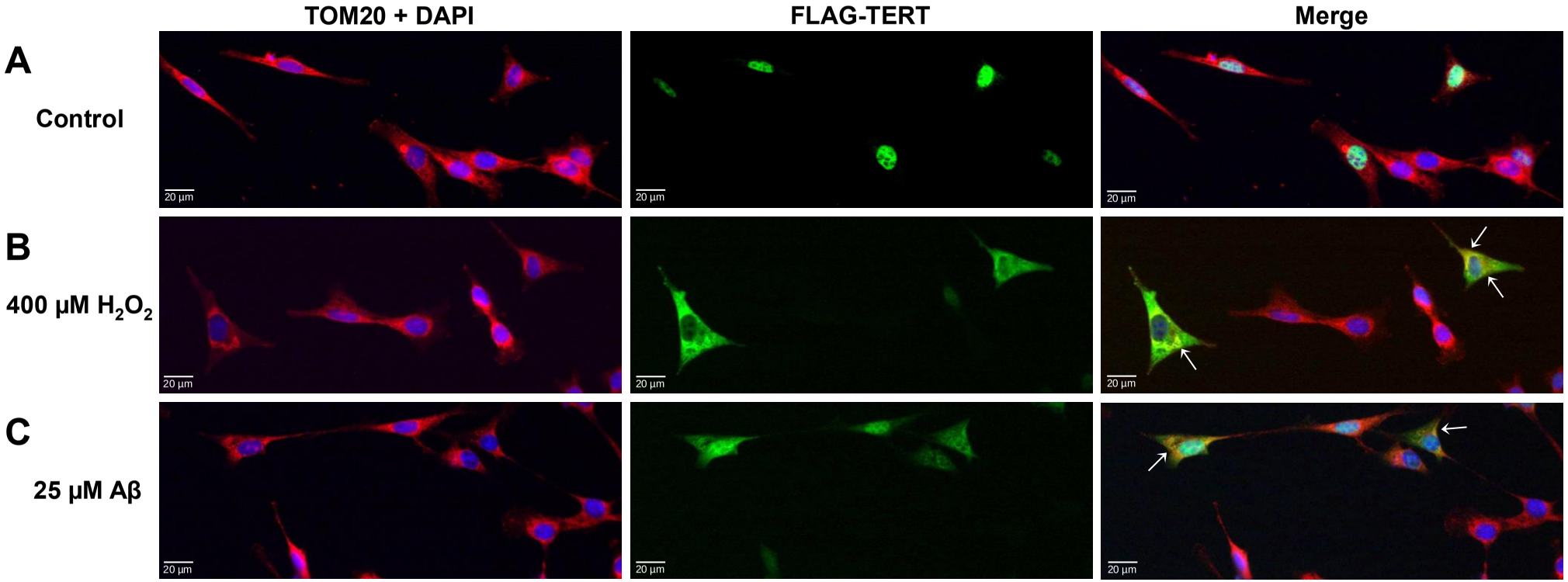

Figure 1: TERT partially localizes to the mitochondria following H_2O_2 or Aβ exposure.

Immunofluorescence staining of HT22 cells transfected with a FLAG-TERT expression construct. Cells were exposed to H_2O_2 (400 µM) or $A\beta_{25-35}$ (25 µM) for 12 hours post-transfection and stained with anti-FLAG and anti-TOM20 antibodies. (A) TERT localizes to the nucleus under control conditions. (B) White arrows indicate partial co-localization of TERT with the mitochondrial marker TOM20 occurred after 400 µM H_2O_2 and (C) 25 µM Aβ exposure. Green (FLAG-TERT), Red (TOM20), blue (DAPI). 40X objective lens. Scale bars are 20 µm.

3.2 TERT expression promotes a shift towards glycolysis under oxidative stress

To assess the effect of TERT expression on metabolic enzyme levels, HT22 cells were transfected with TERT or pcDNA plasmids, followed by a 4 hour exposure to low and high concentrations of H_2O_2 (100 μM, 400 μM) and 25 μM $A\beta_{25-35}$. No toxicity was observed following a four hour exposure to these stressors (data not shown). Protein levels of enzymes involved in metabolism were assessed via Western blotting (Figure 2A). Pyruvate dehydrogenase (PDH) is the rate-limiting enzyme that catalyzes the conversion of pyruvate to acetyl-CoA to fuel the TCA cycle. Increased phosphorylation of PDH at serine 232 by pyruvate dehydrogenase kinase 1 (PDK1) restricts OXPHOS by rendering PDH inactive, resulting in a metabolic shift towards glycolysis to satisfy the cell's energy requirements. TERT transfected cells had elevated levels of phospho-PDH/total PDH and PDK1 when stressed with 100 μM H_2O_2, 400 μM H_2O_2 and 25 μM $A\beta_{25-35}$ (Figure 2B), indicative of less OXPHOS. Another important enzyme involved in glycolytic metabolism is lactate dehydrogenase A (LDHA). LDHA catalyzes the conversion of pyruvate to L-lactate while oxidizing NADH to NAD^+ as a by-product. TERT transfected cells had elevated levels of LDHA when stressed with 100 μM H_2O_2, 400 μM H_2O_2 and 25 μM $A\beta_{25-35}$ (Figure 2B), indicative of enhanced glycolytic metabolism. Finally, the levels of alternatively spliced M1 and M2 isoforms of pyruvate kinase (PK) were quantified. PK catalyzes the irreversible reaction between phosphoenolpyruvate (PEP) and ADP to produce pyruvate and ATP in the last step of glycolysis. While PKM1 is constitutively active, the dimeric form of PKM2 is expressed in tissues with high anabolic functions, such as cancer cells, and has a low affinity for PEP[139]. This results in the accumulation of glycolytic intermediates, which enter the glycerol synthesis and pentose phosphate pathways (PPP), limiting the production of pyruvate to fuel OXPHOS[139]. A decrease in the ratio of PKM1/PKM2 enzymes was observed in TERT transfected cells when stressed with 100 μM H_2O_2, 400 μM H_2O_2 and 25 μM $A\beta_{25-35}$ (Figure 2B), indicating higher PKM2 levels. Altogether, TERT overexpressing cells demonstrate a shift towards glycolytic metabolism by altering protein levels of metabolic enzymes.

Figure 2A

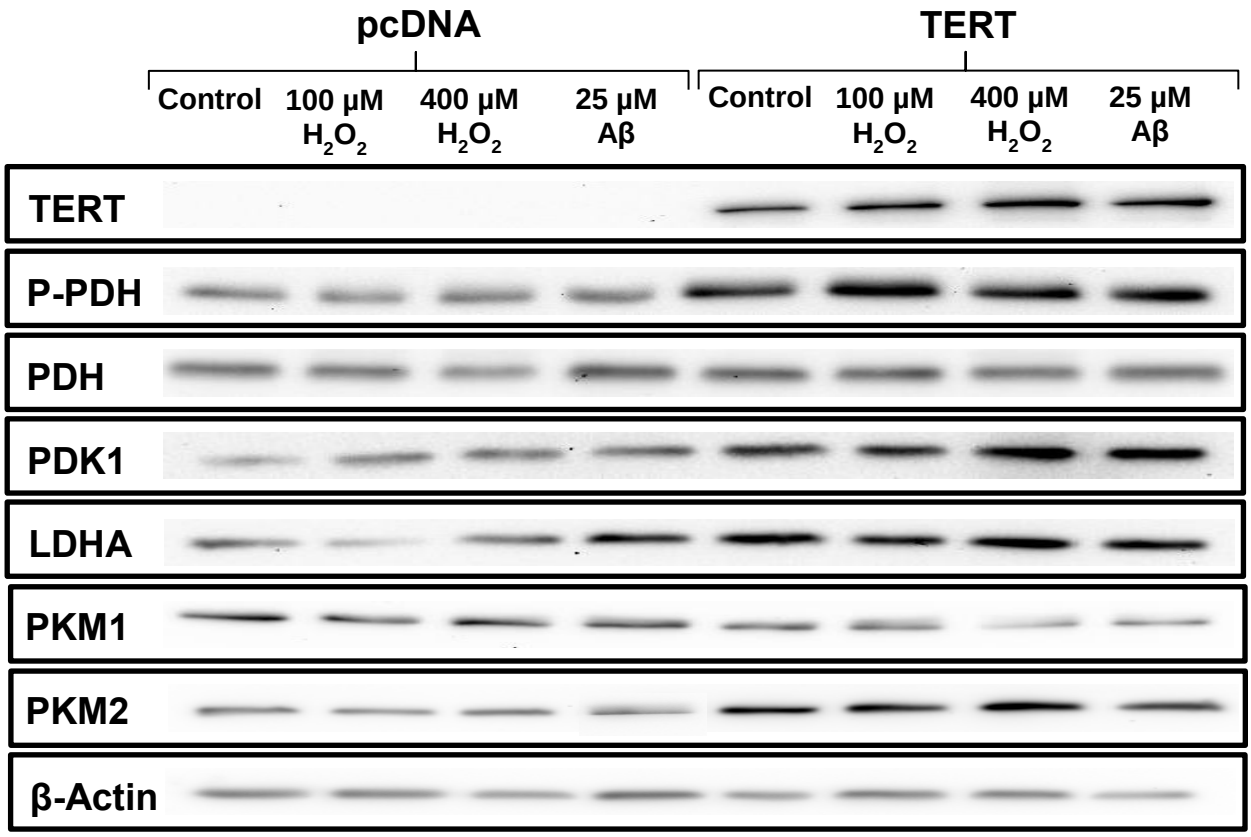
pcDNA
TERT
Control
100 μM H_2O_2
400 μM H_2O_2
25 μM Aβ
Control
100 μM H_2O_2
400 μM H_2O_2
25 μM Aβ
TERT
P-PDH
PDH
PDK1
LDHA
PKM1
PKM2
β-Actin

