ABSTRACT

Title of Document: TELOMERE DYNAMICS AND REGULATION: EFFECTS OF CHRONIC EXERCISE, ACUTE EXERCISE, AND OXIDATIVE STRESS

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This dissertation research is comprised of three studies each examining the effects of chronic exercise, acute exercise, or oxidative stress on telomere biology. Exercise training and physical activity have previously been associated with telomere maintenance, but the underlying mechanisms of this association are unclear. The majority of studies to date have been performed in immune cells; however, the findings from these cells may not reflect telomere biology in other tissues. Since exercise is a multi-organ stimulus we sought to describe the effect of exercise on telomere biology in multiple tissues, with a particular focus on skeletal muscle. Study #1 showed that the effect of chronic voluntary exercise on telomere length in CAST/Ei mice is tissue specific. Exercise was 'telo-protective' (i.e., maintained telomere length) in cardiac and liver tissues, while telomere shortening was observed in skeletal muscle of exercised animals compared to sedentary and young mice. Study #2 was performed to elucidate the responses to acute exercise that could underlie the paradoxical response of telomere

length in skeletal muscle to exercise training. This study revealed that the MAPK pathway appears to be related to the expression of telomere binding proteins in response to acute exercise. In skeletal muscle, p38 MAPK mediated a decrease in gene expression of telomere binding proteins, providing insight into a possible mechanism for eventual telomere shortening in response to chronic exercise. The results of study #2 indicate that the early cellular responses to exercise may accumulate (i.e., repeat bout effect) and underlie the shortened telomere length in skeletal muscle. Study #3 sought to determine if reactive oxygen species were a plausible mechanism of telomere shortening in adult skeletal muscle fibers, as no mechanism to date has been elucidated for telomere shortening in this tissue. Study #3 showed that oxidative stress is a potent telomere-shortening stimulus in skeletal muscle fibers of mice and that telomere binding protein expression was also significantly affected by oxidative stress. In total these results indicate that although chronic exercise attenuates telomere shortening in most tissues, skeletal muscle demonstrates a unique contradictory response likely due to its reaction to oxidative stress.

TELOMERE DYNAMICS AND REGULATION: EFFECTS OF CHRONIC EXERCISE, ACUTE EXERCISE, AND OXIDATIVE STRESS

By

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Chapter 1: Introduction and Specific Aims

Background

As of 2009, 39.6 million people over the age of 65 years live in the U.S. (1) representing about 13% of the total population. By the year 2030 this group is expected to grow to about 19% of the population, due in large part to advances in hygiene and modern medicine. Despite these advances and the ability to delay diseases of aging, interventions to delay or prevent the aging process itself are lacking and the underlying science of aging remains incompletely understood. Some interventions can impact the aging process, including caloric restriction, which increases mean lifespan in rodents and humans (2), and long-term voluntary exercise training, which can delay aging in rodents (3). Exercise training has also been shown to reduce all-cause morbidity and mortality, though evidence is lacking for the cellular and molecular determinants of how exercise delays aspects of aging thus leading to an improved 'health-span'. Understanding how exercise alters the trajectory of aging may elucidate new therapeutic targets to intervene on the aging process.

Telomeres are recognized as a biomarker of aging due to the end-replication problem, in which chromosomal DNA is shortened slightly with each replication. Telomeres and the many related proteins that assist in maintaining telomere length have been the focus of intense research in aging and cancer. Telomeres not only play a role in determining the replicative capacity of cells but also maintain chromatin structures that are vital to transcriptional control (4), thus having relevance to both replicating and non-replicating tissues (e.g., skeletal muscle). Environmental factors (e.g., diet, physical activity, psychological stress) have recently been shown to alter telomere biology.

Evidence is mounting that physical activity is a telomere-maintaining stimulus (5-9), which may explain one means by which chronic exercise delays aging and age-related disease. Much of the research on environmental influences on telomeres has focused on replicating cell types such as peripheral blood mononuclear cells; however, in post-mitotic tissues the role of telomeres and their response to environmental stimuli is less understood. In post-mitotic tissues such as skeletal muscle (10,11) telomere shortening may occur due to non-replication linked mechanisms, such as oxidative stress.

In preliminary studies leading to this dissertation, we observed diametric responses of telomere length in skeletal muscle compared to cardiac tissue in animals that voluntarily exercised for 44 weeks (1 year old animals). Skeletal muscle telomere length decreased in the exercised animals compared to age-matched sedentary and young animals, while in cardiac tissue telomere length was maintained in exercised animals compared to age-matched sedentary animals. These data led to several questions: Why does a post-mitotic tissue such as skeletal muscle respond to endurance type exercise with telomere shortening while telomere length is maintained in response to exercise in cardiac tissue (also traditionally viewed as post-mitotic)? Could the response of skeletal muscle be due to repeated bouts of exercise, with the initial bouts resulting in unrepaired damage to the telomere or alterations in telomere biology leading to subsequent telomere shortening after many bouts? What are the regulatory factors or signals activated in acute exercise that might lead to this counterintuitive response in skeletal muscle?

The overall aim of this dissertation research was to determine the effects of chronic and acute exercise or oxidative stress on telomere dynamics in multiple tissues with a focus on skeletal muscle and its paradoxical telomere shortening in response to

chronic exercise. The studies examined telomere length, several telomere length maintaining proteins (i.e., shelterin components), and proteins known to be associated with telomeres. In addition we investigated the possible involvement of exercise-induced signaling proteins in telomere responses. First, we focused on how telomere length, telomerase, and shelterin components changed in response to chronic exercise. Next, we determined the response of shelterin to acute exercise and possible signaling mechanisms that could explain why skeletal muscle telomere lengths responded differently to chronic exercise. Finally, we tested oxidative stress as a possible mechanism of telomere shortening in isolated adult skeletal muscle fibers.

Specific Aims

Specific Aim 1. To determine the effect of chronic exercise on telomere length and telomere maintaining proteins in mouse cardiac, liver, and skeletal muscle tissues.

Study #1 explored the effects of chronic exercise in short telomere mice (CAST/Ei) across multiple tissues (Ludlow et al. under review at *Journal of Gerontology: Biological Sciences*). Three groups of animals were used to determine the effect of age and chronic voluntary exercise (44 wks of exposure by 1 yr of age) on telomere length, telomerase enzyme activity, telomere binding protein content, and gene expression of several factors associated with telomere biology in skeletal muscle, cardiac muscle and liver tissue. Animals were separated into groups of baseline (8wk, sedentary), 1 year old sedentary (SED-1yr) and 1 year old exposed to 44 wks of voluntary wheel running (EX-1yr). We hypothesized that telomere length would decrease in the sedentary animals after one year in myocardium and liver, and that exercise would

attenuate decreases in these tissues. In skeletal muscle we hypothesized that telomere length would not change with age but rather remain unchanged with exercise training.

Specific Aim 2: To determine the effect of acute exercise on telomere maintaining proteins such as telomere binding proteins in mouse skeletal muscle tissues.

Study #2 investigated the effects of acute endurance type exercise (treadmill running) on the expression of telomere length regulating genes and mitogen-activated protein kinases in skeletal muscle. This study was done to begin to understand why telomere length in skeletal muscle shortens in response to exercise training. Since the adaptation of tissues to exercise is in part the accumulation of repeated bouts of exercise and transient alterations in homeostasis, understanding the signals generated during acute exercise may provide insights into the underlying mechanism of the observations in study #1. The initial gene expression response of key telomere length regulating protein to acute exercise is unknown. The transient changes in gene expression induced by each bout of exercise may translate into altered protein levels and ultimately alter telomere lengths over time. We hypothesized that acute exercise would increase MAPK activation and be associated with decreased shelterin component gene expression in mouse skeletal muscle. We used an *in vitro* cell culture model to determine potential relationships between MAPK signaling and alterations in gene expression of the shelterin components.

Specific Aim 3: To determine the effects of reactive oxygen species on telomere length, telomerase enzyme activity and shelterin in skeletal muscle of two strains of mice differing by telomere length.

Study #3 investigated the effects of reactive oxygen species (ROS) on skeletal muscle telomere length and telomere regulating proteins in two strains of mice differing in telomere length (CAST/Ei = short telomeres; C57BL/6 = long telomeres). ROS has been shown to be a potent telomere-shortening stimulus in other tissues, but no data to date exist about the effect of ROS on telomeres in skeletal muscle fibers. Telomeres not only play a role in the proliferative capacity of cells but also in genomic stability and maintenance of chromatin structure that is vital to transcription. Thus understanding the effect of ROS in skeletal muscle fibers is an important step in elucidating the possible impact of ROS in the telomere shortening observed in adult skeletal muscle in response to chronic exercise. Isolated skeletal muscle fibers were treated with an oxidant, a combination of antioxidant and oxidant, or control conditions to determine if the observed effects were specific to the oxidative stress treatment. We hypothesized that in isolated skeletal muscle fibers oxidative stress would decrease telomere length, increase telomerase enzyme activity, and increase protein content of telomere binding proteins. We also hypothesized that oxidative stress would induce less relative telomere shortening in CAST/Ei compared to C57BL/6 mice.

Chapter 2: Review of Literature

This review of literature is structured into several sections to give an overview of basic telomere biology, the role of telomeres in aging, and the possible impact of environmental factors, in particular chronic exercise, on telomere length and telomere biology. First, a historical perspective outlines the first observations leading to the discovery of telomeres and the importance of telomeres in the aging process. Following is a review of the basic biology of telomeres, the end-replication problem and the end-protection problem. Finally, a review of the literature concerning evidence of the associations of telomeres with age-related disease and physical activity is discussed, as well as the role of physical activity as a modifier of telomere dynamics.

Historical Perspective of Telomeres

Alexis Carrel, a Nobel laureate, was interested in cells grown in culture. He established a line of chicken fibroblasts in 1908 that he cultured for 34 years, leading to the belief that cells in culture grew indefinitely (12). This idea was challenged in 1961 by results from Hayflick and Moorehead, when they observed that human skin fibroblasts grew in culture for 50 or so population doublings before undergoing what they termed cell senescence, or the Hayflick limit (13). Further work by Hayflick showed that cells from older compared to younger individuals divided fewer times in culture (14). Other scientists tried to reproduce Carrel's fibroblast observations and were unsuccessful, which when combined with the results from Hayflick and other investigators, led to the acceptance of the idea that cells in culture can only divide a limited number times. This also raised two important questions in cell biology, 'What is the role of cellular senescence in human cells? And is the limited capacity of cells to divide related to aging,

or is it a tumor suppressing mechanism?' These basic questions, and the evidence from the investigations trying to answer these questions will be outlined in the following section.

Working separately but at about the same time, James Watson (1972) (15) in the United States and Alexel Olovnikov (1973) (16) in then the Soviet Union, both hypothesized that the ends of linear chromosomes should shorten due to the action of DNA polymerase, a process later known as the 'end-replication problem'. Alexel Olovnikov hypothesized that the chromosome end shortening may be responsible for the Hayflick limit.

Barbara McClintock initially recognized the importance of chromosome ends in that without some protective mechanism, chromosomes would stick together and cause cellular dysfunction. At about the same time but working separately Hermann Muller recognized that something at the end of a chromosome gave the DNA stability and coined the term, 'telomere' from the Greek words meaning 'end' (*telos*) and 'part' (*meros*) (17). Chromosome ends where later sequenced by Nobel winner Elizabeth Blackburn who was interested in the structure that allowed DNA to be maintained as a linear chromosome (18). In 1985 working in Elizabeth Blackburn's lab, Carol Greider discovered the enzyme that maintains chromosome ends known as telomerase (19). In addition, it was observed that telomeres were conserved across many species and that in mammals telomere length is tissue specific (20). Since these early observations our understanding of telomeres and telomere biology has greatly increased.

Basic Aging Cell Biology

The continuity of life rests on the fact that cells must divide to reproduce. A cell undergoes an orderly process in which it duplicates all of its cellular components and then splits in two. The cycle of duplication followed by division is known as the cell cycle. The cell cycle consists of five distinct stages that are tightly regulated depending upon several factors not limited to nutrient availability and mitogen-stimulation. The five stages are defined as M phase or mitosis, G_1 phase or growth/ gap 1, S phase or synthesis, G_2 phase or growth/ gap 2, and G_0 or quiescence. Collectively G_1 through G_2 are termed 'interphase'.

Cyclins - Numerous proteins and protein kinases control the cell cycle and its progression. The proteins consist of a family of proteins called cell division cycle genes (*Cdc*) or the cyclins. The kinases that dictate the action of the cyclins are a family of proteins called the cylin-dependent kinases (*Cdk*). To enter and exit each phase of the cell cycle a specific *Cdc* and *Cdk* interaction must take place in a highly ordered fashion for appropriate cell division. In addition to the *Cdc – Cdk* interaction, a Cdk-activating complex is necessary for full activation of cell cycle specific events (21,22). The major *Cdc* and *Cdk* partners are shown in Table 2.1.

Table 2.1. Cyclins and cyclin-dependent kinase partners in specific cell cycle phases. Adapted from Table 17-1 from (23). Only vertebrate genes are shown.

Cyclin (Cdc)	**Cdk partner**	**Cell phase**
Cyclin D	Cdk4, Cdk6	G_1
Cyclin E	Cdk2	G_1/S
Cyclin A	Cdk2, Cdk1	S
Cyclin B	Cdk1	M

Cell cycle inhibition – In certain cell types and under homeostatic stress (e.g., DNA damage) the cell cycle must be inhibited to allow for cellular damage to be repaired or for cells to undergo cell death, either spontaneous or programmed. Two major families of proteins regulate inhibition of the cell cycle: the cip/kip family (CDK interacting protein/Kinase inhibitory protein) and INK4a/ARF (Inhibitor of Kinase 4/Alternative Reading Frame). The cip/kip family consists of p27, p57, and p21, which bind to cyclin-cdk complexes and arrest the cell cycle in G_1. The INK4a/ARF family consists of p16INK4a, p14ARF and p15, p18 and -19, which cause the cell to arrest in G_1 and are important in the response to cellular damage, particularly DNA damage. Cell cycle inhibition is accomplished by these proteins binding to D-type cyclins. An important cell cycle regulator that plays a role in cycle inhibition is tumor protein 53 (p53) and proteins, p21 and p14 ARF that are activated by and prevent the degradation of p53, respectively. The importance of these proteins lies in their tumor suppressor qualities; they have also been found to be highly associated with the DNA damage response at critically short telomeres (24,25).

Cell cycle checkpoints – Cell cycle checkpoints have evolved to prevent damaged or incomplete DNA from being passed onto daughter cells. The checkpoints allow the cell to verify that the necessary processes have accurately occurred before the cell progresses onto the next phase. Important functions of some of the checkpoints are to recognize DNA damage via a sensor-signal-effector mechanism. Since most cells in adult mammals are terminally differentiated, they must leave the cell cycle and enter G_0. This permanent cell cycle withdraw occurs late in G_1 at a point termed the restriction point (RP). In cells that divide continuously throughout the life of a mammal, such as

hematopoietic and gut epithelial cells, RP is overcome via growth factor-induced expression of cyclin D that allows the enhanced interaction with CDK4 and continued cell division (23). The G_1 to S phase transition is governed by cyclin dependent kinase inhibitor p16 (p16 INK4a), which inhibits the interaction of CDK4/6 with cyclin D. In replicating cells CDK4-cyclin D phosphorylates retinoblastoma protein (RB) relieving its inhibition on E2F that controls the expression of cyclin E. Once cyclin E is expressed at sufficient levels (i.e., under mitogen stimulation) cyclin E interacts with CDK2 and the cell can transition to S phase. The next checkpoint is the G2 to M phase, in which the cell must ensure many factors are correctly in place, one of which being faithful replication of DNA. Often DNA is damaged and the cell is arrested until this damage is repaired or the cell is removed via cell death. This checkpoint is governed by the actions of checkpoint 1 and checkpoint 2 (*Chek/Chk1* and *2*). *Chk1* and *2* control the phosphorylation status of cdc25, which controls the transition from G_2 to M phase. In addition, CHK2 and p53 interact and play an integral role in detecting DNA damage and determining cell fate (i.e., repair and transition or cell death) (21-23).

Mechanisms of Aging – Many hypotheses of aging exist, most of which have focused on the role of metabolism and oxidative stress (26-28). Oxidative stress, the production of reactive oxygen species (ROS) by oxygen metabolism, leads to damage of cellular components including DNA, lipids, and proteins (29). Oxygen metabolism produces reactive oxygen species (ROS) such as hydrogen peroxide, superoxide radical, singlet oxygen, and hydroxyl (30). Recently exciting new evidence has linked the free radical hypothesis and cellular senescence hypothesis (31). The free radical and cellular senescence hypotheses will be discussed below.

Oxidative stress and aging – ROS has been implicated in several pathologies including cardiovascular disease, cancer, and recently in the aging process of skeletal muscle, as well as in several skeletal muscle pathologies (specifically cachexia) (32-34). Organisms are equipped with enzymatic and non-enzymatic defense systems to scavenge and deal with ROS (30). These systems must maintain a balance between antioxidant and pro-oxidant stimuli and when pro-oxidants overwhelm antioxidants, damage to cellular components can occur. Even in the healthiest organisms, oxidative damage is apparent. Evidence from several model organisms indicates that life span can be extended with over-expression of one or more key enzymes involved in the protection against ROS (35,36). In aged and diseased skeletal muscle an increase in superoxide production can occur due to an uncoupling at complexes I and III (37). As a result of the increase in superoxide, mitochondrial bound Mn-superoxide dismutase converts the excess superoxide into hydrogen peroxide. Also, a portion of the superoxide created at complex III is released into the mitochondrial intermembrane space and converted to hydrogen peroxide by Cn,Zn superoxide dismutase. It has also been hypothesized that younger individuals can better or more efficiently respond to neutralize oxidative damage than older individuals. Therefore, older cells will have a higher vulnerability to oxidative stress and tissue damage (30). In essence, the accumulation of oxidative stress and the deleterious effects of this damage to tissues lead to aging. This hypothesis was suggested by D. Harman nearly 60 years ago and is supported by many recent investigations (30). When a cell's DNA and other biomolecules become damaged, programmed cell death, apoptosis, and the cessation of cellular proliferation leading to cell senescence may occur, resulting in an aged phenotype at the tissue and organ level.

Oxidative Stress and DNA - DNA within all cells of an organism is constantly under assault from endogenous damaging agents, estimated at tens of thousands of lesions per day (38). Oxidative stress is known to induce multiple forms of DNA damage including base oxidation (specifically guanine) and single-strand and double-strand breaks (39). When two single-strand breaks (SSB) occur in close proximity or when the DNA replication machinery encounters a SSB, double strand breaks (DSB) can occur (40). In addition to strand breaks, oxidative insults result in various chemical modifications of guanine, such as the commonly studied 8-oxoguanine (8-oxodG) (41). Unrepaired 8-oxodG can lead to altered base pairing and mutations during DNA replication (42). DNA insults that result in DNA damage can result in cellular senescence and apoptosis via p53 and p21 tumor suppressor pathways (39). DNA damage repair is a series of complex systems that consist of sensors, mediators, transducers or effectors, and cellular responses (40). Typical repair mechanisms include: base excision repair (BER), nucleotide excision repair (NER), non-homologous end-joining (NHEJ) and homologous recombination (HR) (40). DNA damage repair is regulated differently between cell types, progenitor cells preferring apoptosis over repair while terminally differentiated cells, such as adipocytes, neurons and skeletal muscle cells undergoing DNA damage repair more readily (43). Terminally differentiated skeletal muscle myotubes seem to be deficient in BER compared to myoblasts, which leads to skeletal muscle DNA being hypersensitive to oxidative DNA damage (44). Narciso et al. (44) treated terminally differentiated skeletal muscle cells with H_2O_2 and observed significant increases in SSB, phosphorylated $^{(Serine\ 139)}$ histone 2A.X (γH2A.X) foci and inefficient repair. These results indicate that myotubes (terminally differentiated

muscle cells) may accumulate DNA damage, which may become genotoxic and result in nuclei death that could lead to loss of muscle cell size, which could have important implications in aging and muscle wasting diseases.

Certain regions of the genome are more sensitive to oxidative DNA damage, such as chromosome ends or telomeres. Telomeres are particularly susceptible to DNA damage because telomeres are G-rich (guanine triplet) and thus sustain more oxidative damage than the rest of the genome (45,46) and damage at the telomere has been implicated in aging related diseases and progeria conditions (41,47). Further, SSB repair is deficient at telomeres (48); for these reasons telomeres are especially susceptible to oxidative-induced damage compared to the rest of the genome (45,46,49,50). Deciphering the role of oxidative stress in DNA damage, specifically at the telomeres, could have a profound impact on our understanding of several diseases, aging and progeria conditions.

Cellular Senescence and Aging - In contrast to the ROS model, another model of aging has been proposed by Kipling (51). In his model there are three components to aged tissues: aged extracellular environments, aging of cells that are non-mitotic, and the aging of mitotic cells. Each of these areas contributes to overall human aging. When cells that undergo division (i.e., mitosis) are removed from the body, they can only replicate a finite number of times before entering a senescent state (14). This senescent state should not be confused with cell death, necrosis, or apoptosis. In this mitosis-arrested state, (senescence) the cell is still metabolically active and can survive for many months or longer but is unable to replicate or repair damage (66).

Moorehead and Hayflick were the first to describe the cellular state of senescence,

though the mechanism was unknown. Harley et al. (52) were the first to show that the mitotic clock was housed at the ends of linear chromosomes in the repetitive DNA element termed a telomere. During *in vitro* aging, telomeres shorten with each population doubling until a critical length is reached and replicative senescence occurs (53). Telomere length has been associated with aging *in vivo* as well (54-56). Telomeres are said to act as a mitotic clock, keeping track of cell divisions until the critical length is reached and the cell enters replicative senescence (53). Thus, telomere length has been indicated as a novel biomarker of cellular aging.

End-replication Problem

Telomeres - Telomeres (5'-TTAGGG$_N$-3' up to several kilobases (kb) depending on species) are found on ends of linear chromosomes and protect the ends of DNA from damage and being recognized as damaged DNA (Figure 2.1). Following the double stranded telomeric DNA region is a 50-400-nucleotide single-stranded 3' overhang that is critical in end protection (57). Telomerase, an enzyme that maintains telomere length homeostasis by adding 5'-TTAGGG$_N$-3' repeats to telomeres, uses the guanine (G) rich 3' overhang as a primer sequence in S phase of the cell cycle (58). Telomere length, in cells lacking telomerase, is reduced with each cell division due to the inability of DNA polymerase to replicate telomere repeats, termed the 'end-replication problem (57). Thus, telomere length has been proposed as a mechanism of cellular aging, due to the fact that when telomeres reach a certain length, cells in culture enter telomere-dependent senescence (critically short telomeres lead to senescence, where a cell is metabolically active but unable to repair damage or divide) (53). Telomere length is tissue specific (i.e., telomere length within an organism will vary depending upon the tissue or cellular

population of investigation) and species specific (59). Human telomeres are typically 5-12kb in length (60) and mouse telomeres (consisting of the same sequence) are much longer (depending on inbreeding status and strain) up to 150kb (60). In humans, the rate of telomere attrition is estimated at 30-150bp per year in leukocytes and fibroblasts (61). Telomere length is not a simple switch dictating cellular fate, but rather a dynamic system controlled by external cellular stimuli and numerous proteins. Understanding the interplay of telomere length and telomere-associated proteins is critical to predicting cellular outcomes in disease states and understanding how this system responds to and deals with external stressors.

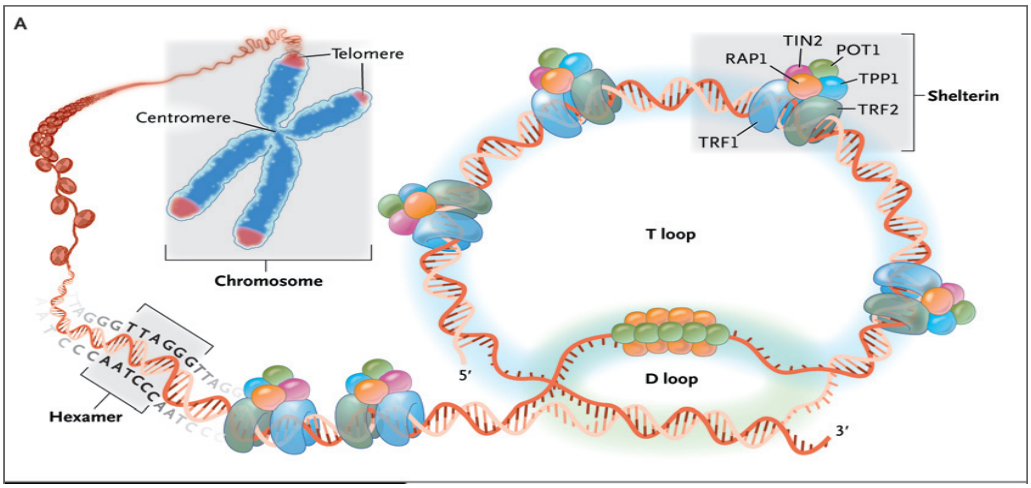

Figure 2.1. This figure shows a diploid chromosome, and an enlarged image of the chromosome end or telomere. T-Loop and D-loop conformations are also presented along with various telomere-binding proteins. Adapted from Figure 1 of Calado and Young (62).

Telomerase – Telomeres and their length are not, however, static entities, but rather are dynamic (58). In certain cells, the enzyme, telomerase, maintains and lengthens telomeres, allowing continued cell division beyond what would be expected in cells without telomerase. Discovered in 1985 by Carol Greider working in Elizabeth Blackburn's lab, telomerase is a reverse transcriptase that overcomes the end replication

problem and inability of DNA polymerase to replicate or maintain telomeres (19). Telomerase (Figure 2.2) is a specialized reverse transcriptase consisting of two components; an RNA component, encoded by the gene *Terc* (telomerase RNA component) and a protein component, encoded by the gene *Tert* (telomerase reverse transcriptase) (63). For telomerase to be functional and active both components must be expressed and present in the cell. The *Terc* gene is typically expressed ubiquitously by cells while *Tert* is only expressed in cells that have telomerase activity (63). In adult human skeletal muscle homogenates only very low levels of telomerase are detectable within the multinucleated fibers (64), while in C2C12 myoblasts telomerase is detectable (65). In C2C12 cells differentiation of myoblasts to myotubes is believed to down-regulate telomerase (66); however, under oxidative stress conditions the activity of telomerase is unknown in skeletal muscle. In tissues other than skeletal muscle, telomerase has been observed to have alternative, non-nuclear functions under stressful conditions (67).