Figure 2B

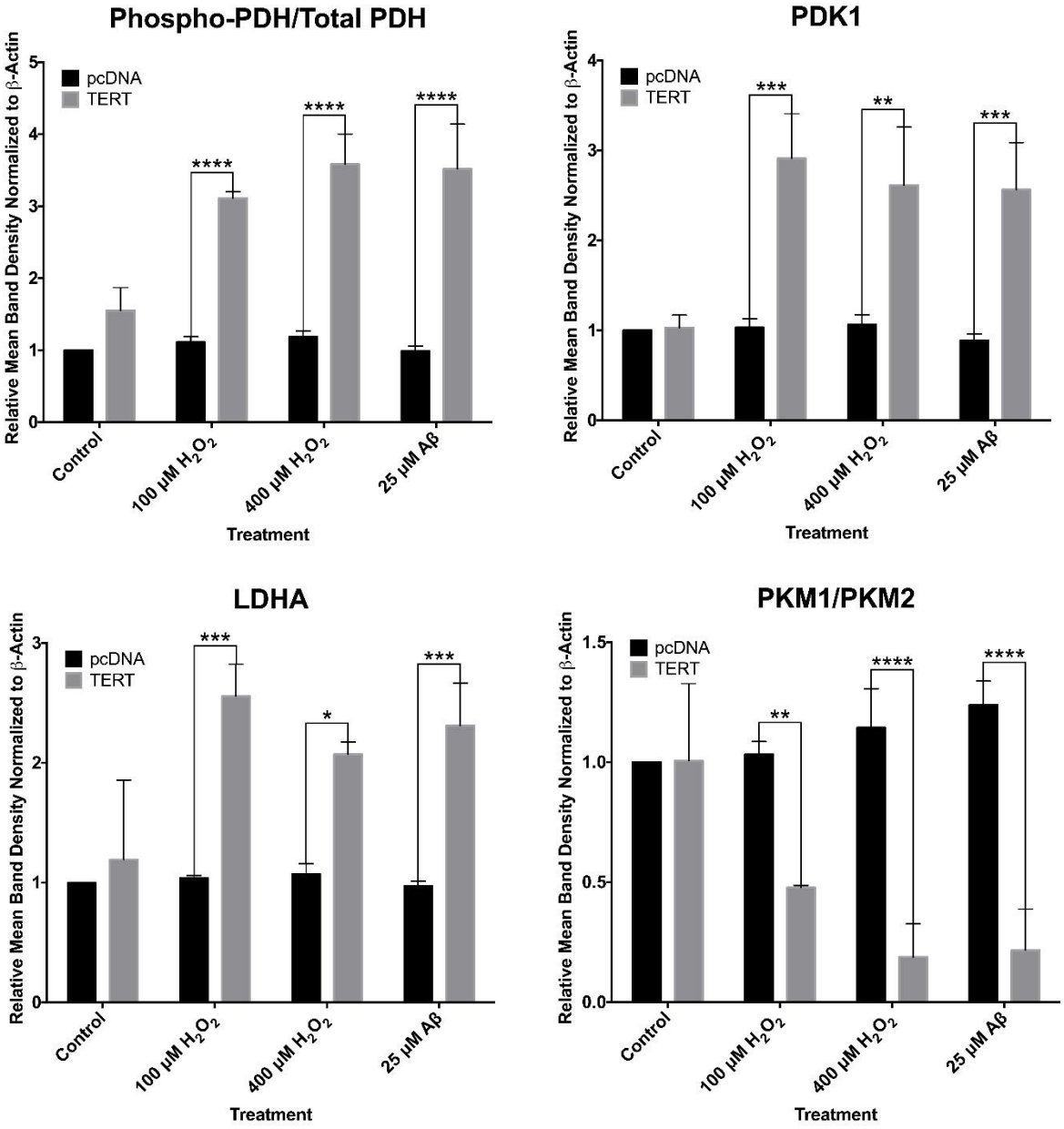

Figure 2: Immunoblot analysis of metabolic markers reveals a shift towards glycolysis in TERT expressing cells following H_2O_2 and Aβ exposure.

(A) HT22 cells were transfected with pcDNA or TERT plasmids followed by a 4 hour exposure to H_2O_2 (100 μM, 400 μM) or $A\beta_{25\text{-}35}$ (25 μM). Protein levels of metabolic enzymes (phospho-PDH/total PDH, PDK1, LDHA, PKM1/PKM2) were assessed by immunoblot analysis with the indicated antibodies. (B) Densitometric analysis of band intensity revealed significantly higher levels of phospho-PDH/PDH, PDK1 and LDHA and a lower protein ratio of PKM1/PKM2 in the TERT transfected cells compared to controls after oxidative stress treatments (100 μM H_2O_2, 400 μM H_2O_2, 25 μM $A\beta_{25\text{-}35}$). The data presented are from three independent experiments ± SEM. Asterisk indicates significant difference (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$) tested by One-Way ANOVA followed by a Tukey test.

3.3 TERT expression prevents increases in mitochondrial ROS under oxidative stress

Since the TERT overexpression plasmid did not have a fluorescent tag, cell cultures were co-transfected with a GFP plasmid at a 2:1 ratio (TERT:GFP) in order to identify transfected cells. It is presumed that cells with green fluorescence (GFP) will also contain the TERT overexpressing plasmid. HT22 cells were co-transfected with TERT + GFP and pcDNA + GFP plasmids, followed by a 12 hour exposure to 400 µM H_2O_2 or 25 µM $A\beta_{25-35}$. Transfected cells were stained with MitoTracker Red CMXROS, a mitochondrial localized dye that emits red fluorescence in proportion to mitochondrial ROS levels, and visualized by fluorescence microscopy (Figure 3A,B). MitoTracker Red CMXROS fluorescence intensity of GFP positive cells was analyzed to determine differences between treatments (Figure 3C). Under control conditions, pcDNA and TERT transfected cells had comparable levels of ROS. However, when cells were exposed to 25 µM $A\beta_{25-35}$, TERT transfected cells had significantly lower levels of ROS compared to pcDNA transfected cells. Therefore, under oxidative stress conditions elicited by $A\beta_{25-35}$ exposure, cells overexpressing TERT have lower mitochondrial ROS levels.

Figure 3A

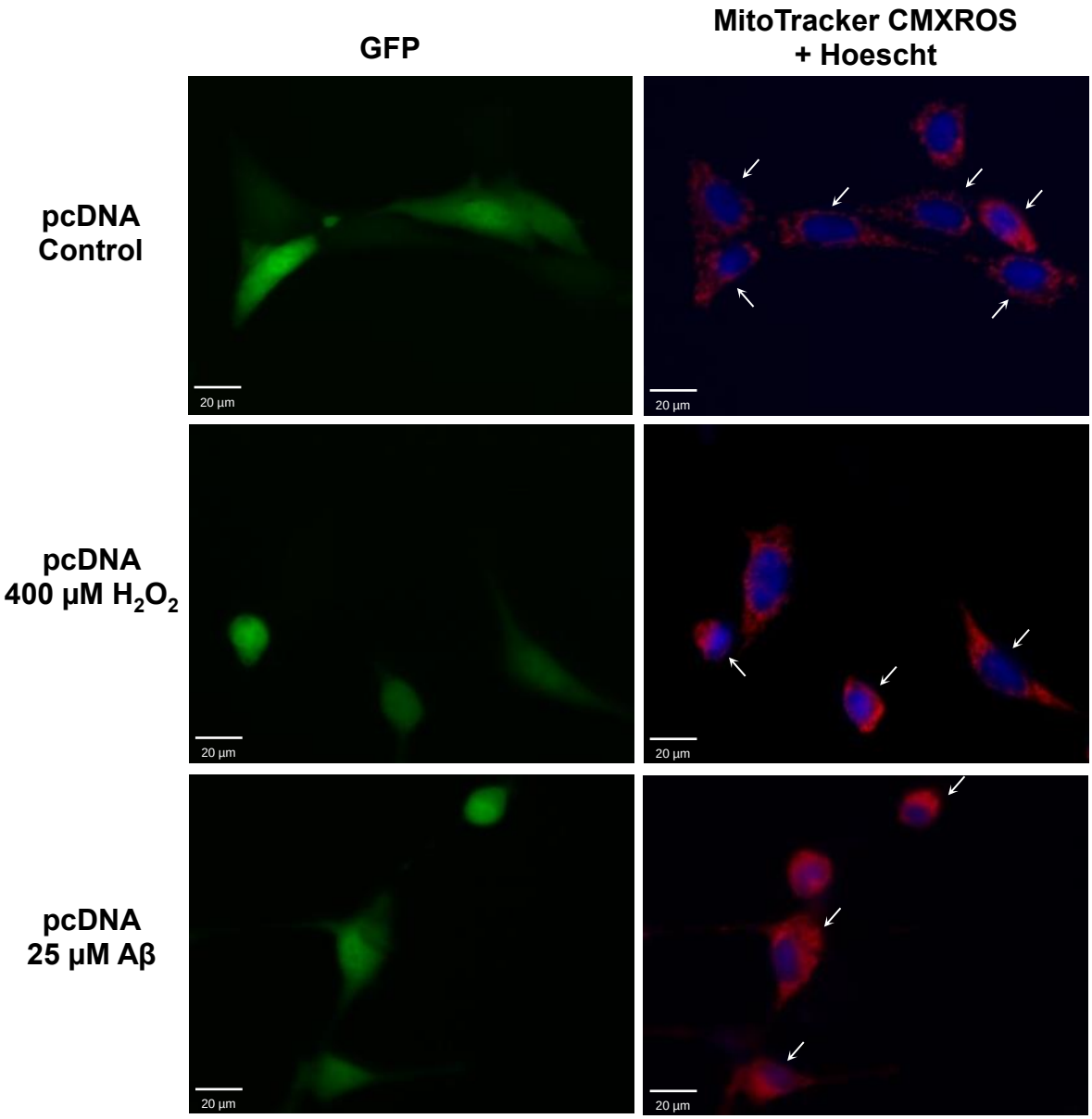
GFP
MitoTracker CMXROS
+ Hoescht
pcDNA
Control
pcDNA
400 µM H_2O_2
pcDNA
25 µM Aβ
20 µm

Figure 3B

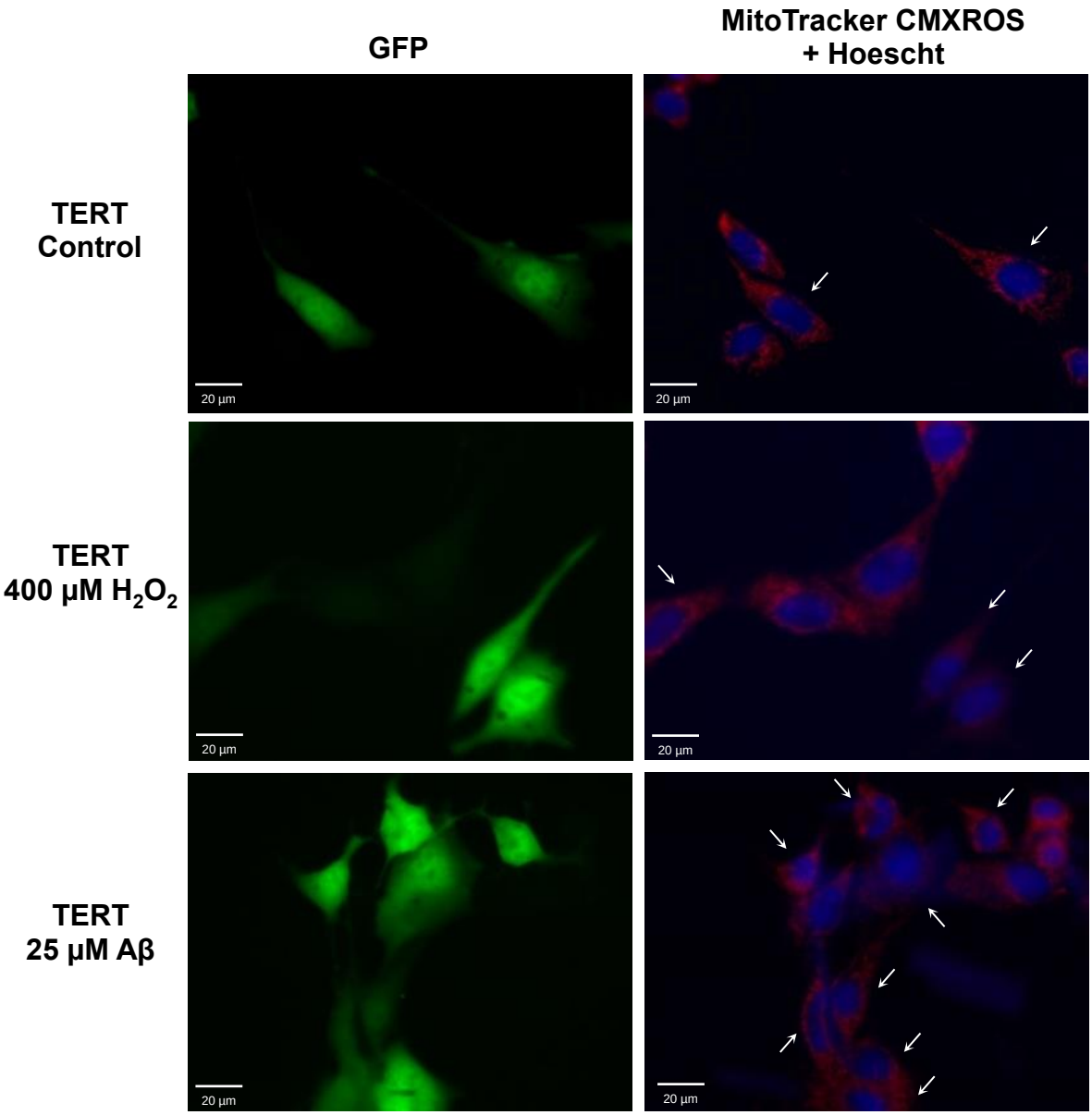
GFP
MitoTracker CMXROS
+ Hoescht
TERT
Control
TERT
400 μM H_2O_2
TERT
25 μM Aβ
20 μm
20 μm
20 μm
20 μm
20 μm
20 μm

Figure 3C

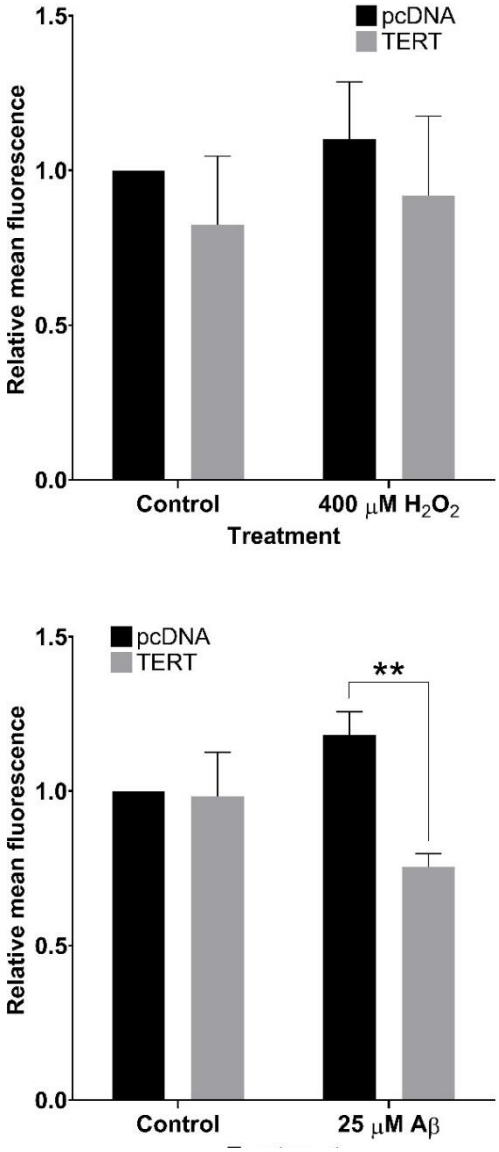

Figure 3: TERT overexpression reduces mitochondrial ROS levels following Aβ exposure.

HT22 cells were co-transfected with GFP along with pcDNA or TERT plasmids followed by a 12-hour exposure to H_2O_2 (400 μM) or $A\beta_{25-35}$ (25 μM). TERT transfected cells were stained with MitoTracker Red CMXROS and visualized by fluorescence microscopy. Red (MitoTracker CMXROS), blue (Hoescht), white arrows (GFP positive cells). Representative photomicrographs of pcDNA **(A)** and TERT transfected cells **(B). (C)** Fluorescence intensity of GFP positive cells was quantified using Image J software. TERT overexpressing cells have a reduction in ROS levels under 400 μM H_2O_2 treatment and a significant reduction in ROS levels under 25 μM $A\beta_{25-35}$ treatment compared to pcDNA transfected cells. The data presented are from three independent experiments ± SEM. Asterisk indicates significant difference (**, $p \leq 0.01$) tested by One-Way ANOVA followed by a Tukey test. Scale bars are 20 μm.

3.4 TERT overexpression promotes increased cell viability during oxidative stress

HT22 cells were transfected with TERT or pcDNA plasmids, followed by a 24 hour exposure to 400 µM H_2O_2 or 25 µM $A\beta_{25-35}$. Live cells were visualized by phase contrast microscopy (Figure 4A). When cells were cultured under control conditions, TERT transfected cells had a higher confluency compared to pcDNA transfected cells. When stressed with 400 µM H_2O_2 or 25 µM $A\beta_{25-35}$, TERT transfected cells maintained healthy morphology characterized by neuronal-like elongated processes protruding from the cell body. In contrast, increased cellular debris and morphologically stressed cells characterized by shrunken processes and rounding was observed in oxidant challenged pcDNA transfected cells. The MTT cell viability assay revealed that TERT transfected cells had significantly more cell viability under control and oxidative stress conditions compared to pcDNA transfected cells (Figure 4B). These results confirm that TERT confers a survival advantage following oxidative stress induced by H_2O_2 or Aβ exposure.

Figure 4A

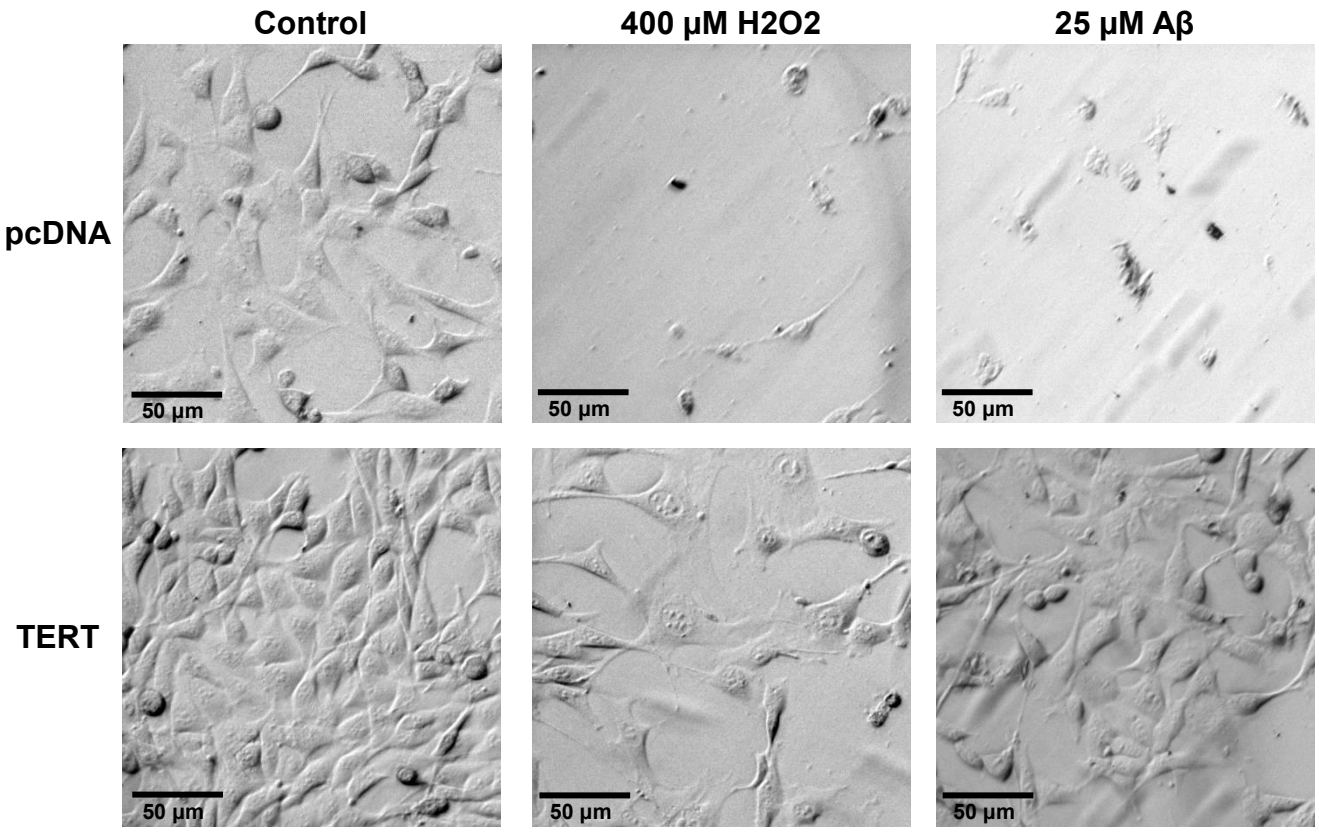
Control
400 µM H2O2
25 µM Aβ
pcDNA
TERT
50 µm
50 µm
50 µm
50 µm
50 µm
50 µm