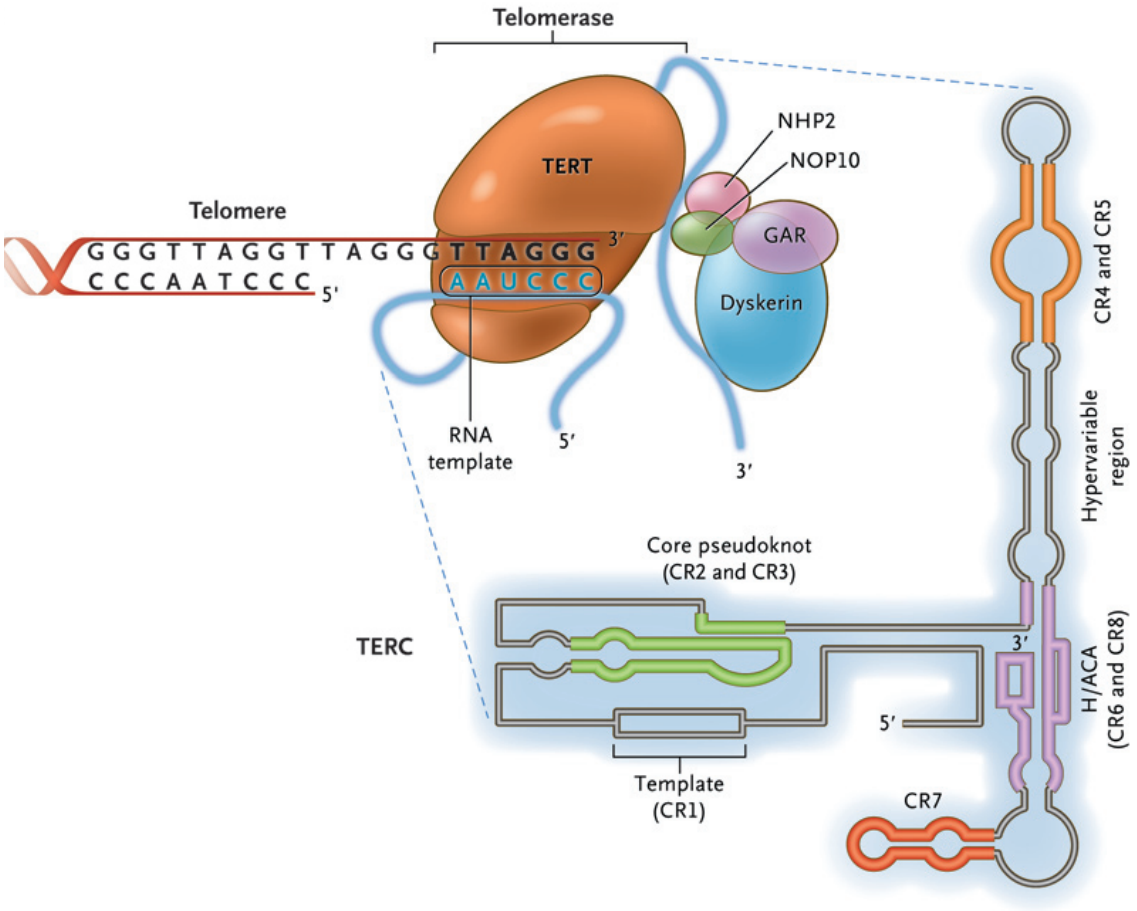

Figure 2.2. The telomerase holoenzyme and various accessory proteins. Telomerase is shown with TERC adding telomeric DNA to a chromosome end. The telomerase holoenzyme and binding partners dyskerin, GAR, NOP10, and NHP2 are also shown. In addition TERC is shown in its native RNA structure. Adapted from Figure 3 of Calado and Young (62).

End-protection problem

The end-protection problem concerns how chromosome ends are recognized. McClintock described how normal chromosome ends were protected from being stuck together but broken chromosomes were not protected from joining (68). Muller observed that chromosomes lacking their natural ends tended to fuse (68). The next observations came much later when it was observed that when foreign linear DNA was introduced into cells it was unstable and recombined into the host's genome (68). This revealed that two

important mechanisms existed to deal with broken or exposed DNA ends: homology-directed repair (HDR), and non-homologous end-joining (NHEJ) (68). Next, it was observed that some mechanism prevented the natural ends of chromosomes from being recognized as broken DNA (68). Thus the natural ends of linear chromosomes, telomeres, possess the ability to suppress kinases involved in recognizing broken DNA (ATM; ataxia telangiectasia mutated and ATR; ataxia telangiectasia and Rad3 related). In summary, the end-protection problem refers to that chromosome ends must have defenses against four separate pathways: ATM, ATR, NHEJ and HDR. Failure to do so results in cell cycle arrest (ATM or ATR mediated), end-to-end fusions (NHEJ), or sequence exchanges (HDR). Telomeres solve the end protection problem by associating with a six-protein complex termed shelterin. Shelterin creates a protective DNA-protein cap on the end of chromosomes (68). Telomeres are able to switch between a capped and uncapped state (58). When the telomeres are in a capped state telomere length is sufficiently long and able to fold back upon itself and bind to telomere binding proteins, forming a T-loop (69). Telomerase is only able to add telomere repeats from the RNA template when the telomere is in an uncapped state (i.e., unfolded or no T-loop conformation) and the telomere is accessible to telomerase (69-72). T-loops are formed by telomeres at the ends of chromosomes when telomere-repeat binding factor 2 (TRF2) is present (73). T-loops are sequences of 5'-TTAGGG$_N$-3' repeated in a loop. The 3' single stranded Guanine-rich (G-rich) section of 150-300 nucleotides invades the telomere at complimentary sequences (termed D-Loop), forming a protective structure (73). It has been shown that as little as 400bp are needed to assemble a fully functional telomere (69). Further experiments have concluded that telomere length must be below

1kb to induce senescence in cancer cells and at least thirteen 5'-TTAGGG repeats are needed to prevent end to end fusions (69). However, none of the above experiments tested whether these telomere features were sufficient to suppress a DNA damage response. Attached to and essential for the formation of T-loops is the six-protein complex shelterin (69).

Shelterin - Shelterin is a six-protein complex that protects chromosome ends from being recognized by the DNA damage response proteins (DDR), cell cycle checkpoints and inappropriate telomerase activity (Figure 2.3). Specifically telomere-repeat binding factors 1 and 2 (TRF1 and TRF2), play critical roles in telomere biology and chromosomal stability. TRF1 is a negative regulator of telomere length and is involved in mitotic progression (74), while TRF2 is crucial in maintaining the telomere 't-loop' structure and preventing chromosome end-to-end fusions and chromosome abnormalities (69). TRF2 is known to inhibit telomere end recognition by the DDR protein ATM, as well as inhibit recognition by the non-homologous end-joining (NHEJ) pathway (75). POT1 (with isoforms a and b in the mouse) is a telomere single-strand overhang binding protein essential for T-loop formation and is known to inhibit DDR recognition by ATR (76). Other shelterin components that do not interact directly with telomeric DNA but interact through protein-protein inactions with TRF1, TRF2, and POT1 are TIN2 (TRF1 – interacting nuclear protein), RAP1 (RAP1 GTPase activating protein) and TPPI/ACD (adrenocortical dysplasia homolog), respectively (69) (Figure 2.3). Interestingly, shelterin can act as a negative regulator of telomere elongation, by inhibiting telomerase or activating telomere deletion (77,78). If the integrity of the shelterin complex or the telomere cap is compromised, telomere shortening, cellular senescence and apoptosis can

occur (73,79,80). As telomeres shorten, chromosomal destabilization and lack of recruitment of shelterin proteins results in loss of T-loop formation and uncapped chromosome ends (81). This situation resembles double-stand DNA breaks and may activate the p53 or $p16^{ink4a}$ pathways leading to senescence or apoptosis (82).

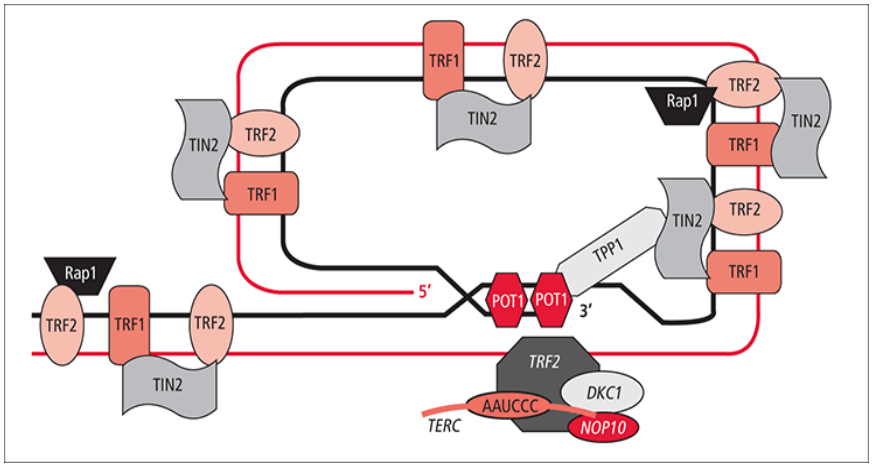

Figure 2.3. Shelterin complex bound to telomeres in a T-loop conformation. Shown are the six-shelterin proteins. Telomere repeat binding factors 1 and 2 (TRF1 and 2), protection of telomeres 1 (POT1), telomere repeat binding factor 1 interacting protein 1 (TIN2), RAP1 GTPase activating protein (RAP1), and Adrenocortical dysplasia protein homolog (ACD or TPP1) which interacts with TIN2 and POT1. Image adapted from S. Savage (83).

Non-replicative telomere shortening – Several studies have linked telomere shortening to high levels of ROS regardless of age of cell donor (84,85). For example, Richter and von Zglinicki (86) used several well-established cell culture systems from donors of various ages (embryonic to adult) and showed that placing cells in different oxygen concentrations (a condition known to induce different levels of oxidative stress) resulted in differential rates of telomere shortening and eventually cell cycle arrest. At very high ambient oxygen concentrations (40%) the cell line from the oldest donor (human skin fibroblasts from a 91yr old female) with the lowest antioxidant capacity was observed to only undergo three population doublings before perishing. It has also been

demonstrated that over-expression of extracellular superoxide dismutase can slow telomere shortening in a cell line with low antioxidant capacity and oxidative stress-induced telomere shortening thus extending the culture's viability (87). Under oxidative stress conditions it has been shown that in telomerase positive cells telomeres shorten as fast as telomerase negative cells; the reasons are suspected to be nuclear exclusion of telomerase and localization of TERT to the mitochondria, which protects mtDNA and lowers superoxide production resulting in less oxidative stress (88).

As telomeres shorten, either from damage or replication, chromosome ends become unprotected (or uncapped) leading to a DNA damage response and growth arrest (52,77). Interestingly when telomeres shorten, gene expression of several shelterin components is down-regulated (78). That being said, all telomeres in a cell or nucleus do not need to be short for senescence to occur, as shown by Hemann et al. (89), as the shortest telomeres within the nucleus dictate cell fate. These shortest telomeres become uncapped and are recognized by the DNA damage response machinery (90), referred to as telomere-damage induced foci (TIF) (80). Telomeres become uncapped or dysfunctional when TRF2 is inhibited and excessive telomere shortening occurs in the absence of telomerase, thus creating TIFs that are indicated by DDR factors such as 53BP1, γH2AX, Rad17, ATM and Mre11 localizing at the telomere (80). Telomere dysfunction is related to a DNA damage response that is mediated by tumor protein 53 (p53) leading to the transcriptional activation of CDK p21 (91-94) or p16 (95-97) both of which are mediators of senescence. In a recent study by Chebel et al. (78), it was shown in serial cultures of lymphocytes that shelterin gene expression was markedly reduced with senescence, with TRF1 mRNA and protein decreased the most of any shelterin

component, while TRF2 did not change. Also, the authors monitored the formation of TIFs throughout the cultures and observed an increased number of foci over time. Thus, one could imagine a situation where oxidative damage and shortening of telomeres in a nucleus results in uncapping, loss of shelterin gene expression, and eventually senescence or cell death. A number of studies have indicated that telomere dysfunction plays a main role in the stress-induced senescence program and in cell death (39,98).

Recently in neuronal cell culture (U373 cells), Pollicitia et al. (84) showed that HIV infection resulted in telomere shortening. They hypothesized that the telomere shortening in neuronal cells after HIV infection was due to oxidative stress. They were able to rescue the telomere shortening with the antioxidant *N*-acetyl-L-cysteine, confirming their hypothesis. Telomerase activity was not affected by either HIV infection or antioxidant treatment. Makpol et al. (99) cultured human skin fibroblasts from young and aged donors and showed that treatment with H_2O_2 resulted in significant oxidative DNA damage, telomere shortening and decreased telomerase enzyme activity. The telomere shortening and telomerase activity-lowering effects of H_2O_2 were blunted by pretreatment with alpha-tocopherol (vitamin E). The authors speculated the alpha-tocopherol could be activating transcription factors that up-regulate telomerase gene expression (c-Fos and Ap-1 binding to DNA sites) and thus maintain telomere length via telomerase. Further, Spallarosa et al. (100) showed that several members of the MAPK family, specifically p38 MAPK and JNK, are involved in the regulation of shelterin gene expression in cardiomyocytes. Specifically, p38 MAPK inhibition resulted in up-regulation or rescue of TRF2 gene expression after cells were exposed to doxorubicin (an anticancer drug known to have cardiotoxic effects). In summary, cells respond to

telomere dysfunction in a cellular stress mediated pathway (i.e., MAPKs) resulting in senescence, which leads to a loss of functional tissue and nuclei that could add to an aging or disease phenotype. In two recent studies by the Depinho group (101,102) further evidence for the importance of telomere length and aging phenotypes was demonstrated. In a first study, they showed that when telomerase was re-expressed in short telomere mice (G4-6 TERT knock-out mice) tissue dysfunction was overcome. Interestingly they observed significant alterations in non-mitotic tissues such as the brain (101). In the second study, short telomeres were linked to mitochondrial dysfunction. Extensive testing confirmed that short telomeres were the first step, prior to mitochondrial dysfunction, leading to increased ROS production and enhanced ROS induced telomere shortening and altered gene expression of important mitochondrial biogenesis genes (102). These results underscore the importance of telomeres in genome stability and provide evidence of a unified hypothesis of aging.

Telomere length has also been proposed as a potent tumor suppressing mechanism as well. Since cells in culture have only a limited capacity to divide (20 – 40 population doublings; (13)) and since it likely takes about 100 doublings or so to create a colony of cells with a second mutation, only having the ability to double 40 or so times would act as a potent suppression mechanism against tumor formation and outright malignancy (103). Also the finding that telomeres are shorter from cells of older donors *in vivo* and that telomeres shorten *in vitro* with increased cell doublings (52,104) indicated that telomeres might count or keep track of the number of cell divisions a cell undergoes. This finding was strengthened by evidence that overexpression of the catalytic subunit of hTERT was able to restore telomerase activity, maintain telomere

length, and eliminate a limit of cellular replication (105). Additional evidence was provided when telomerase activity was abolished in tumor cells and proliferative capacity was reduced (105). It is hypothesized that telomeres represent the crossroads for cells between proliferative aging and cancer. In certain tissues, such as skeletal muscle, the telomere dogma is different.

Telomeres and Skeletal Muscle - Skeletal muscle is unique in that it consists of multinucleated muscle fibers and multiple niche populations of singularly nucleated cell types, the most well characterized being satellite cells (106). Skeletal muscle is also considered to be post-mitotic, with only the satellite cells actively dividing when new nuclei are needed within the skeletal muscle fiber. When skeletal muscle satellite cells are activated to divide and incorporate into muscle fibers as new myonuclei, the fibers' average telomere length is reduced (106,107). The newly added satellite cell nuclei represent the nuclei with the shortest telomeres since these newly added nuclei would have been actively dividing over time (106,107). Minimum telomere length in skeletal muscle is thus thought to represent the replicative history of satellite cells (108). As such, over time skeletal muscle telomere length can change despite its post-mitotic condition (106,109,110).

Thus, when measuring skeletal muscle telomere length, mean and minimum telomere length (i.e., the shortest of the lengths observed in a tissue sample), representing fiber telomere length and satellite cell telomere length, respectively, must be analyzed and interpreted correctly (106). A recent investigation showed that skeletal muscle telomere length was reduced in elderly individuals compared to young controls, as well as a modest trend for shorter skeletal muscle homogenate telomere lengths in sarcopenic

compared to non-sarcopenic individuals (111). Other studies in skeletal muscle have focused on the replicative potential and regenerative capacity of satellite cells rather than skeletal muscle per se. For example, Wernig et al. (112) studied the regenerative capacity of satellite cells and showed a slight decrease in mean telomere length with age in those cells, corresponding to reduced regenerative capacity. Data from several different muscular dystrophies indicate shortened satellite cell and skeletal muscle homogenate telomeres and telomere-related dysfunction associated with the muscle degradation and atrophy (110,113,114). When considered together, these studies indicate that skeletal muscle telomere length can change, and moreover that using diseases such as muscular dystrophies as extreme skeletal muscle remodeling models may provide an avenue to understand the complex skeletal muscle telomere biology landscape.

Methods of telomere length determination – Several methods of determining telomere length have been developed over the years. Terminal restriction fragment (TRF) analysis by Southern blot, quantitative in-situ hybridization (Q-FISH), Flow-FISH, and quantitative PCR (qPCR). There is an ongoing debate in the field as to which method is the most accurate, with each method having several limitations. For instance the TRF Southern method is hindered by the remaining sub-telomeric DNA in the variable sequence region of the chromosome end, thus leading to inaccurate telomere length estimates (115). The qPCR method has also been recently probed and found that a curvilinear relationship to the 'gold-standard' TRF Southern may be more accurate (116). The Q-FISH and Flow-FISH techniques are hindered by several factors including the need for high numbers of cell nuclei and labor intensive and highly sensitive techniques.

As of right now the two most common telomere length measurements are the qPCR and TRF Southern blotting techniques.

Genetic component – Telomere length is highly variable from individual to individual, suggesting a strong genetic component. Heritability estimates range from 30 to 90%, with the lower estimates likely being more accurate as they were derived from better-controlled studies (117,118). Several genetic diseases are associated with shortened telomeres and recently have been shown to be linked to telomere-related genes.

Monogenic diseases such as dyskeratosis congenita and idiopathic pulmonary fibrosis have been identified as 'telomere diseases' (62). In addition several of the shelterin components have recently been shown be mutated in several genetic diseases (119). In addition studies have shown that promoter polymorphisms in the hTERT gene are related to telomerase enzyme activity, telomere length and to cardiovascular disease (120).

Aging and Physical activity

Aging is a complex biological phenomenon and the factors governing the process of aging and longevity are only beginning to be understood. Physical inactivity increases the risk of several age-related diseases such as cardiovascular disease (CVD), hypertension, osteoporosis, stroke and type 2 diabetes (121). Becoming or remaining physically active in old age has been shown to reduce the risk of morbidity and mortality from these age-related conditions (122,123). Moreover, multiple reports have revealed the lifespan extending potential of physical activity (PA) (3,124-126). Consistently performed PA appears to slow the rate of cellular and molecular damage accumulation and blunt the decline in physiological function that is characteristic of the aging process

(127,128). Despite these findings, the causal molecular and genetic links between PA and the reduction in age-related disease risk remain elusive. A potential link between aging and increased disease risk is shortened and/or dysfunctional telomeres. The role of telomeres in several diseases of physical inactivity has recently been elucidated (129) and recent epidemiological and experimental evidence points to PA as being able to influence telomere biology (5,9,130).

Telomeres and Physical Activity

Recently, several reports, including one from our laboratory, have associated PA or exercise training with alterations in telomere length and/or the network of proteins that interact with telomeres (5-8,106,109,130). These results provide evidence of a link between PA and aging at the cellular level, and indicate the possibility of a mechanistic link between the influences of PA in the attenuation of age-related diseases.

Collins et al. (109) were the first to demonstrate an association of telomere length with physical activity when they reported excessive telomere shortening in skeletal muscle of endurance athletes with severe fatigued athlete myopathic syndrome (FAMS) compared to age- and training volume-matched athletes. In a follow-up study by the Collins group, shorter minimum telomere lengths were observed in those endurance athletes with the highest number of years and hours spent training (7). These results indicate that long-term endurance training by highly-trained athletes may be a significant stressor to skeletal muscle and/or satellite cell telomeres, as indicated by the shorter minimum telomere lengths.

Beginning in 2008, a number of groups began to study the association of typical, moderate physical activity with telomere length in humans. Ponsot et al. (131)

investigated skeletal muscle telomere length in physically active and inactive men and women in two age groups, young and old, and observed that telomere length was similar within an age group regardless of activity level, indicating that moderate PA is not detrimental to skeletal muscle telomere length. In epidemiological studies by Cherkas et al. (5) and Ludlow et al. (130) a positive association between PA and leukocyte telomere length was observed up to moderate levels of typical PA, while high-levels of PA were associated with shorter PBMC telomere lengths (130). Though these results were not replicated in a large Chinese cohort (132), the two studies provided the first evidence of a potential benefit of moderate PA on telomere biology. Additional evidence was provided in a recent study in which physically active women had longer leukocyte telomere lengths compared to sedentary individuals, which the authors hypothesized was related to PA diminishing the potential influence of perceived psychological stress (54) on leukocyte telomere length (133). A recently published study investigating telomere length and PA in adolescent males and females observed that African American females who spent more time performing vigorous PA had significantly longer telomeres than less active peers (134).

Other investigations have attempted to associate telomere alterations to resistance exercise. Kadi et al. (135) studied competitive powerlifters compared to recreationally resistance-trained individuals and both average and minimum telomere lengths of vastus lateralis muscle in the powerlifters were shown to be longer compared to the resistance-trained controls. In contrast, within the group of powerlifters minimum telomere length was inversely associated to personal records in the squat and deadlift, indicating that the greater the weight lifted in a maximum effort the shorter the minimum telomere length.

Though speculative, the long-term training in the powerlifters seems to have resulted in a protective effect on telomere length in skeletal muscle, while excessive training associated with elite-level performance may result in accelerated telomere shortening.

Animal models have also provided evidence of a role of physical activity in modifying telomere biology. Although rodents tend to have significantly longer telomeres compared to humans (60), the associated proteins and overall telomere biology between the species are similar, thus providing useful insights into possible regulatory mechanisms. Werner et al. (9) recently showed that the cardioprotective effects of voluntary wheel running on cardiomyocytes was conferred by an exercise-induced increase in telomere binding proteins (shelterin) and telomerase enzyme activity mediated by the presence of telomerase protein component TERT, as well as IGF-1 and eNOS. Exercise was also shown to decrease markers of apoptosis and reduce gene expression of several cell cycle associated genes, showing that exercise induces an anti-apoptotic environment thus preserving functional tissue and delaying an aged phenotype (9). Cardiomyocyte telomere length itself was not different between exercise and sedentary groups but was different with age (3-months versus 18-months), indicating age-related telomere shortening. In a follow-up study in aortic tissues of mice and mononucleocytes of humans, Werner and colleagues (8) again showed that exercise training increased telomerase enzyme activity, increased telomere binding protein and mRNA expression, and decreased levels of Chk2, p16, and p53 in mouse aorta. These gene expression changes were again shown to be mediated by TERT, IGF-1, and eNOS in the aorta. Similar to the cardiomyocyte data, aortic telomere length was not altered by 3-weeks or 6-months of voluntary wheel running but demonstrated age-related shortening (3-months

versus 18-months). In the human arm of the study, the authors investigated young sedentary, young athletes, aged sedentary, and aged athlete groups for telomere-related outcomes. Mononuclear cell telomere length was preserved in the aged athletes and was shortest in the aged sedentary individuals, and telomerase activity was greater in young and aged athletes compared to the age-matched sedentary individuals. Telomere-repeat binding protein 2 (TRF2) expression was increased in the athletes compared to sedentary individuals, while cell cycle genes (e.g., p16, p53, Chk2) were reduced in the athletes compared to the inactive groups (8).

In summary, these human and animal experimental and epidemiological results, most notably the papers by Werner and coworkers, indicate that exercise training is a potent stimulus to the telomere biology system, though influences on telomere length itself may not be seen unless the training is for a long duration (See Table 2.2). The data also indicate the possibility that moderate PA may provide the greatest positive influence on telomere biology, while higher levels of exercise training may have a negative effect.

Table 2.2. Summary of studies investigating physical activity and telomeres.

Author and Year	Major Findings
Human Skeletal Muscle	
Collins et al. (109)	Shorter telomeres in FAMS subjects (VL)
Ponsot et al. (131)	Equal telomere length between sedentary and active individuals (VL)
Kadi et al. (135)	Longer telomeres (mean and minimum) in powerlifters compared to sedentary (VL)
Human Leukocytes (WBC unless otherwise noted)	
Cherkas et al. (5)	Longer telomeres in more active in individuals
Ludlow et al. (130)	Longer telomeres in moderately active individuals compared to both sedentary and high active (PBMC)
Woo et al. (132)	No difference in telomere length between active and sedentary
Shin et al. (136)	No difference in obese middle age women who under went 6 months of aerobic exercise training compared to untrained obese controls
Werner et al. (8)	Longer telomeres in older athletes compared to sedentary older individuals (PBMC)
Puterman et al. (133)	Longer telomeres in active individuals with lowest psychological stress
Zhu et al. (134)	Longer telomeres in active adolescent African American females compared to less active peers
Larocca et al. (6)	Longer telomeres in older active individuals compared to sedentary peers
Song et al. (137)	Longer telomeres negatively correlated with biomarkers of aging and positively with lifestyle factors such as PA level, BMI and smoking status
Rodent Tissues	
Radak et al. (138)	No change in telomerase activity with swimming in rat skeletal muscle or liver
Werner et al. (9)	Increased telomere protection, reduced apoptotic signaling, elucidation of possible mechanisms with VWR in myocardium
Werner et al. (8)	Increased telomere protection, reduced apoptotic signaling, confirmation of possible mechanisms with VWR in aorta tissue

VL = vastus lateralis. PBMC = peripheral blood mononuclear cells. PA = physical activity. BMI = body mass index. VWR = voluntary wheel running.

Possible Mechanisms of Physical Activity-Induced Modifications of Telomere Biology

Few studies have defined the direct cellular and molecular mechanisms of the effect of PA on the biology of aging, though as outlined above a slowing of telomere degradation may act as one potential mechanism. The direct signaling pathways by which PA interacts with the telomere are not clearly delineated, though the work of Werner and colleagues has provided some initial insights. In general, regular PA is thought to prevent and delay inactivity- and age-related disease through multiple mechanisms, with reductions in either oxidative stress (125) or inflammation (139) or both being key potential mediators. These same pathways can be linked to changes in telomere biology, providing potential mechanisms by which PA influences telomere biology with downstream influences on disease development and progression.

For example, exercise training increases antioxidant capacity via an increase in antioxidant enzyme activity (140-142). Telomere shortening is exacerbated in numerous cell types due to oxidative stress (84,86,87,143). Thus, telomere length may be maintained in moderately physically active individuals due to reductions in oxidative damage occurring to the telomere. Telomerase is also responsive to oxidative stress and may be key to protecting cells from stress insults (67,144), so this component of the telomere system may also benefit from the reduction in oxidative stress associated with PA.