Figure 4B

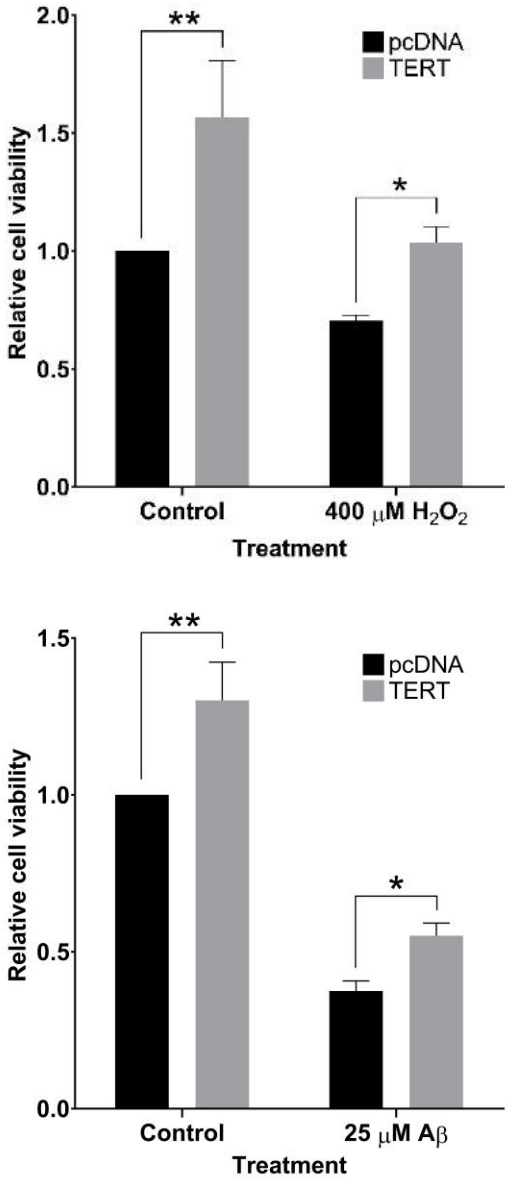

Figure 4: TERT confers a cell survivability advantage under H_2O_2 and Aβ induced stress.

(A) Representative photomicrographs (200X magnification) of HT22 cells transfected with pcDNA or TERT plasmids followed by a 24 hour exposure to H_2O_2 (400 µM) or $A\beta_{25-35}$ (25 µM). (B) Cell viability was assessed using the MTT assay. TERT overexpressing cells had significantly higher cell viability under control, 400 µM H_2O_2 and 25 µM $A\beta_{25-35}$ treatments. The data presented are from three independent experiments ± SEM. Asterisk indicates significant difference (*, $p \leq 0.05$; **, $p \leq 0.01$) tested by One-Way ANOVA followed by a Tukey test. Scale bars are 50 µm.

Chapter 4: Discussion

The present study provides evidence that TERT partially localizes to the mitochondria under H_2O_2 and Aβ-induced oxidative stress and alters metabolism in favour of glycolysis. By limiting mitochondrial metabolism, ROS production is significantly reduced and cell viability is improved. TERT promotes a shift towards glycolysis within the first 4 hours of oxidative stress exposure, which is supported by changes in expression of metabolic enzymes such as an increase in the phospho-PDH/total PDH ratio, elevated levels of PDK1 and LDHA, and a decrease in the PKM1/PKM2 ratio. This is the first demonstration that TERT expression can promote altered levels of glycolytic enzymes during oxidative stress. Up to 12 hours later, TERT overexpressing cells have decreased mitochondrial ROS production and have greater cell survivability at 24 hours after oxidative stress treatment compared to controls. These results indicate that aerobic glycolysis may be an important feature for TERT's protective effects against oxidative stress. As previously discussed, elevated aerobic glycolysis allows neurons to become resistant to Aβ toxicity. This study offers evidence of a novel pro-survival mechanism mediated by TERT involving upregulation of aerobic glycolysis enzymes and a concomitant reduction of mitochondrial ROS production. A proposed mechanism is outlined in Figure 5.

4.1 HT22 cell culture is an appropriate model to study AD

The HT22 cell line is an immortalized mouse hippocampal neuronal cell line, developed from HT4 cells that were originally immortalized from primary mouse hippocampal neuronal cultures[140]. AD patients experience severe neurodegeneration and a decrease of volume of the hippocampus, which has critical functions in learning and memory[141]. The HT22 cell cultures recapitulate features of hippocampal neurons such as expression of cholinergic markers and glutamate sensitivity[142,143]. In addition, HT22 cells have been used extensively as a neuronal model to study oxidative stress induced by glutamate and H_2O_2 exposure[144–148]. Furthermore, Aβ induced oxidative stress in HT22 cells has been previously used as a model to study neurotoxicity in Alzheimer's disease[149–153]. However, there are some limitations of using an immortalized cell line to study the function of

neurons. HT22 cells are rapidly dividing cells that have a doubling time of approximately 15 hours which may subject the cells to genetic drift as the cells divide, whereas neurons in the brain are post-mitotic. In addition, the brain is a complex structure consisting of various cell types, such as astrocytes and glia, and molecules that can act on neurons, such as neurotransmitters, which makes the physiology of neurons in the brain different from HT22 cell cultures. Future studies should use primary neuronal culture as a more physiologically relevant model to determine the protective effects of TERT.

Figure 5

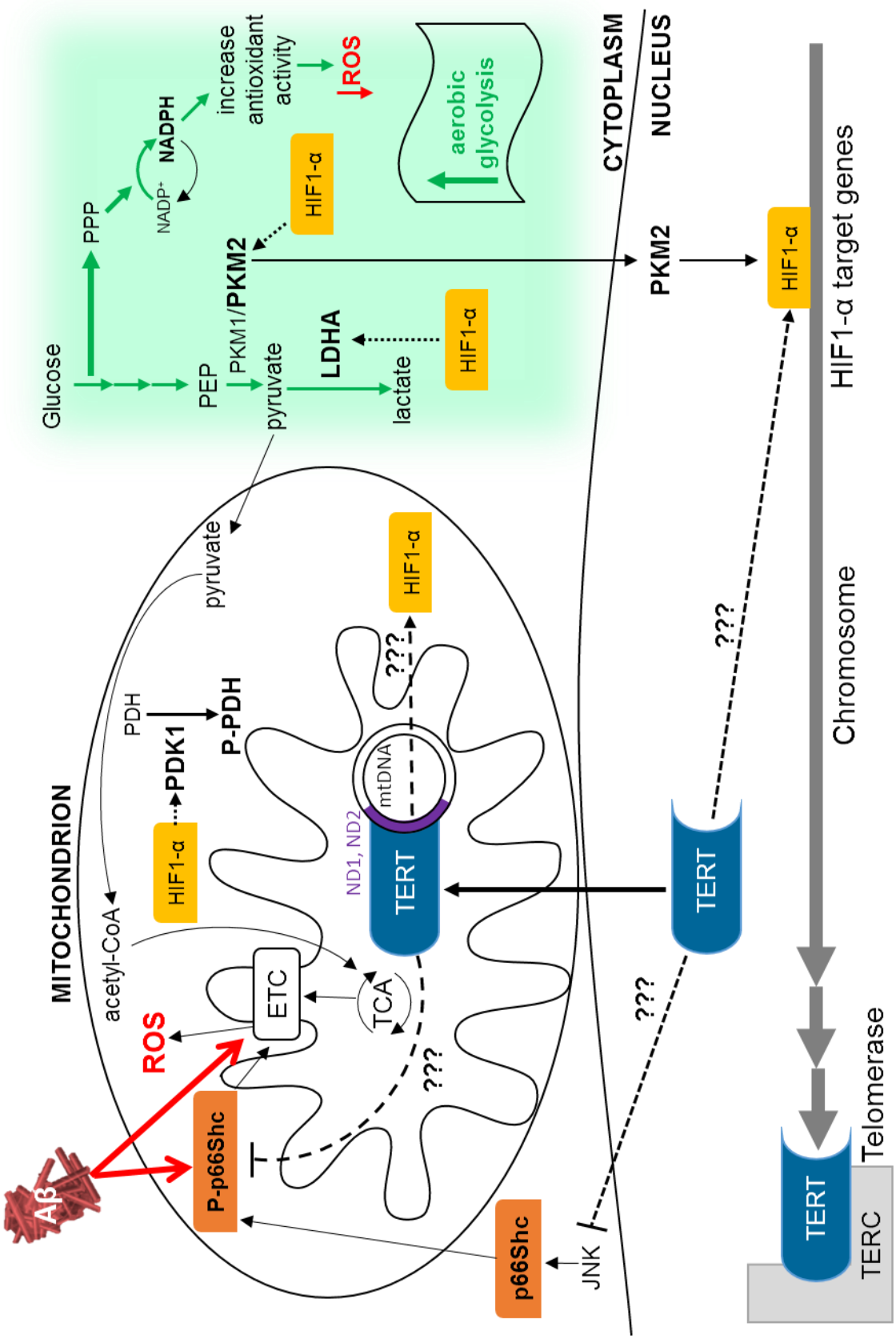
Glucose
PPP
NADP+
NADPH
increase antioxidant activity
ROS
HIF1-α
aerobic glycolysis
PEP
PKM1/PKM2
pyruvate
LDHA
lactate
HIF1-α
CYTOPLASM
NUCLEUS
PKM2
HIF1-α
HIF1-α target genes
Chromosome
pyruvate
PDH
P-PDH
PDK1
HIF1-α
HIF1-α
???
mtDNA
ND1, ND2
TERT
TERT
???
MITOCHONDRION
acetyl-CoA
ROS
ETC
TCA
???
Aβ
P-p66Shc
p66Shc
JNK
???
TERT
Telomerase
TERC

Figure 5: Proposed mechanism of TERT mediated induction of aerobic glycolysis and protection from Aβ toxicity.

Aβ can promote increased mitochondrial ROS by either activating p66Shc or directly interfering with the electron transport chain (ETC). Elevated aerobic glycolysis confers protection from Aβ toxicity by reducing mitochondrial respiration while increasing the pentose phosphate pathway (PPP) with a resulting increase in antioxidant activity. An increase in aerobic glycolysis enzymes including PDK1, phospho-PDH/PDH and LDHA along with a decrease in PKM1/PKM2 protein levels was observed in TERT transfected cells exposed to Aβ. HIF1-α is known to promote aerobic glycolysis by increasing expression of the same metabolic enzymes induced by TERT expression. Hence, it is proposed that TERT may activate HIF1-α in the nucleus, or TERT may translocate to the mitochondria and bind to ND1 and ND2 coding regions which activates HIF1-α and prevents oxidative stress-induced damage. Alternatively, TERT may prevent activation of p66shc through JNK, or may directly interact with P-p66Shc in the mitochondria to prevent mitochondrial dysfunction.