In the studies by Werner and colleagues, a role for endothelial nitric oxide synthase has been described, such that the presence of eNOS, and assumed nitric oxide

(NO) bioavailability, is critical for the exercise-induced alterations to the telomere binding proteins in cardiomyocytes and aortic tissue in mice. These findings indicate that exercise, mediated by the beneficial effects of NO, is able to confer oxidative stress resistance and lower levels of apoptosis in multiple tissue types via genome stabilization by an increased expression of telomere binding proteins and telomerase via activation of cell survival pathways. Nitric oxide signaling via cGMP is able to activate several pro-survival protein kinases including phosphatidylinositol 3′ kinase (PI-3K) and protein kinase B (AKT) (145). These studies by Werner and colleagues also showed a role for IGF-1 in the activation of telomerase. Recent epidemiological evidence indicates that older individuals with low circulating IGF-1 and free of overt disease have shorter leukocyte telomere lengths (146). Since acute exercise stimulates expression of IGF-1 from both skeletal muscle and liver, IGF-1 may be playing a role in the augmentation of telomere biology by exercise (147). How the ”exercise signal” is transmitted via pro-survival pathways to the telomere-related genes has not been defined; however, these recent findings have highlighted possible molecular underpinnings of the beneficial effects of exercise training on vascular health.

Stress hormones such as cortisol and stress responsive pathways such as those of the mitogen-activated kinases (MAPK) have also been implicated in telomere biology (100,148). For instance Spallarossa et al. (100) demonstrated that p38 MAPK was involved in the regulation of *Trf2* gene expression in response to doxorubicin treatment in cardiomyocytes. Acute exercise is known to activate MAPK signaling, though the effects of exercise training on MAPK are less clear (149). So acute exercise may result in a repression of telomere binding protein expression, which is consistent with longer

telomeres (150). The MAPK pathway should be investigated as a possible mechanism by which exercise can influence telomere biology.

In addition to oxidative stress-related pathways, short telomeres may contribute to the chronic inflammatory phenotype associated with aging, as demonstrated recently in a study showing shortened telomeres implicated in the regulation of interferon-stimulated gene 15 (ISG-15) (151). Lou et al. (151) used a customized microarray analysis of cells with short, normal and artificially elongated telomeres to elucidate genes differentially expressed in human skin fibroblasts and ISG-15 was clearly differentially expressed in relation to telomere length. Several studies have linked the expression and secretion of ISG-15 to inflammation through its stimulation of the pro-inflammatory interferon, IFNγ (152,153). These results point to a possible mechanism whereby shortened telomeres are causing an increase in inflammation (a condition associated with many age-related diseases), thus contributing directly to the disease process. A recent study using *mTert-/-* cells with shortened telomeres showed enhanced toll-like receptor 4 (TLR4) expression (154). TLR4 mediates the inflammation process by stimulating the expression of NF-κB, which initiates the transcription of several pro-inflammatory genes such as Il-6, COX2 and TNF-α. Thus, shortened telomeres have been linked to a pro-inflammatory cellular environment and point to a possible protective effect of PA against telomere shortening through reduced inflammation-related gene expression.

Both acute and chronic moderate exercise training are associated with beneficial changes in inflammatory makers (155,156). Several inflammatory cytokines have been shown to be augmented in age-related diseases (e.g., TNF-α), and PA may blunt these age-related changes in inflammatory cytokines (157). Short telomeres may initiate a

feed-forward mechanism resulting in the expression of both inflammatory and oxidative stress pathways that could accelerate telomere shortening and enhance age- and inactivity-related disease phenotypes. PA may be exerting its anti-aging effects by protecting telomeres from shortening by improvements in antioxidant capacity and chronic inflammation.

Determining whether or not telomere shortening is causing cellular dysfunction and age-related disease or if telomere length is simply a bystander reflecting the hostile cellular environment associated with age-related disease is a challenge. The phenotypes of early generation *Tert-/-* and *Terc-/-* knock-out mice do not display telomere shortening as expected, but later generations of these animals have shortened telomeres and overt phenotypes such as CVD, indicating a direct link between telomere shortening and disease (4,158). These and other knock-out animal models may provide insights into whether or not shortened telomeres are responsible for diseases of aging and inactivity. Other possibilities include engineering tissue-specific knock-out animals for components of the shelterin complex. Whole-body knock-outs for *Trf1* and *Trf2* have been attempted but have not survived past the embryonic stage (74,80), showing vital roles for these proteins in development. In contrast, tissue-specific (e.g., skin) shelterin component knock-out mouse strains produce viable offspring that display aging phenotypes and cancer (150,159). Tissue-specific knock-out animals could be exposed to PA intervention studies to further elucidate the role of PA in telomere biology.

Summary of Literature Review

In general there is sufficient evidence from the literature to conclude the following: 1) telomere length decreases with age in most cell/tissue types and this is

associated with many age-related phenotypes and diseases including cardiovascular disease and most cancers; 2) environmental factors such as psychological stress and physical activity/ exercise training are associated with altered telomere states (Figure 2.3), with physical activity generally viewed as a 'telo-protective' stimulus. What is unclear is how exercise (or any environmental factor) is affecting telomere biology across a range of tissues, not just in immune cells. Therefore a priority for advancing the field is to discern the effect of chronic and acute exercise on telomere biology in different tissues, both rodent and human. In addition determining the mechanisms (signaling transduction pathways) by which exercise is affecting telomere biology could lead to novel therapeutic approaches to the aging process and possibly common age-related diseases such as cardiovascular disease. The aims of the present dissertation contribute to our understanding of the impact of acute and chronic exercise on telomere biology in multiple tissues, in particular skeletal muscle.

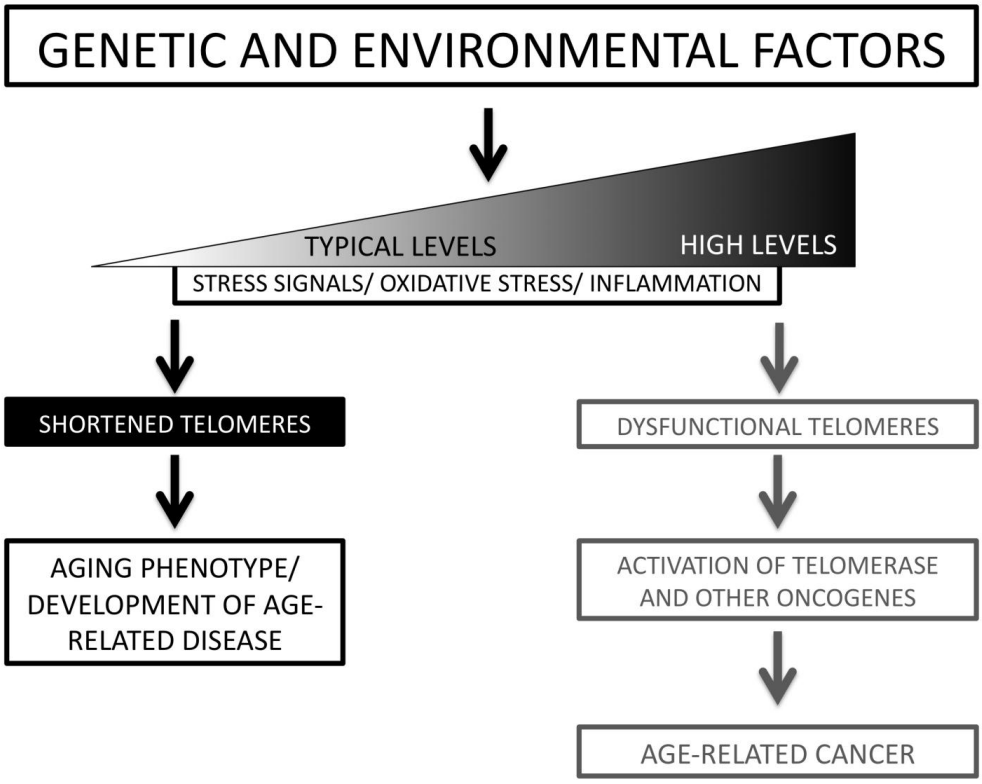

Figure 2.4. The major factors leading to age-related disease or cancer phenotypes are shown. Interaction of genetic and environmental factors can lead to varying levels of DNA damage, oxidative stress, and inflammation, all of which can contribute to shortened or dysfunctional telomeres, depending on the extent of damage. Typically-shortened telomeres can result in cell cycle arrest, tumor suppression, and loss of functional tissue via senescence or apoptosis/necrosis and an aging phenotype. At the extreme, DNA damage (e.g., gene mutations) and subsequent activation of oncogenes, such as TERT and telomerase activity, can lead to age-related cancer. Becoming or remaining physically active may prevent or delay the onset of many age-related diseases and even some cancers, potentially through protection of telomeres. Figure reprinted from Ludlow and Roth (160).

Rationale for the Present Dissertation Studies

In order to partially inform the issues outlined above, the major goal of this dissertation work was to describe how telomere length and telomere associated proteins were responding to chronic and acute exercise. In addition we sought to describe acute exercise-associated alterations in signaling proteins that have been related to telomere

binding protein expression, as these acute alterations may be critical to determining the outcome of chronic exercise on telomere length. Finally we sought to determine if oxidative stress is a telomere-shortening stimulus in skeletal muscle fibers in the absence of satellite cells.

We have chosen to use two strains of mice for some of these experiments due to the large difference in telomere length between the two strains and the fact that biological differences between these two strains may exist in the response to ROS insult. The suspected reason behind the large telomere length difference is the recent inbreeding of the CAST/Ei strain compared to the long-term inbreeding of the C57BL/6 strain (60). During stress, the regulation of genes that control telomere length may be differentially regulated in the two strains based on genotype and telomere length. By using these two strains we can test for possible biological differences in the response to an oxidative challenge.

Hydrogen peroxide is a relatively stable compound and has only weak oxidizing potential, but with metal catalysis, such as Fenton's reaction, hydroxy (OH-) radicals are formed which are highly reactive and damaging. During contraction H_2O_2 production is increased from various cellular sites including but not limited to the mitochondria (161) and xanthine oxidase (162). Physiological levels of H_2O_2 in skeletal muscle are achieved with extracellular doses ranging from 10μM to 100μM (163,164). Also it has been reported that only approximately 7 to 10% of extracellular concentration of H_2O_2 actually permeates the cell membrane of skeletal muscle in culture (163,165), therefore an extracellular dose of 100μM would result in an intracellular concentration of 10μM. Similar treatment durations (5 days) have been used to investigate exercise-like

treatments *in* vitro (166) and single muscle fibers have been shown to be viable for this duration in culture (165). Interestingly in a report by Anderson et al. (161) differences between rat skeletal muscle fiber type and production of H_2O_2 were observed. At basal respiration rate, it was observed that H_2O_2 production was 3.5-fold higher in type IIb muscle fibers, and there were significant differences in the ability of the different fiber types to scavenge H_2O_2. These data indicate that rodent skeletal muscle, particularly the mainly fast twitch muscles such as the EDL or FDB that may be particularly susceptible to oxidative insult via H_2O_2. Also many studies have used exogenous H_2O_2 to stimulate exercise like effects such as glucose uptake (167,168). While the application of exogenous H_2O_2 may not mimic all aspects of ROS induced exercise signaling, it is a useful tool in pinpointing certain aspects of signaling cascades, particularly in skeletal muscle considering that H_2O_2 is produced both at rest and during contraction. The goal of this study is not to determine where the H_2O_2 originated from within the skeletal muscle, but to determine if ROS (H_2O_2) is a telomere-damaging compound in skeletal muscle.

N-acetylcysteine (NAC) is a non-specific antioxidant that supports glutathione production (169). NAC has been commonly used in studies using skeletal muscle and has proven to be a potent ROS scavenger in skeletal muscle (170). NAC has been observed to reduce the effects of ROS as potently or more potently than the addition of SOD plus catalase (superoxide and H_2O_2 scavengers), ebselen (a glutathione peroxidase mimetic), and MnTBAP (an SOD mimetic) (171). Therefore NAC should be a sufficient ROS scavenger during my treatments to confirm or not that ROS is associated with the observed changes. Also since H_2O_2 application can induce other forms of ROS (NO,

superoxide production) using a non-specific antioxidant will also prevent harmful effects from these unwanted treatment side-effects.

Chapter 3: Chronic exercise modifies age-related telomere dynamics in a tissue-specific fashion.

The following article is currently under review at *Journal of Gerontology: Biological Sciences A.*

Chronic exercise modifies age-related telomere dynamics in a tissue-specific fashion.

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Running Title – Effects of chronic exercise on telomeres

Key words –telomere length, telomerase, Cast/Ei mice, exercise, shelterin, aging

Abstract

We evaluated the impact of long-term exercise on telomere dynamics in wild-derived short telomere mice over one year. We observed significant telomere shortening in liver and cardiac tissues in sedentary 1-year old mice compared to young (8 wk) baseline mice that was attenuated in exercised 1-year old animals. In contrast, skeletal muscle exhibited significant telomere shortening in exercise mice compared to sedentary and young mice. Telomerase enzyme activity was increased in skeletal muscle of exercise compared to sedentary animals but was similar in cardiac and liver tissues. We observed significant age-related decreases in expression of telomere related genes that were attenuated by exercise in cardiac and skeletal muscle but not liver. Protein content of TRF1 was significantly increased in plantaris muscle with age. In summary, long-term exercise altered telomere dynamics, slowing age-related decreases in cardiac and liver tissue, but contributing to shortening in skeletal muscle of exercised animals.

Introduction

Telomeres are stretches of repetitive DNA (5'-TTAGGG$_n$) at the ends of chromosomes that, in combination with telomere binding proteins, serve to protect DNA in this region from being detected as damaged (79). DNA polymerase cannot fully copy telomere DNA during replication resulting in loss of telomere repeats. The loss of telomere repeats over time leads to shortening of telomeres that can reach a critical length resulting in senescence or cell death (52,103). In certain tissues, the ribonucleoprotein telomerase can maintain and/or elongate telomeres (172). In addition telomeres are bound by a six-protein complex termed shelterin that functions to repress DNA damage signaling at the telomere, regulate telomere length, and telomerase action at the telomere (173). Short telomeres have been associated with cancers and many age-related diseases (160). Physical activity and chronic endurance exercise training are associated with delayed cellular aging (3,125) and decreased morbidity and mortality (122). Longer telomere length is found in several tissues in individuals who regularly perform moderate levels of physical activity compared to sedentary peers (8,9,130,174). This suggests a potential "telo-protective" effect of exercise, consistent with the data supporting the benefits of exercise on cellular aging. However, there is a lack of evidence as to which components of the telomere system and other interacting proteins are underlying this observation. Also, to our knowledge only two studies have examined mouse telomere biology and exercise in depth, those being focused on the myocardium (9) and vascular and immune cells (8). Telomere length is tissue-specific (20) and exercise elicits a multi-organ stimulus that may have unique outcomes depending on the tissue studied.

The purpose of the present study was to determine the effect of long-term (44wks) voluntary wheel running on telomere length, telomerase enzyme activity, and shelterin and DNA damage response (DDR) gene expression in three tissues commonly examined in relation to exercise adaptation (skeletal and cardiac muscles and liver). We performed these studies in a unique mouse strain, CAST/Ei, that displays telomere lengths shorter than many strains and similar to humans in multiple tissues (60). In the present study, three groups of mice were studied: baseline young animals (BL-8wk), and mice that either had access to a running wheel (EX-1Y) or no wheel access (SED-1Y) for forty-four weeks from 8 wks of age to 52 wks of age. Telomere length and related outcomes were measured in skeletal muscle, myocardium, and liver in all animals. We hypothesized that telomere length would decrease after one year in myocardium and liver, and that exercise would attenuate any decreases. In skeletal muscle we hypothesized that telomere length would remain unchanged with age and exercise. We found that both telomere length and expression of telomere-related genes were altered in a tissue-specific fashion following chronic exercise exposure compared to the young and one-year sedentary animals.

Methods

Animals and Exercise – All animal experiments were approved by the University of Maryland Institutional Animal Care and Use Committee and conformed to the National Institutes of Health's Guide for the Use and Care of Laboratory Animals (NIH Pub. No. 85-23, revised 1996). Tissues from ten young (8-10 wk old) male CAST/Ei J mice were purchased for the BL-8wk group, and thirty additional (20 male and 10 female) seven-week-old CAST/Ei J animals were purchased for the EX-1Y and SED-1Y groups (Jackson Laboratory, Bar Harbor, ME). Animals were acclimated to the animal facility for one-week prior to being randomly assigned to either an individual sedentary cage (no wheel access, n = 15) or an individual exercise cage (wheel access, n = 15). The animals were housed at 25ºC on a 12 hour light-dark cycle. Animals were fed *ad libitum* laboratory mouse chow (Prolab RMH 3000, 5P00, LabDiet, Nestle Purina, Vevey, Switzerland) and given free access to water. The exercise cage was equipped with a voluntary running wheel and monitor system (Lafayette Instruments, Lafayette IN). Body mass was monitored bi-weekly. Only 16 males and 5 females survived the full year intervention thus 10 EX-1Y animals (8 males and 2 females) and 11 SED-1Y animals (8 males and 3 females) were analyzed. The running wheels were locked for 48 hrs and food was removed four hours prior to euthanasia to minimize any effects of acute exercise or feeding on outcome parameters. Animals were isoflurane anesthetized and euthanized by heart dissection. Tissues were weighed, frozen in liquid nitrogen and stored at -80ºC for subsequent analyses.

Telomere Length Measurement – We used slightly modified qRT-PCR methods to measure telomere length (175,176), using genomic DNA isolated and quantified using

standard procedures (130). Between 12.5 and 20 ng of total genomic DNA was added to a reaction mixture containing 1XSybr Green Master mix (Applied Biosystems, Carlsbad, CA), 220nM forward and reverse primers for the T (telomere) PCR and 301nM forward and 502nM reverse for the S (single copy gene) PCR. Cycling conditions for the T PCR were 50ºC for 2 min, 95ºC for 10 min followed by 30 cycles of 95ºC for 10 sec and annealing at 56ºC for 1 min with data collection. Conditions for the S PCR were 50ºC for 2 min, 95ºC for 10 min, 35 cycles of 95ºC for 15 sec, annealing at 56ºC for 1 min with data collection, extend 72ºC for 1 min. For the T PCR we used the following primers (Tel 1 Forward 5'-GGT TTT TGA GGG TGA GGG TGA GGG T; Tel 2 Reverse 5'- TCC CGA CTA TCC CTA TCC CTA TCC CT) and for the S PCR (acidic ribosomal phosphatase PO, 36B4; Forward 5'- AAC AAG GCA GGA GTG AGA CTG; Reverse 5'- CCA GGG ATA CGG GAG AAA A). All samples were run in triplicate, a standard curve was run on every plate, and a reference sample was included to ensure linearity and consistency of assays. Intra-assay coefficients of variation for T and S PCRs are as follows for each tissue: liver T PCR 1.1%, S PCR 0.9%; skeletal muscle T PCR 1.8%, S PCR 1.2%; Cardiac muscle T PCR 1.32%, S PCR 0.7%. Inter-assay coefficient of variation for T PCR was 6.9% and for S PCR was 12.2%.

To confirm the qRT-PCR method mean telomere length was measured by terminal restriction fragment (TRF) length analysis as described previously (177). We used samples from heart and liver that represented the two longest and two shortest from the qRT-PCR method. Briefly, 2μg of genomic DNA was digested overnight with Rsa1 and HpaIII (Roche, Indianapolis, IN) and electrophoresed on 0.7% agarose gels for 2hrs and visualized via ethidium bromide staining. The gel was then electrophoresed for

16hrs at 60v to ensure adequate separation of DNA fragments. The DNA was blotted onto positively charged Nylon membranes (Roche, Indianapolis, IN), heat cross-linked and hybridized overnight at 50ºC with the telomeric probe (digoxigenin 3′-end-labeled 5′-$(CCTAAA)_3$) and visualized using a chemiluminescence detection system (Syngene Bio Imaging, Frederick MD; Supporting Figure 3.1). Mean, median, minimum and maximum TRFs were determined according to previous methods (178).

Telomerase Enzyme Activity - Telomerase enzyme activity was measured using a commercially available kit (Quantitative Telomerase Detection Kit; US Biomax, Rockville, MD) that utilized the Telomere Repeat Amplification Protocol (TRAP) (179). To ensure reliability and validity of the assay, triplicate samples, as well as internal controls provided in the kit, were assayed. Inter- and intra-assay coefficients of variation were calculated and compared to published data that used the same kit (intra- and inter-assay coefficients of variation for gastrocnemius (10.1%, 12.6%), heart (3.7%, 5.5%) and liver (7.1%, 7.3%). In addition we ran heat-treated negative samples and if the heat-treated sample was at least three standard deviations above the positive sample, the data were accepted. The telomerase data was analyzed as directed by the manufacturer and as previously performed in our lab (130). Tissue samples were normalized for protein content prior to performing the telomerase assay. Briefly, we used the bicinchoninic acid protein assay (Pierce, Rockford, IL) to normalize each sample to 0.43 μg of protein, as previously described (130,180). Analysis was performed in one gastrocnemius, one-quarter of powdered heart tissue (~20mg) and liver (~50mg) of all animals.

Reverse Transcription-Polymerase Chain Reaction (PCR) – Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). Reverse transcription was performed

with 1μg of total RNA using the high-capacity cDNA kit (Applied Biosystems, Carlsbad, CA). PCR was normalized to *Gapdh* for all target genes and expressed relative to the baseline group. Primer sequences for *Trf1, Trf2, Ku70, Ku80, Chk2* and *p53* are from (9) (Supporting Table 1). Analysis was performed in one EDL, PLT, one-quarter of heart tissue (~20mg), and liver (~50mg).

Western Blotting – For GAPDH, TRF1, and TRF2, one EDL (extensor digitorum longus), one PLT (plantaris), one-quarter of powered heart (~20mg), and liver tissue (~50mg) were homogenized in lysis buffer (100 mmol/ l Tris [pH 6.8], 4% sodium dodecyl sulfate [SDS], 20% glycerol and protease inhibitor cocktail (complete mini EDTA-free, Roche, Indianapolis, IN). The protein concentration of the lysate was determined as described above and 30μg of protein was separated on 10% SDS-polyacrylamide gels. Proteins were transferred onto PVDF membranes (Immunobilon-P, Millipore, Billericka, MA) blocked in 3% NFDM for 30 min and exposed to primary antibodies overnight at 4ºC as follows: TRF1 (C-19, SC 1977, 1:200 in 1% NFDM, Santa Cruz Biotechnologies, Santa Cruz, CA), TRF2 (H-300, SC 9143, 1:200 in 1% NFDM, Santa Cruz Biotechnologies, Santa Cruz, CA) and GAPDH (Cell signaling, 14C10 Rabbit mAb # 2118 1: 1000 in 5% BSA, Beverly, MA). In a portion of the gastrocnemius muscle from a subset of animals, mTERT was immunoprecipitated by using 500μg of protein and mTERT (SC-7212 H-231 rabbit polyclonal 1:200 in 1 % BSA, Santa Cruz Biotechnologies, Santa Cruz, CA) with agarose-A protein goat anti-rabbit IgG (Sigma Aldrich, St. Louis, MO). All products were visualized using enhanced chemiluminescence (Pierce, Rockford, IL) and HRP as a substrate on the Gene Gnome (Syngene Bio Imaging, Fredrick, MD).

Statistical Analysis -

Relative band intensities from agarose gels and immunoblots were analyzed using NIH ImageJ software (181). All values, unless otherwise stated, are presented as mean ± standard error of the mean. Pearson product-moment correlations were calculated to correlate the percent change of telomere length and TRF1 and TRF2 percent change in protein content in SED-1Y and EX-1Y animals. Generalized linear models were used to analyze the data with SAS version 9.2. Only omnibus p values are reported in the text of the results, specific contrast p values are reported in the figure legends. We compared BL-8wk animals to SED-1Y animals to determine the effect of age and the effect of exercise was determined by comparison of SED-1Y and Ex-1Y animals. All analyses were repeated considering sex as an independent variable and similar tendencies were observed (data not shown), thus we collapsed all data by sex for presentation purposes. We acknowledge that the small sample of females in our cohort should be considered exploratory, but we did not observe any effects of sex for any analysis. Differences were considered significant at $p < 0.05$.

Results

Chronic exercise modifies age-related changes in telomere length and telomerase enzyme activity

Twenty-seven CAST/EiJ mice were analyzed at two different ages and with or without access to a cage-mounted, computer-monitored running wheel for forty-four weeks. Skeletal muscles (gastrocnemius, EDL and PLT), cardiac muscle, and liver were studied from young 8-week old (BL-8wk, n = 6) mice, one-year old sedentary animals (SED-1Y, n = 11, standard housing conditions), and one-year old exercised animals (EX-1Y, n = 10). Mean voluntary running distance in the EX-1Y group was 6475 ± 1396 m/d (mean ± standard error) over the 1-yr intervention. Body and heart masses were significantly smaller in the BL-8wk animals compared to the SED-1Y animals; gastrocnemius and liver masses were not different among groups (Table 3.1). The differences in body and heart masses were expected and likely due to post-natal growth. Exercise did not change body mass, heart, liver, or gastrocnemius mass in comparison to SED-1Y (Table 3.1).

Skeletal muscle (gastrocnemius) telomere length was not significantly different due to age ($p = 0.7$), but was significantly shorter in the EX-1Y animals compared to SED-1Y ($p = 0.007$; Figure 3.1.A). In contrast, cardiac muscle and liver telomere lengths were shorter due to age (BL-8wk v. SED-1Y, $p < 0.01$ heart; $p < 0.01$ liver), and exercise significantly attenuated those decreases (EX-1Y v. SED-1Y, $p < 0.01$ heart; $p < 0.01$ liver; Figure 3.1.A). Telomerase enzyme activity was maintained at baseline levels in gastrocnemius muscle of EX-1Y animals compared to a significant reduction in SED-1Y mice ($p = 0.02$; Figure 3.1.B). Telomerase reverse transcriptase (mTERT) protein

content was detected in skeletal muscles of EX-1Y and SED-1Y but was not statistically different between groups ($p = 0.30$, data not shown). Telomerase enzyme activity was not altered by age or exercise in either cardiac muscle or liver (Figure 3.1.B).

Chronic exercise alters the gene expression and protein content of some shelterin components in skeletal muscle

To determine if telomere-protecting components were altered in skeletal muscle in response to exercise, we measured gene expression of several telomere length-regulating genes. We examined two different skeletal muscles, the PLT (mixed oxidative and glycolytic phenotype, located in the triceps surae and activated in wheel running behavior) and the EDL (glycolytic phenotype, anterior of leg, minimal activation with wheel running; (182).