4.2 TERT has partial localization to the mitochondria

Immunofluorescence experiments illustrated that TERT localizes to the nucleus under control conditions and has partial localization with the mitochondria after 12 hours of 400 μM H_2O_2 and 25 μM Aβ treatments. This is in agreement with previous studies that identified TERT expression in cultured hippocampal neurons as well as in control adult human hippocampal neurons[46,135]. Mitochondrial localization of TERT has also been previously confirmed under different cell types exposed to H_2O_2 stress and in hippocampal neurons derived from AD brains[73,80,83,135,154]. However contrary to previous research, the current study demonstrated that TERT is not completely excluded from the nucleus in the cells that exhibit mitochondrial localization. This effect may be due to the use of the FLAG-TERT plasmid that contains the FLAG epitope at the N-terminus of the TERT coding sequence, which may interfere with mitochondrial targeting and import of TERT.

It has previously been determined that TERT has a maximum exclusion level of 60% from the nucleus after 24 hours in cancer cells[154]. Although it is suggested that oxidative stress promotes translocation of existing nuclear TERT to the mitochondria, this study does not address the possibility of cytosolic TERT that bypasses nuclear localization and is directly targeted to the mitochondria instead. Future studies should quantify FLAG-TERT co-localization patterns in HT22 cells to determine if there is significant mitochondrial localization following H_2O_2 and Aβ oxidative stress treatments. In addition, subcellular fractionation and subsequent immunoblotting of nuclear, cytosolic and mitochondrial lysates could be performed to provide further support to localization patterns of TERT in HT22 cell cultures transfected with the TERT plasmid. Furthermore, time course experiments would provide insight to the maintenance of TERT in the mitochondria in HT22 cells and may eliminate the variability of nuclear exclusion observed in this study. Finally, analysis of the localization patterns of endogenous TERT in a neuronal cell line would be ideal to rule out the possibility of mis-localization of highly expressed ectopic TERT following transient transfection.

4.3 TERT affects metabolism under oxidative stress

4.3.1 *TERT shifts metabolism towards aerobic glycolysis*

Enzymes involved in glycolysis were upregulated in TERT overexpressing cells exposed to low or high concentrations of H_2O_2 and Aβ induced oxidative stress. There was an increase in PDK1 protein levels and a concomitant increase in phospho-PDH/PDH ratio, indicating that upregulation of PDK1 expression promoted PDH phosphorylation. Phosphorylated PDH is inactive, thereby preventing the conversion of pyruvate to acetyl-CoA, which is needed for the TCA cycle and the generation of NADH to fuel the electron transport chain in the inner mitochondrial membrane. There was also an increase in LDHA protein levels, which catalyzes the conversion of pyruvate to L-lactate and limits the use of pyruvate for mitochondrial metabolism. Finally, there was a decrease in the PKM1/PKM2 ratio indicating higher PKM2 levels. Allosteric regulation allows PKM2 to switch from a high-activity tetrameric state to a low-activity dimeric state[139]. The dimeric form of PKM2 causes the accumulation of glycolytic intermediates and a shift towards the PPP, an alternative pathway for glycolysis that generates NADPH, a reducing agent used by antioxidant enzymes, ribose 5-phosphate needed for the synthesis of nucleotides, and erythrose 4-phosphate needed for the synthesis of aromatic amino acids. Altogether, these results demonstrate that TERT overexpressing cells combat H_2O_2 and Aβ induced oxidative stress by limiting mitochondrial OXPHOS and shifting towards a glycolytic phenotype. In addition, the increased growth of TERT-overexpressing cells under control conditions may occur by TERT-mediated induction of aerobic glycolysis and anabolic metabolism typical of cancer cells.

4.3.2 *TERT may promote aerobic glycolysis by activation of HIF1-α*

A proposed mechanism for TERT's ability to shift metabolism during oxidative stress may be through the activation of the alpha subunit of hypoxia induced factor 1 (HIF-1α). Many types of cancers have an upregulation of HIF1-α activity which is a transcription factor that increases expression of glucose transporters and enzymes within the glycolytic pathway[155]. HIF1-α is known to induce the expression of LDHA, which is critical for maintaining a reducing environment in order to combat ROS since it regenerates NAD^+

during the conversion of pyruvate to lactate, an essential co-factor for GAPDH, a key glycolytic enzyme that converts glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate[156]. HIF1-α also directly elevates PDK1 levels which limits the conversion of pyruvate to acetyl-CoA to be used for OXPHOS, thereby shifting towards glycolysis[156]. Furthermore, PKM2 has been reported to translocate to the nucleus to promote the activation of HIF1-α and increased transcription of PKM2 in a feed forward mechanism to alter metabolism[157]. Previously, HIF1-α has been linked to TERT as a transcriptional regulator and can bind to the hTERT proximal promoter region through two HIF-1α consensus binding sites to activate TERT transcription and enhance TERT expression[158–160]. In addition, murine embryonic stem cells rely on HIF-1α to promote TERT expression and maintain telomere length[161].

However, the relationship between TERT and HIF-1α is yet to be explored in neurons and other post-mitotic cells. In the current study, oxidative stress induced with H_2O_2 or Aβ was unable to increase endogenous TERT expression in the HT22 cells, given that there were no immunoreactive bands on Western blots probed with anti-TERT antibodies in cell extracts from pcDNA transfectants. Therefore, it is possible that TERT acts as an activator of HIF1-α instead. Mutations in the subunit ND2 of complex I has been shown to increase ROS levels leads to a subsequent increase in HIF1-α levels in head and neck squamous cell carcinoma[162]. Interestingly, TERT binds to mitochondrial DNA at the coding region for ND2 and protects against oxidative stress-induced damage in human umbilical vein endothelial cells, however the mechanism is unknown[80]. It is possible that the binding of TERT to mitochondrial DNA at the coding region for ND2 may activate HIF1-α to initiate metabolic changes that are protective against oxidative stress. Future research should determine whether TERT can affect HIF-1α activity during oxidative stress to elucidate potential feed-forward mechanisms for the metabolic changes caused by TERT overexpression.

4.3.3 *TERT may promote aerobic glycolysis by preventing activation of p66Shc*

Another mechanism for the TERT-induced shift in metabolism may be via inhibition of the 66-kilodalton isoform of the adaptor protein Src homology 2 domain containing

protein (p66Shc), which plays major roles in redox balance, oxidative stress and apoptosis[163]. Following phosphorylation on serine 36, p66Shc translocates to the mitochondria where it increases mitochondrial ROS production and perturbs mitochondrial membrane potential in favour of cytochrome C release[164]. Furthermore, p66Shc has been linked to neurodegeneration via the opening of the permeability transition pore (PTP) located on the OMM, which is a key event to initiate apoptosis[165]. Phosphorylation of p66Shc can be achieved by kinases PKCβ and Jun NH_2-terminal kinase (JNK) which are activated by oxidative stressors such as H_2O_2 and Aβ respectively[166,167]. In addition, p66Shc activation leads to a reduction in antioxidant enzymes such as SOD, GPx and catalase, which further renders the cell susceptible to toxic levels of ROS[168]. Interestingly, recent studies demonstrate that cells lacking p66Shc shift their metabolism away from mitochondrial OXPHOS and towards aerobic glycolysis, along with an increase in lactate production[169,170]. TERT may be able to shift metabolism towards glycolytic pathways by preventing phosphorylation and subsequent activation of p66Shc under oxidative stress conditions. Studies in our lab have shown that Aβ can trigger phosphorylation and activation of p66Shc in HT22 cells (unpublished observations). Moreover, inhibition of p66Shc expression leads to elevated aerobic glycolysis and decreased sensitivity to Aβ toxicity. TERT has been shown to supress caspase mediated apoptosis, and this is dependent on phosphorylation of JNK[171]. TERT inhibition of JNK activation, and subsequent p66Shc phosphorylation, may promote a shift towards aerobic glycolysis, decreased ROS production, and increased cell viability under Aβ-induced oxidative stress. In addition, since localization of p66Shc and TERT converge in the mitochondria, it is possible that TERT may directly interact with p66Shc to prevent its phosphorylation and subsequent activation. Future studies should explore whether TERT directly or indirectly influences p66Shc activation and explore possible interactions between TERT and p66Shc within the mitochondria.

4.3.4 *Metabolic alterations in TERT-deficient and TERT-overexpressing mice*

Although the current study focuses on a cell culture model to examine the metabolic effects of TERT under oxidative stress conditions, *in vivo* studies support the finding that

TERT may alter metabolism and mitochondrial function. Metabolic changes in TERT-deficient and TERT-overexpressing mice are reviewed below.

First generation TERT knockout mice have no changes to fertility or identifiable phenotypic change[172]. However, later generations have a significant reduction in telomere length and lifespan compared to wild-type, leading to atrophy and functional decline of proliferative (ex. intestine) as well as quiescent (ex. liver) organs, and infertility by the fourth to sixth generation (G4-G6)[173,174]. Interestingly, G4 TERT-deficient mice have severe metabolic and mitochondrial changes, especially in networks regulated by the alpha and beta of peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1α and PGC-1β), which control mitochondrial biogenesis and metabolic processes such as OXPHOS, gluconeogenesis, fatty acid metabolism and β-oxidation[175]. When the liver and heart mitochondria of G4 mice were examined, there was repression of OXPHOS genes from all five ETC complexes, reduced activity of ETC complexes, reduced oxygen consumption, decreased ATP synthesis, decreased levels of antioxidant enzymes (catalase, GPx, SOD1) and increased mitochondrial ROS production[174]. In a separate study, when G4 mice were fed a high fat, high sucrose diet for 8 weeks, there was increased insulin resistance and glucose intolerance in liver and muscle tissue compared to controls[176].

TERT overexpressing mice exhibit improvement in metabolic decline during aging. Constitutive expression of TERT in a mouse with a cancer resistant background was shown to have delayed telomere shortening and an extension of median lifespan accompanied by a youthful physiological profile, including delayed degenerative and inflammatory age-related pathologies of organs such as the skin, intestine, kidney, liver and brain[177]. In addition, these mice had improved glucose tolerance compared to aged wild type controls[177]. When a metabolic profile of wild-type young and aged mice was compared to the same TERT overexpressing cancer-resistant mice, aged TERT overexpressing mice had a younger metabolic age compared with wild-type mice of the same chronological age[178]. The same effect held true for aged mice receiving TERT gene therapy via adeno associated virus vectors (AAV) in various stratified epithelia[179]. When adult and old mice were given two months of TERT gene therapy, researchers observed

improved insulin sensitivity and glucose tolerance, along with a reversal of the metabolic signature associated with aging compared to age matched wild-type mice[178,179].