Plantaris –

Gene expression of *Trf1*, *Trf2*, *Pot1a* and *Pot1b* (telomere repeat binding factor 1 and 2, protection of telomeres 1a and 1b, respectively) were not different across the three groups (data shown in Figures 3.2.A, 3.3.A, 3.4.A and 3.4.B). In addition to these shelterin components, several other proteins have been found to be transiently associated with telomeres, such as DDR heterodimer KU (KU70/KU80) (183). Gene expression of *Ku70* was not different among groups, while *Ku80* gene expression was lower in SED-1Y compared to BL-8wk, and chronic exercise tended to attenuate this reduction (*Ku70*, $p = 0.10$; *Ku80*, $p < 0.01$; Figures 3.5.A and 3.5.B; significance reported as omnibus ANOVA p value, contrast specific significance for age and exercise effects are reported in the figure legends for all gene expression and protein data). These results reveal that exercise may up-regulate the expression of genes associated with DDR. To investigate

whether chronic exercise resulted in altered gene expression of proteins critical to the cell cycle we measured *p53* and *Chk2*; gene expression was not different across groups for either gene (Figures 3.6.A and 3.6.B). We measured protein content of TRF1, TRF2 and GAPDH (normalization/ loading control) in plantaris. TRF1 protein content tended to be higher in SED-1Y compared to BL-8wk ($p = 0.06$; Figures 3.2.B and 3.2.C); no differences were observed for protein content of TRF2 (Figures 3.2.B and 3.3.C).

We also correlated the change in telomere length with the change in TRF1 and TRF2 protein content. We hypothesized that as telomere length decreased with age, shelterin protein content would also decrease (184). We did not observe any significant correlations of percent change in telomere length with percent change in TRF1 or TRF2 protein content in the plantaris.

Extensor Digitorum Longus –

Trf1 and *Trf2* gene expression was lower in SED-1Y compared to BL-8wk, and exercise blunted this age-related reduction (*Trf1*, $p < 0.01$; *Trf2*, $p = 0.01$; Figures 3.2.A and 3.3.A). *Pot1a* and *Pot1b* gene expression was also lower in SED-1Y compared to BL-8wk, but no exercise effect was observed (*Pot1a*, $p < 0.01$; *Pot1b*, $p = 0.14$; Figures 4.A and 4.B). *Ku70* and *Ku80* gene expression was lower in SED-1Y compared to BL-8wk, and exercise blunted this reduction (*Ku70*, $p < 0.01$; *Ku80*, $p = 0.05$; Figures 3.5.A and 3.5.B). *p53* and *Chk2* gene expression tended to be lower in SED-1Y compared to BL-8wk, with a tendency for exercise to attenuate the age-related reduction (*p53*, $p = 0.08$; *Chk2*, $p = 0.06$; Figures 3.6.A and 3.6.B). TRF1 and TRF2 protein content (normalized to GAPDH) were not different among groups ($p = 0.62$ and $p = 0.31$, respectively; Figures 3.2.B and 3.3.B). These data indicate that long-term voluntary

exercise is a potent telomere-altering stimulus both for telomere length and telomere-related gene expression in skeletal muscle. The different responses between skeletal muscle types likely stems from intrinsic (muscle fiber type composition) and extrinsic factors (neuronal activation levels) during wheel running (182).

We observed a significant negative correlation between the percent change in telomere length and percent change in TRF2 protein content ($r = -0.71$, $p = 0.02$, data not shown) in the EX-1Y EDL. This result did not support our hypothesis and indicates that the animals that lost the greatest percentage of telomere length maintained or increased TRF2 protein content. This finding supports data showing that when TRF2 is overexpressed *in vitro* it results in telomere shortening (185). We did not observe any significant correlations between the change in telomere length and the percent change in TRF1 protein content in EDL.

Chronic exercise attenuated age-related alterations in gene expression of shelterin components in cardiac muscle

In general, cardiac muscle showed reduced gene expression of telomere-related genes with age that were attenuated with long-term voluntary exercise. Specifically, gene expression of *Trf1, Trf2*, *Pot1a*, and *Pot1b* were lower in SED-1Y compared to BL-8wk and this age-related reduction was blunted in EX-1Y for all four genes (all $p < 0.05$; Figures 3.2.A, 3.3.A, 3.4.A, and 3.4.B). *Ku70*, *Ku80*, and *p53* exhibited similar responses, with BL-8wk gene expression higher than SED-1Y but not different from EX-1Y (all $p < 0.05$; Figures 3.5.A, 3.5.B, 3.6.A). A similar pattern was seen for *Chk2* gene expression, but the differences did not reach significance ($p = 0.07$; Figure 3.6.B). We measured TRF1 and TRF2 protein content (normalized to GAPDH) and did not observe

any differences among groups ($p = 0.76$ and $p = 0.59$, respectively; Figures 3.2.B and 3.3.B).

Several significant correlations were observed in the heart for percent change in telomere length and percent change in TRF1 and TRF2 protein content in SED-1Y or EX-1Y animals. We observed in SED-1Y animals a significant negative correlation ($r = -0.76$, $p = 0.01$) for telomere length and TRF1 protein content, and a significant positive correlation ($r = 0.74$, $p = 0.01$) for telomere length and TRF2 protein content. In the EX-1Y animals we observed a significant negative correlation ($r = -0.74$, $p = 0.02$) between change in telomere length and change in TRF1 protein content with no correlation for TRF2. These data offer partial support for the hypothesis that as telomere length is reduced, shelterin components are also reduced; TRF2 and telomere length in the SED-1Y animals were positively correlated, supporting our hypothesis. However, similar to the skeletal muscle data, TRF1 protein content changed in the opposite direction of telomere length.

Chronic exercise altered gene expression and protein content of some shelterin components and DNA damage response genes in the liver

In liver, no significant differences were observed for gene expression of *Trf1*, *Trf2*, or *Pot1a* across any groups (Figures 3.2.A, 3.3.A and 3.4.A). Gene expression of *Pot1b* tended to be lower in BL-8wk compared to SED-1Y ($p = 0.06$) with no difference in EX-1Y (Figure 3.4.B). Gene expression of *Ku70* was lower in EX-1Y compared to both BL-8wk and SED-1Y ($p < 0.01$; Figure 3.5.A) and a similar tendency was seen for *Ku80* ($p = 0.07$; Figure 3.5.B). No differences were observed for *p53* or *Chk2* in any comparison (Figures 3.6.A and 3.6.B). We measured TRF1 and TRF2 protein content

and TRF1 tended to be higher in SED-1Y compared to EX-1Y ($p = 0.09$; Figure 3.2.C), while TRF2 was not different among groups (Figure 3.3.C).

We did not observe any significant correlations between the change in telomere length and the change in shelterin components in liver tissue.

Discussion

We show for the first time that chronic voluntary exercise prevented age-induced decreases in telomere length in liver and cardiac tissue, but not skeletal muscle in CAST/Ei mice. Rather, chronic exercise contributed to accelerated shortening in skeletal muscle. We also observed significant alterations in gene expression of telomere-related genes providing evidence that physical activity is able to attenuate age-related changes in telomere dynamics in a tissue-specific manner. When considered together, these findings help provide cellular explanations by which chronic exercise may attenuate the aging process. Our results highlight the importance of the environment in the aging process and in particular the need to study telomere dynamics in tissues other than immune cells in response to exercise. In addition this is the first time that CAST/Ei mice have been used to investigate the role of exercise on telomere dynamics and these results show that short telomere strains of mice may respond differently to exercise than long telomere strains of laboratory animals (8,9).

Exercise attenuated age-related telomere reductions in cardiac and liver tissues yet induced telomere length loss in skeletal muscle

The use of telomere length as a biomarker of tissue aging has become widely accepted in the literature. Several studies have demonstrated reduced telomere length with age in both humans and rodents (56,186-188). While is it well accepted that in most tissues telomere length decreases with age, limited information exists about interventions to blunt or slow this reduction. Our results indicate that exercise may prevent or delay the reduction in telomere length in certain tissues. In cardiac tissue of Wistar rats telomere length decreases to a greater extent compared to other tissues such as liver, lung,

kidney and brain (187). The authors speculated that the telomere shortening was due to unrepaired oxidative damage to telomeres (189) or due to sub-populations of dividing cardiomyocytes (190). In C57BL/6 mice significant telomere shortening was observed in cardiac tissue of 18-month-old compared to 6-month-old animals (9). Our results are in agreement with these previous reports that cardiac tissue does undergo age-related telomere shortening in rodents and we report for the first time that long-term exercise exposure is able to attenuate the age-related telomere shortening in cardiac tissue of CAST/Ei mice. We also observed age-related telomere shortening in liver of CAST/Ei mice, specifically. Others have shown age-related telomere shortening in liver of rodents (186). Similar to cardiac tissue, our data reveal that exercise attenuated the age-related telomere shortening observed in the liver of sedentary animals. These data demonstrate that long-term exercise is able to alter the aging process at the cellular level and provide the impetus to pursue future mechanistic studies to determine how telomere length is maintained with age in certain tissues of exercised CAST/Ei mice.

In the skeletal muscle of the exercised animals we observed significant telomere shortening. Our data and that of others demonstrate that skeletal muscle telomeres do not shorten with age given the tissue's post-mitotic status (191,192). However, Collins et al. (109) measured telomere length in age-and training-matched endurance athletes with one group of individuals displaying fatigued athlete myopathic syndrome (FAMS) and observed shorter skeletal muscle telomere length in the FAMS group compared to the healthy athletes. Rae et al. (7) reported similar findings in healthy individuals with skeletal muscle telomere length shorter in endurance-trained individuals who had been training for the longest (number of years) and for the greatest duration of time per week

(hours spent running). Our data in the skeletal muscle of the CAST/Ei mice that had long-term access to running wheels support these previous findings that chronic exposure to exercise is related to decreased skeletal muscle telomere length. Further, these data are evidence that telomere dynamics of skeletal muscle between humans and CAST/Ei mice are qualitatively similar.

Several factors could have caused the tissue-specific response of telomere length to long-term exercise exposure. A common hypothesis for non-replicative associated telomere shortening is unrepaired oxidative damage to telomeres (143,193). Intrinsic differences in antioxidant enzyme levels could have resulted in poor skeletal muscle reactive oxygen species (ROS) scavenging while cardiac muscle and liver, which have higher antioxidant capacity than skeletal muscle, maintained redox balance and prevented oxidative damage to telomeres (194). Future investigations are needed to directly test the tissue-specific response of telomere length to oxidative stress.

To our knowledge this is the first investigation to report an increase in skeletal muscle telomerase enzyme activity with any type of exercise stimulus. Our results are in contrast to those previously observed in rat skeletal muscle, where no alteration in skeletal muscle telomerase enzyme activity was observed in response to swimming training (138); however, differences in the nature of the exercise stimulus and the model organism could account for the different results. Telomerase enzyme activity is known to be very low in resting skeletal muscle (112,195), but an increase in response to exercise could reflect satellite cell proliferation or other cells infiltrating exercised muscle. Increased telomerase activity could represent an adaptation to exercise, such that as telomeres shorten in exercised muscle the muscle cells may counteract the shortening by

transiently up-regulating mTERT and telomerase activity. Additionally, recent evidence has shown nuclear-independent actions of mTERT, including a role in protecting mitochondrial DNA from oxidative insults (67,88). Exercise training is known to exert powerful beneficial effects on mitochondrial function (196), suggesting an intriguing possibility for an effect of exercise on mTERT being involved in skeletal muscle mitochondrial health. Our results indicate that long-term exercise may be a telomerase-activating stimulus in skeletal muscle.

No change in telomerase activity was detected in liver or cardiac muscle with chronic exercise in CAST/Ei mice. In contrast, Werner et al. (9) observed that six-months of exercise increased telomerase activity in cardiac muscle in C57BL/6 mice. Several differences between the studies should be noted that may account for contrasting telomerase results: 1) we used a different strain of mice (short telomere versus long telomere mice), 2) our exercise intervention was six-months longer, and 3) our measurements were made in older mice. Further, a recent report by Gomes et al. (197) provides evidence that species with longer telomeres respond differently to oxidative stress compared to species with shorter telomeres, highlighting the influence of telomere length on telomeric responses to various stimuli. With regard to liver, ours is the first report to measure telomerase enzyme activity after a long-term exercise intervention. Radak et al. (138) measured liver telomerase enzyme activity in rats after short term forced exercise training and also observed that telomerase enzyme activity was not different. Therefore we speculate that exercise is acting through a telomerase-independent mechanism to maintain telomere length in liver tissues in short telomere mice.

Gene expression of shelterin is reduced after 1 year of age, and exercise blunted this age-related change

Shelterin is a six-protein complex that binds and protects telomere ends (173). Telomere-repeat binding factor 1 (TRF1) is important in telomere length homeostasis and TRF2 is critical for T-loop formation, while both are negative regulators of telomere length (185). The literature concerning shelterin genes and the effect of age is limited. Since it is well established that telomere length diminishes with age, we hypothesized that shelterin levels would show similar decreases. We observed significant decreases in skeletal muscle and cardiac muscle gene expression of shelterin components that were counteracted by chronic exercise. Although the magnitude of decrease in TRF1 and TRF2 protein content were not quantitatively significant in the EDL or cardiac muscle, the direction of change was similar to the change in gene expression. This could indicate that at later ages the decrease in gene expression becomes apparent at the protein level, however we were unable to detect a decrease in protein content at our chosen time-point.