Studies using TERT-deficient and TERT-overexpressing mice have provided evidence supporting TERT's ability to delay aging and to alter the metabolic profile of an organism. However, research into the effect of TERT-deficiency or TERT-overexpression on the metabolism of brain cells in *vivo* is lacking. In addition, it is difficult to determine whether the protective effects observed in TERT-overexpressing mice rely on restoring telomerase activity or if non-canonical functions of TERT are at play. Based on the results of the present study, TERT-overexpression may be able to improve age-related oxidative damage and neurodegeneration by altering metabolism in favour of aerobic glycolysis, however this is yet to be confirmed *in vivo*.

4.4 TERT alleviates mitochondrial ROS production

The decrease in ROS production under H_2O_2 and Aβ oxidative stress treatments in TERT overexpressing cells corroborates previous research, although a mechanism is still speculative. As previously discussed, TERT can decrease ROS through improving respiratory chain activity by decreasing succinate-linked respiration, complex I and IV activity and UCP2 expression[73,80,81]. TERT has also been shown to increase the availability of glutathione for GPx activity as well as promote more rapid recovery of peroxidases from a hyperoxidized state[81].

The change in PKM1/PKM2 protein levels in TERT overexpressing cells during H_2O_2 and Aβ treatments levels suggests a metabolic mechanism for alleviating ROS production under oxidative stress treatment. As previously described, allosteric regulation allows PKM2 to switch from a high-activity tetrameric state to a low-activity dimeric state[139]. The dimeric form of PKM2 causes the accumulation of glycolytic intermediates and a shift towards the PPP, which is an alternative pathway for glycolysis that promotes the synthesis of nucleotides and aromatic amino acids[180]. The PPP also produces the reducing factor NADPH that can be used for antioxidant enzymes and to recycle the anti-oxidant glutathione needed for GPx activity, which may further contribute to the decreased ROS effect observed in this study. Additional support arises from previous studies that

demonstrate TERT overexpressing cells have higher reduced versus oxidized glutathione compared to control cells[81].

Future experiments should assess levels of NADPH in TERT overexpressing cells under oxidative stress conditions to confirm if there is an upregulation of the PPP. In addition, levels of reduced glutathione and GPx activity should be assessed to determine if changes in antioxidant activity contributes to a reduction in ROS levels observed under Aβ exposure in TERT overexpressing cells.

4.5 TERT improves cell viability

The MTT assay was used to reveal that TERT overexpressing cells have increased cell viability and proliferative capacity under control and H_2O_2 or AB oxidative stress. Immunofluorescence experiments illustrated that TERT localizes to the nucleus under control conditions and has partial localization with the mitochondria under H_2O_2 and AB oxidative stress treatments. This suggests that TERT may have nuclear as well as mitochondrial functions that contribute to cell viability and proliferation.

4.5.1 *TERT overexpression may reconstitute telomerase activity*

Since TERC expression or telomerase activity was not tested in the current study, it is possible that overexpression of TERT may reconstitute telomerase activity in combination with endogenous TERC. This may lead to telomere elongation and a subsequent increase in proliferative capacity of cells, as is expected with TERT's canonical function. Recently, an alternative TERC has been identified in the mouse brain and has been shown to interact with both mouse and human versions of TERT, leading to an increase in telomerase activity and increased cell survivability under H_2O_2 oxidative stress[181]. Therefore, there is also the possibility of an alternative TERC present in the mouse HT22 cell line that may reconstitute telomerase activity and contribute to increased cell viability and proliferation in TERT overexpressing cells. Additional experiments should be performed to ascertain TERC expression in HT22 cells and whether overexpression of TERT restores telomerase activity and extends telomeres.

4.5.2 *TERT may have nuclear functions that contribute to cell viability*

While it is possible that telomerase activity may contribute to improved cell viability, previous research suggests that TERC expression is absent from hippocampal neurons which express TERT[46]. Therefore, it is possible that TERT may influence cell viability in HT22 cells in a TERC-independent manner. As previously discussed, TERT may act as a transcription factor in the nucleus to increase the expression of genes involved in cell growth and proliferation[48]. TERT interacts with p65 and p50 to influence transcription of target genes of the redox sensitive transcription factor NF-κB[55]. Furthermore, estrogen receptor activation was shown to increase TERT's association with p65 and NF-κB and protect against Aβ toxicity[182]. It is known that NF-κB can influence transcription of antioxidants such as Mn SOD, metallothionein and glutathione-S-transferase[183]. Therefore, TERT may be exerting its pro-survival effects through upregulating the transcription of antioxidants targeted by NF-κB. Alternatively, TERT can increase transcription of genes in the Wnt/β-catenin signalling pathway which is involved in stem cell proliferation and self-renewal[57]. In addition to transcriptional regulation, TERT may contribute to telomere capping and chromosomal structure, independent of telomere lengthening[58–61]. To determine if TERT acts as a transcription factor to increase the expression of pro-survival genes, future studies could use DNA microarrays or RNA-Seq to identify genes that are upregulated or downregulated under H_2O_2 and Aβ induced oxidative stress in TERT overexpressing cells.

4.5.3 *TERT may have mitochondrial functions that contribute to cell viability*

It is highly probable that TERT's effect on cell viability arises from its mitochondrial localization, since the mitochondria controls the cell's intrinsic apoptosis pathway. As previously discussed, TERT inhibition increases CC3 expression, decreases Bcl-2/Bax ratio expression and reduces mitochondrial membrane potential in cultured neurons under oxygen and glucose deprivation[98]. In addition, it was shown that TERT upregulated Bcl-2 and downregulated Bax and p53 in rat spinal cord motor neurons; events linked to TERT-mediated anti-apoptotic effects[99]. Furthermore, it was established that TERT maintained mitochondrial membrane potential in mouse hippocampal neurons exposed to Aβ[133].

Therefore, levels of Bcl-2/Bax, p53 and cleaved caspases as well as mitochondrial membrane potential should be measured to determine if supressing the intrinsic apoptosis pathway is a mechanism owing to the observed increase in cell viability in TERT overexpressing cells under H_2O_2 and Aβ oxidative stress.

4.6 TERT is a potential therapeutic for Alzheimer's Disease

4.6.1 *Aβ$_{25-35}$ treatment has the same effect as other Aβ species*

It has been established that $A\beta_{1-42}$ and $A\beta_{1-40}$ are the predominant forms of Aβ within insoluble extracellular plaques that interfere with cognitive function in AD[184,185]. However, racemization of $A\beta_{1-40}$ causes the *in vivo* conversion to the toxic $A\beta_{25-35}$ fragment, which is also present in senile plaques and degenerated CA1 neurons in AD brains[186]. It has been proposed that $A\beta_{25-35}$ is the biologically active region responsible for Aβ toxicity, since it is the shortest fragment able to form stable B-sheet aggregates and induce neurotoxicity[40–42]. Similar to $A\beta_{1-42}$, $A\beta_{25-35}$ is able to spontaneously transform from soluble oligomers to aggregated fibrils and the monomeric form of $A\beta_{25-35}$ is also cytotoxic[190–192]. A previous study has also characterized the effect of Aβ species on rat brain mitochondria and reported similar effects with exposure to $A\beta_{1-42}$ or $A\beta_{25-35}$. Both Aβ peptides accumulated in the mitochondria, caused declines in respiratory chain complexes I, II and IV activity, and increased mitochondrial membrane viscosity, all resulting in a strong inhibition of ATP synthesis[193]. In addition, there was an increase of cytochrome C release upon $A\beta_{1-42}$ or $A\beta_{25-35}$ exposure[193]. Furthermore, additional studies observed that $A\beta_{1-40}$ or $A\beta_{25-35}$ exposure results in pro-apoptotic effects such as increased cleaved caspase 3 and 8, reduced Bcl-2/Bax protein ratio, loss of mitochondrial membrane potential and mitochondrial DNA damage in murine cerebrovascular endothelial cells[132,194]. Therefore, $A\beta_{25-35}$ is an appropriate model to study the toxic

effects of full length $A\beta_{1-42}$ and it is expected that results generated with $A\beta_{25-35}$ in this study would yield parallel findings if other toxic species of Aβ were used. However, it would still be worthwhile to compare the ability of exogenously expressed TERT to protect against toxicity elicited by various species of $A\beta_{1-42}$.

4.6.2 *Metabolic alterations in AD may be mitigated with TERT*

As previously described, patients with AD have reduced glucose uptake, altered glucose metabolism and damage to mitochondrial components of OXPHOS[110,111,113]. These metabolic characteristics are also reflected in transgenic mouse models of AD that express the mutant forms of human genes encoding APP or PSEN1 to promote amyloidogenic processing of the Aβ peptide. Researchers observe a downregulation of mitochondrial biogenesis, decrease in activity and expression of OXPHOS complexes, impaired glucose tolerance, and reduced insulin sensitivity in the hippocampus of 3 and 6 month old transgenic AD mice[195]. Thus, targets that combat the early metabolic changes in hippocampal neurons caused by Aβ accumulation may prove to be effective in delaying the progression of AD. The current study demonstrates that TERT is protective against Aβ induced oxidative stress in a hippocampal neuronal cell line by altering metabolism away from OXPHOS and towards aerobic glycolysis, leading to reduced mitochondrial ROS production and improved cell viability. Given the results of this study and previous studies that show mouse models using TERT gene therapy improves glucose tolerance and insulin sensitivity, it stands to reason that TERT overexpression may also be beneficial to mitochondrial function of hippocampal neurons exposed to Aβ *in vivo*.

4.6.3 *TERT can be used as a therapeutic agent without increasing cancer*

TERT overexpression in somatic cells increases replicative lifespan of cells, but no transformation to a malignant cancer phenotype occurs, even in the presence of oncogenic HRAS expression[196]. Similar studies have confirmed that TERT overexpressing somatic cells respond normally to contact inhibition, cell-cycle checkpoints and growth arrest treatments and do not form tumors when injected into mice, even when 10 times the amount of cells are used[197–199]. A possible explanation for

this may be that most tumors with elevated hTERT expression occur after a mutation arises in the tumor suppressor *p53* gene, indicating that multiple mutations are needed for a malignant phenotype[37]. These findings also extend to animal models. When adult and old mice were given TERT gene therapy via adeno associated virus vectors (AAV), researchers observed improved insulin sensitivity and glucose intolerance, decreased incidence of osteoporosis, improved neuromuscular and object-recognition tasks and extended lifespan, all without increasing tumorigenic activity[179]. Therefore, given the evidence of TERT's protective effects against Aβ toxicity presented in this study, ectopic TERT expression could possibly be used as a therapeutic for AD and other neurodegenerative diseases.

4.7 Final Remarks and Future Directions

The current study demonstrates that TERT partially localizes to the mitochondria under H_2O_2 and Aβ induced oxidative stress and causes a shift in metabolism towards aerobic glycolysis. In addition, mitochondrial ROS production was lessened during toxic Aβ exposure and cell viability was improved with TERT overexpression. While these results illustrate that TERT is protective against H_2O_2 and Aβ toxicity, further research is needed to determine the mechanisms behind this effect.

Although one may argue that aerobic glycolysis is inefficient at producing ATP compared to the mitochondria when measured per unit glucose, studies have proven that the rate of ATP production through aerobic glycolysis is comparable to OXPHOS[200,201]. While changes to metabolic enzymes in TERT overexpressing cells during oxidative stress conditions provides compelling evidence in favour of aerobic glycolysis, future experiments should measure glucose uptake, lactate production and oxygen consumption to confirm. In addition, the rates of glycolysis versus OXPHOS should be measured using other methods (Ex. Seahorse XF24 Analyzer) to confirm an increase in aerobic glycolysis following oxidative stress treatment in TERT overexpressing cells.

It is not entirely clear whether nuclear or mitochondrial localization of TERT is providing the observed benefits against oxidative stress. TERC expression and telomerase activity should be assessed in TERT overexpressing cells to determine if TERT's canonical

function in the nucleus provides protection under H_2O_2 and Aβ induced oxidative stress. In addition, modifications to TERT can be used to alter organelle localization. An exclusively mitochondrial TERT could be engineered with a constitutive mitochondrial targeting sequence at the amino terminus and deletion of the nuclear localization signal at amino acid residues 222-240. Alternatively, nuclear export of TERT can be inhibited by increasing activity of Shp2 or the 14-3-3 signalling protein along with deletion of the nuclear export signal located at the C-terminus of TERT. Furthermore, the RT domain of TERT should be removed or mutated to determine whether the protective effects are dependent on the catalytic function of TERT. Then, protein levels of metabolic enzymes, mitochondrial ROS production and cell viability can be assessed after exposure to toxic levels of H_2O_2 and Aβ in HT22 cell cultures overexpressing either nuclear-/mitochondrial- targeted and inactive/active TERT.

Due to the previous literature on TERT's non-canonical mitochondrial functions, it would be promising if the same metabolic shift, alterations in mitochondrial ROS production, and changes in cell viability are observed with overexpression of a TERT protein engineered to be constitutively mitochondrially located. The observation of TERT's ability to promote aerobic glycolysis under Aβ-induced oxidative stress suggests that TERT gene or protein therapy may be a therapeutic for AD. However, further research is needed to elucidate the mechanisms behind TERT's metabolic and mitochondrial functions in hippocampal neurons *in vitro* and *in vivo*.

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Appendix: Supplemental Figures and Tables

TERT Reverse primer- 5'{AGGTGAACCAGCACGTCG}3'

hCMV Promoter

CCCCCCNTATTGACGTCAATGNNCGGNNAAAATGGCCCGCCTNGNCNNTNATNNCCAGTACATGACCTNNTGNGACTT

TCCTACTNGNCAGTACANNNNACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTNGGCAGTACATCAATGGGCG

NNGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAAT

CAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTC

T7 Promoter

TATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAGGG

FLAG Epitope

AGACCCAAGCTTGGTACCAACATGGACTACAAGGACGACGATGACAAGGGAATTCCCGGGTCGACCCACGCGTCCGGG

hTERT sequence

CAGCGCTGCGTCCTGCTGCGCACGTGGGAAGCCCTGGCCCCGGCCACCCCCGCGATGCCGCGCGCTCCCCGCTGCCGA

GCCGTGCGCTCCCTGCTGCGCAGCCACTACCGCGAGGTGCTGCCGCTGGCCACGTTCGTGCGGCGCCTGGGGCCCCAGG

GCTGGCGGCTGGTGCAGCGCGGGGACCCGGCGGCTTTCCGCGCGCTGGTGGCCCAGTGCCTGGTGTGCGTGCCCTGGG

ACGCACGGCCGCCCCCCGCCGCCCCCTCCTTCCGCCAGGTGTCCTGCCTGAAGGAGCTGGTGGCCCGAGTGCTGCAGAG

GCTGTGCGAGCGCGGCGCGAAGAACGTGCTGGCCTTCGGCTTCGCGCTGCTGGACGGGGCCCGCGGGGGCCCCCCCGA

GGCCTTCACCACCAGCGTGCGCAGCTACCTGCCCAACACGGTGACCGACGCACTGCGGGGGAGCGGG

TERT Forward primer- 5'{CCCTCTGCTACTCCATCCTG}3'

hTERT sequence

CGCTGGGGGCCAGGGCGCCGCCGGCCCTCTGCCCTCCGAGGCCGTGCAGTGGCTGTGCCACCAAGCATTCCTGCTCAA

GCTGACTCGACACCGTGTCACCTACGTGCCACTCCTGGGGTCACTCAGGACAGCCCAGACGCAGCTGAGTCGGAAGCT

CCCGGGGACGACGCTGACTGCCCTGGAGGCCGCAGCCAACCCGGCACTGCCCTCAGACTTCAAGACCATCCTGGACGA

FLAG Epitope **SP6 Promoter**

CTACAAGGACGACGATGACAAGTGATCTAGAGGGCCCTATTCTATAGTGTCACCTAAATGCTAGAGCTCGCTGATCAGC

BGH Terminator

CTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTC

CCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGT

GGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGCTTCTG

pMB1 ori

AGGCGGAAAGAACCAGTGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAA

AGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCA

TCACAAAAATCGACGCTCAAGTCAGANGTGGCGAANCCCGACAGGACTATNAAGATACCAGGCGTTTCCCCCTGGAAG

CTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACNGGATANCTGTNCGCCTTTCTCCCTTNNGGAAGCGNGGCGC

TTTCTCATAGCTCACGCTGNAGGTATCTCAGTTCGGTGTANGTCGTTCGCTCCAAG

Appendix 1: Sequencing Information of FLAG-TERT overexpression plasmid. The 071 plasmid (referred to as FLAG-TERT) overexpression plasmid was provided without a plasmid map so sequencing was performed by creating TERT Forward and TERT Reverse primers to confirm coding of the FLAG epitope and the TERT protein. The resulting sequencing information is provided in the 5' to 3' direction. Sequencing revealed a FLAG epitope at the N- and C- terminus of TERT coding regions. Potential start codons are highlighted in yellow.

TERT Reverse primer- 5'{AGGTGAACCAGCACGTCG}3'

hTERT sequence

GANCATGGAGANCNNNNNNTTTGCGGGGNTTNNNNCGGGACGGGCTNNNTCNTGCNTTTTGNTGGATGANTTTCTNGT

TGGTGACACNNCACNTCACCCCACGCGAAAACCTTCCTCAGGACCNTGNTCCGAGNNTGTCCCTGAGTATGGCTGCGT

GGTGAACTTGCGGAAGACAGTGGTGAACTTNCCCTGTAGAAGACGAGGCCCTGGGTGGCACGGCTTTTGTTCAGATGC

CGGCCCACGGCCTATTCCCCTGGTGCGGCCTGNTGCTGGATACCCGGACCCTGGAGGTGCAGAGCGACTACTCCAGCTA

TGCCCGGACCTCCATCAGAGCCAGTCTCACCTTCAACCGCGGCTTCAAGGCTGGGAGGAACATGCGTCGCAAACTCTTT

GGGGTCTTGCGGCTGAAGTGTCACAGCCTGTTTCTGGATTTGCAGGTGAACAGCCTCCAGACGGTGTGCACCAACATCT

ACAAGATCCTCCTGCTGCAGGCGTACAGGTTTCACGCATGTGTGCTGCAGCTCCCATTTCATCAGCAAGTTTGGAAGAA

CCCCACATTTTTCCTGCGCGTCATCTCTGACACGGCCTCCCTCTGCTACTCCATCCTGAAAGCCAAGAACGCAGGGATG

TCGCTGGGGGCCAAGGGCGCCGCCGGCCCTCTGCCCTCCGAGGCCGTGCAGTGGCTGTGCCACCAAGCATTCCTGCTCA

AGCTGACTCGACACCGTGTCACCTACGTGCCACTCCTGGGGTCACTCAGGACAGCCCAGACGCAGCTGAGTCGGAAGC

TCCCGGGGACGACGCTGACTGCCCTGGAGGCCGCAGCCAACCCGGCACTGCCCTCAGACTTCAAGACCATCCTGGACG

FLAG Epitope

ACTACAAGGACGACGATGACAAGTGATCTAGAGGG

TERT Forward primer- 5'{CCCTCTGCTACTCCATCCTG}3'

hTERT sequence

CGCTGNNGGCNNAGGGCGCCGCCGGCCCTCTGCCCTCCGAGGCCGTGCAGTGGCTGTGCCACCAAGCATTCCTGCTCA

AGCTGACTCGACACCGTGTCACCTACGTGCCACTCCTGGGGTCACTCAGGACAGCCCAGACGCAGCTGAGTCGGAAGC

TCCCGGGGACGACGCTGACTGCCCTGGAGGCCGCAGCCAACCCGGCACTGCCCTCAGACTTCAAGACCATCCTGGACG

FLAG Epitope **SP6 Promoter**

ACTACAAGGACGACGATGACAAGTGATCTAGAGGGCCCTATTCTATAGTGTCACCTAAATGCTAGAGCTCGCTGATCA

BGH Terminator

GCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCAC

TCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGG

GTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGCTTC

pMB1 ori

TGAGGCGGAAAGAACCAGTGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCA

AAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATANGCTCCGCCCCCCTGACGAG

CATCNCAAAANNCGACGCTCAAGTCAGANGTGGCGAANCCCGACAGGANTATAAAGATACCAGGCGTTTCCCCCTGGN

AANCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTA

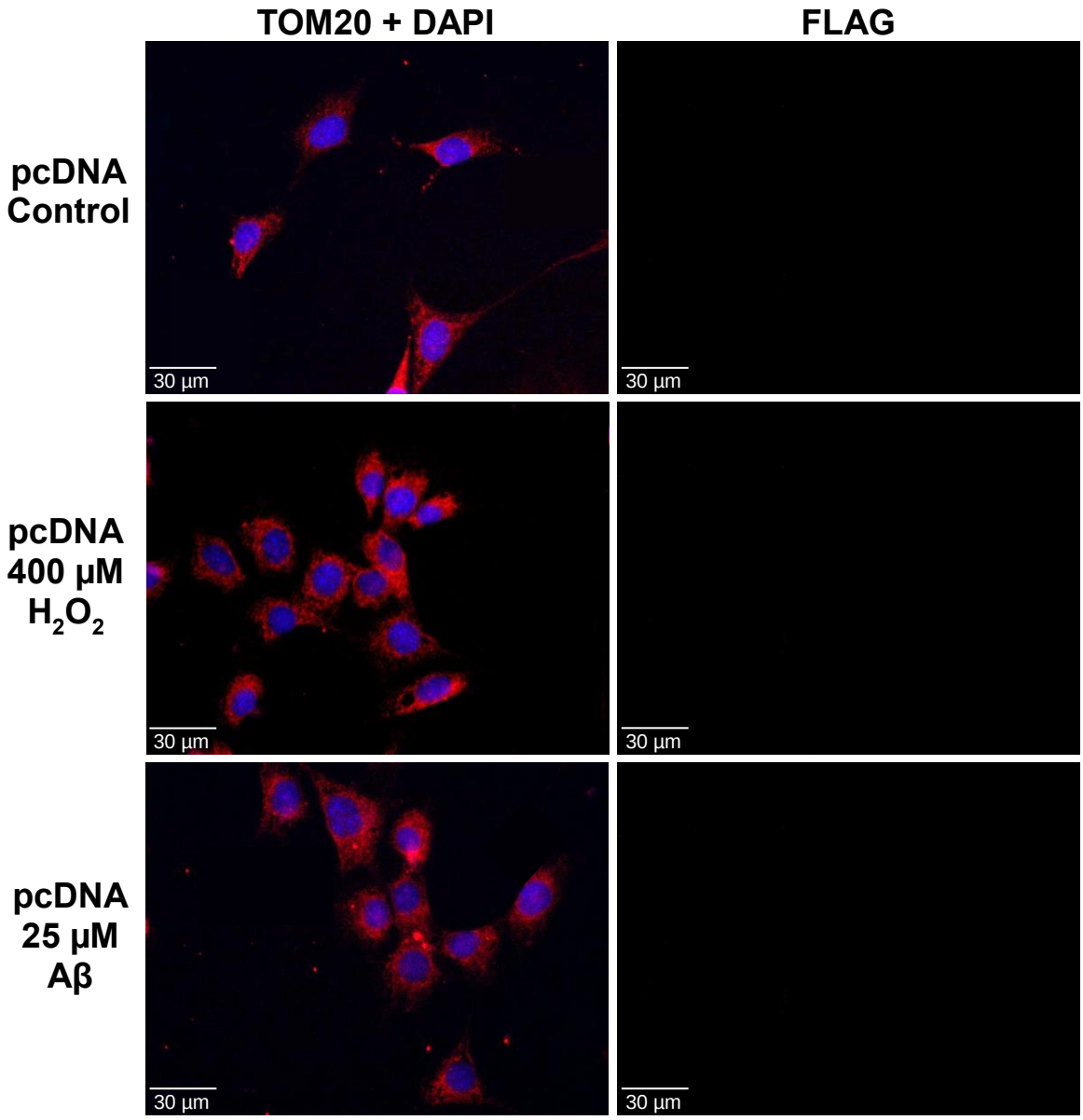
Appendix 2: Sequencing Information of TERT overexpression plasmid.

The 072 (referred to as TERT) overexpression plasmid was provided without a plasmid map so sequencing was performed by creating TERT Forward and TERT Reverse primers to confirm coding of the TERT protein. The resulting sequencing information is provided in the 5' to 3' direction. Although sequencing revealed a FLAG epitope at the C-terminus of TERT, anti-FLAG antibodies could not detect FLAG by Western blot or by immunofluorescence in 072 (TERT) transfected cells.

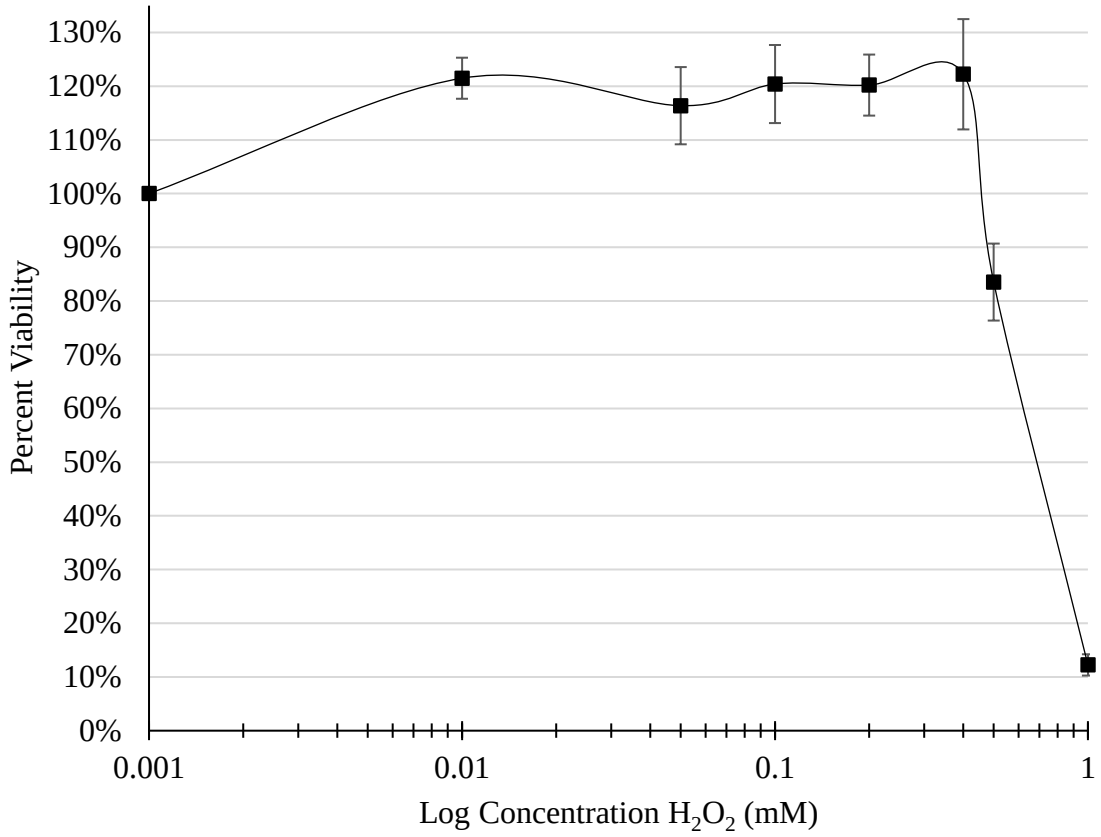
Antibodies and Dyes

Target	Host	Company	Catalogue Number
hTERT	Rabbit polyclonal	Rockland	600-401-252S
FLAG	Mouse monoclonal	Sigma	F3165
TOM20	Rabbit polyclonal	Santa Cruz	sc-11415
PDH	Mouse monoclonal	abcam	ab110334
$pSer^{232}$ PDH	Rabbit polyclonal	EMD Millipore	AP1063
PDK1	Rabbit polyclonal	Enzo Life Sciences	ADI-KAP-PK112-F
LDHA	Rabbit polyclonal	Cell Signalling	#2012
PKM1	Rabbit monoclonal	Cell Signalling	#7067
PKM2	Rabbit polyclonal	Cell Signalling	#3198
HRP-conjugated secondary mouse	Goat	Santa Cruz	sc-2030
HRP-conjugated secondary rabbit	Goat	Santa Cruz	sc-2005
Alexa Fluor 488	Goat anti-mouse polyclonal	Sigma	R37120
Alexa Fluor 594	Goat anti-rabbit polyclonal	Sigma	A-11008
MitoTracker CMXROS	---	Invitrogen	M7512
MTT	---	Sigma	M2003
DAPI (ProLong Antifade Mountant with DAPI)	---	Invitrogen	P36962

Appendix 3: Table 1: List of antibodies and dyes.



Appendix 4: **pcDNA transfected cells do not exhibit anti-FLAG immunoreactivity following immunofluorescence staining.**
Immunofluorescence staining of HT22 cells transfected with the pcDNA expression construct. Cells were exposed to H_2O_2 (400 µM) or $A\beta_{25-35}$ (25 µM) for 12 hours post-transfection and stained with anti-FLAG and anti-TOM20 antibodies. There is no visible FLAG fluorescence in all treatments. (Green (FLAG), Red (TOM20), blue (DAPI). 40X objective lens. Scale bars are 30 µm.



Appendix 5: **Dose Response Curve of HT22 cells following H_2O_2 exposure.**
HT22 cells were seeded in 96 well plates at 7,000 cells/well then exposed to the indicated concentrations of H2O2 for 24 hours. Cell viability was determined using the MTT assay and expressed as a percentage of untreated cells. The LD50 was determined to be 0.67 mM.

Curriculum Vitae

Name: Olivia Singh

Post-secondary Education and Degrees:

University of Toronto
Toronto, Ontario, Canada
2011-2015 Hons. B.Sc.
(Neuroscience & Genome Biology)

The University of Western Ontario
London, Ontario, Canada
2015-2017 MSc.
(Neuroscience)

Honours and Awards:

Western University- Department of Biology Travel Award, 2016

Western University-Graduate Research Forum Poster Award, 2016

Canadian Association of Neuroscience
5th Neurometabolic Meeting Best Poster Award, 2017

Related Work Experience

Teaching Assistant
The University of Western Ontario
2015-2017

Laboratory Assistant
The Hospital for Sick Children
2014-2015

Psychobiology Laboratory Volunteer
The University of Toronto
2013

Poster Presentations

Neurometabolic Meeting at CAN (Montreal, QC), May 2017
London Health Research Day, March 2017
Fallona Family Interdisciplinary Showcase, Feb. 2017
Society for Neuroscience Meeting (San Diego, CA, USA), Nov. 2016
Biology Graduate Research Forum, Nov. 2016
UWO Graduate Neuroscience 25th Anniversary Research Day, May 2016

Publication:

Harris RA, Tindale L, Lone A, Singh O, Macauley SL, Stanley M, Holtzman DM, Bartha R, & Cumming RC. (2016). Aerobic glycolysis in the frontal cortex correlates with memory performance in wild-type mice but not the APP/PS1 mouse model of cerebral amyloidosis. *Journal of Neuroscience* 36(6), 1871-1878.