Gene expression of *Ku70*, *Ku80*, *p53* and *Chk2* are altered with age and chronic exercise

We observed that gene expression of DDR, *p53* and *Chk2* genes were reduced with age and this reduction was attenuated by exercise in skeletal muscle and cardiac muscle, but not in liver. Different responses in gene expression between tissues to the exercise may be due to different intrinsic responses to training that have yet to be identified (194). These gene expression changes may indicate that the risk of cellular dysfunction is increased with age and that exercise was able to attenuate this risk in certain tissues. Interestingly, (137) showed in peripheral blood cells that a combination

of recently elucidated biomarkers of aging and specifically $p16^{Ink4a}$ levels negatively correlated with exercise duration indicating that exercise reduces cellular DNA damage and perhaps delays cellular aging. Our data support these findings but demonstrate a tissue specific response to exercise training.

A limitation of our work is that the majority of our findings are constrained to tissues that largely exist in a post-mitotic state. In addition, the inclusion of additional time points with older animals could expand upon our findings, particularly with respect to the shelterin complex. Future studies should target the relationship between cellular and functional changes with exercise and aging and potential mechanisms such as stress resistance, apoptosis, and various signaling pathways in these specific tissues.

Summary

Understanding how physical activity specifically alters telomere dynamics across a range of tissues and species is important to gain knowledge of how the environment can affect the aging process. We report for the first time significant changes in telomere length, shelterin, DDR, and cell cycle related genes with age that are altered with chronic exercise, but in a tissue specific manner in CAST/Ei mice. These data also support the use of the CAST/Ei mouse strain in the study of telomere dynamics compared to other long-telomere mouse strains. The results of this investigation raise multiple questions about how exercise and age interact at the cellular and molecular levels among tissues and organ systems.

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Table 3.1. Animal tissue weights and other phenotypes

Phenotype	**BL – 8wk**	**EX – 1Y**	**SED – 1Y**	**p - values**		
				Overall	Age	Exercise
Body mass (g)	12.9±0.45 (n = 6)	16.4±0.35 (n = 10)	16.9±0.34 (n = 11)	< 0.01	<0.01	0.28
Voluntary running distance ($m*d^{-1}$)	-	6475±1396 (n = 10)	-	-	-	-
Gastrocnemius mass (g)	0.07±0.006 (n = 6)	0.07±0.007 (n = 4)	0.08±0.080 (n = 3)	0.59	0.35	0.38
Heart mass (g)	0.08±0.006 (n = 6)	0.13±0.007 (n = 4)	0.12±0.008 (n = 3)	<0.01	<0.01	0.60
Liver mass (g)	0.41±0.06 (n = 6)	0.57±0.05 (n = 10)	0.53±0.04 (n = 11)	0.13	0.12	0.59

Data are presented as means ± SEM. Overall = p value for overall ANOVA; Age is the comparison between BL and SED; Exercise is the comparison between EX and SED. BL - 8wk – baseline eight weeks of age; EX = exercise; SED = sedentary; 1Y = one year of age.

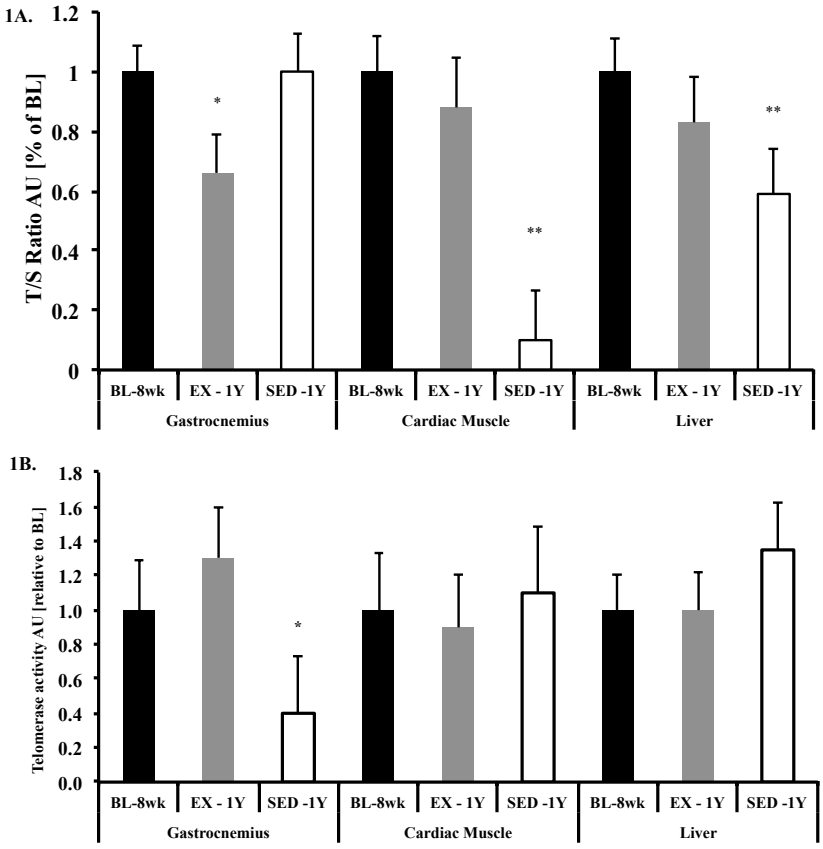
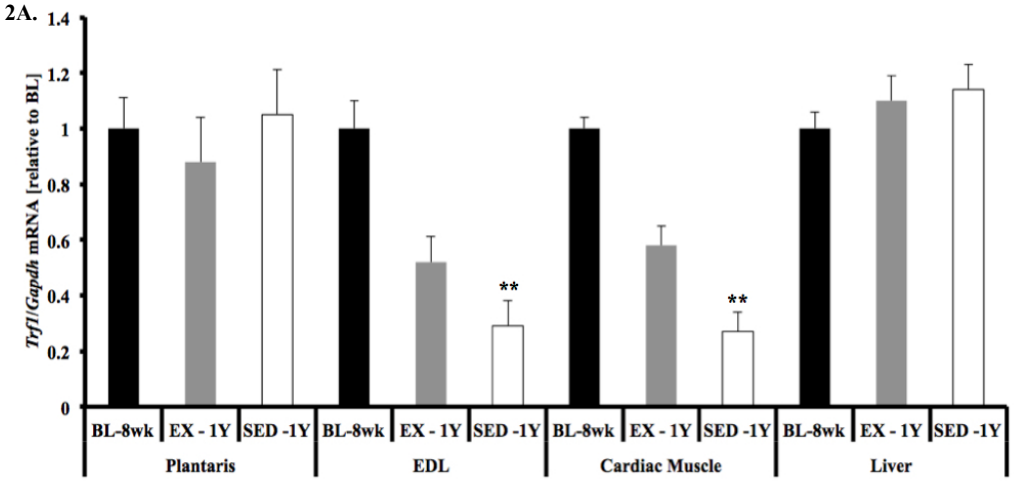
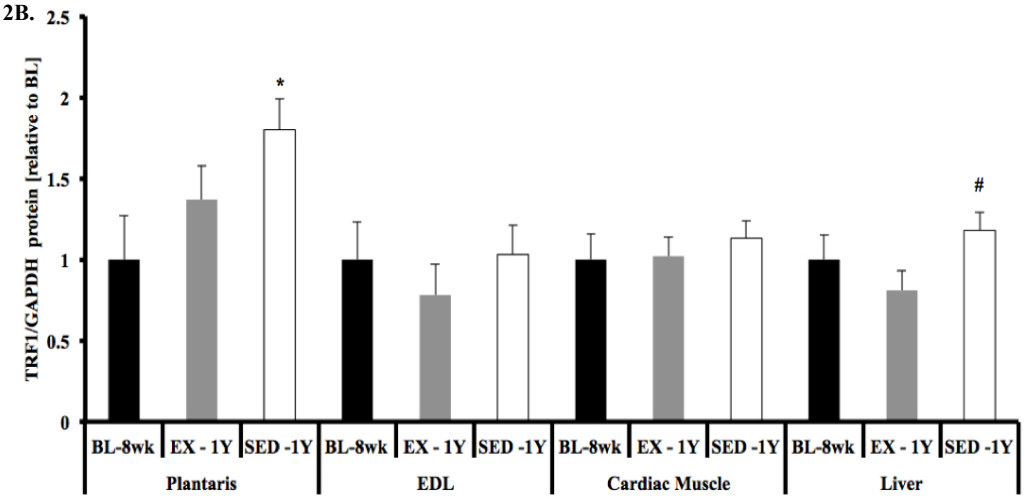


Figure 3.1: Telomere length and telomerase enzyme activity. A. Telomere length (T/S ratio) of skeletal muscle (gastrocnemius), cardiac muscle, and liver from baseline (BL-8wk), exercise (EX-1Y) and sedentary (SED-1Y) animals. Skeletal muscle telomere length was not different between the BL-8wk and SED-1Y animals ($p = 0.7$), but was significantly different between the EX-1Y and SED-1Y animals ($p < 0.001$). Cardiac muscle telomere length was significantly different between BL-8wk and SED-1Y animals ($p < 0.001$) and between EX-1Y and SED-1Y animals ($p = 0.01$). Liver telomere length was significantly different between BL-8wk and SED-1Y animals ($P = 0.005$) and between EX-1Y and SED-1Y animals ($p = 0.003$). * significantly different from BL-8wk and SED-1Y. ** significantly different from BL-8wk and EX-1Y. **B.** Telomerase enzyme activity in skeletal muscle (gastrocnemius), cardiac muscle, and liver from BL-8wk, EX-1Y and SED-1Y animals (values normalized to baseline across tissues). Telomerase enzyme activity was different between EX-1Y and SED-1Y animals in skeletal muscle ($P = 0.03$) but was not different between any groups in heart or liver. * significantly different between EX-1Y and SED-1Y. BL-8wk – baseline eight weeks of age; EX = exercise; SED = sedentary; 1Y = one year of age.

2A.


Trf1/Gapdh mRNA [relative to BL]
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
BL-8wk
EX - 1Y
SED -1Y
Plantaris
EDL
Cardiac Muscle
Liver

2B.


TRF1/GAPDH protein [relative to BL]
2.5
2
1.5
1
0.5
0
*
#
BL-8wk
EX - 1Y
SED -1Y
Plantaris
EDL
Cardiac Muscle
Liver

2C.

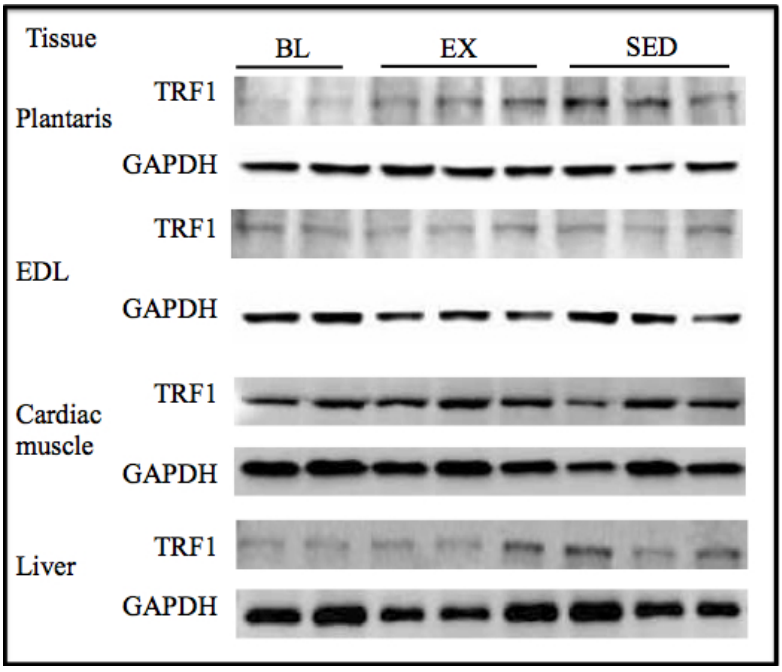
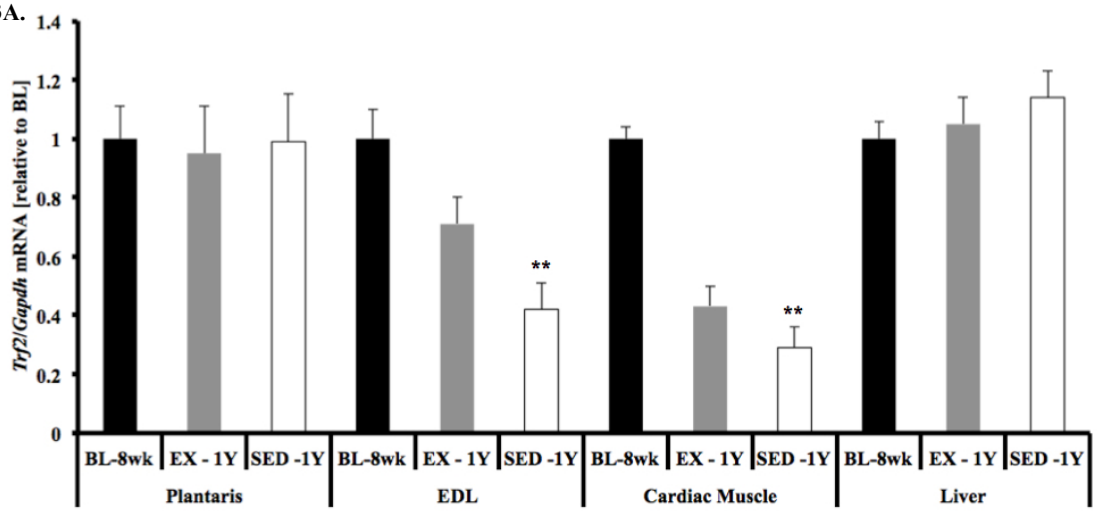
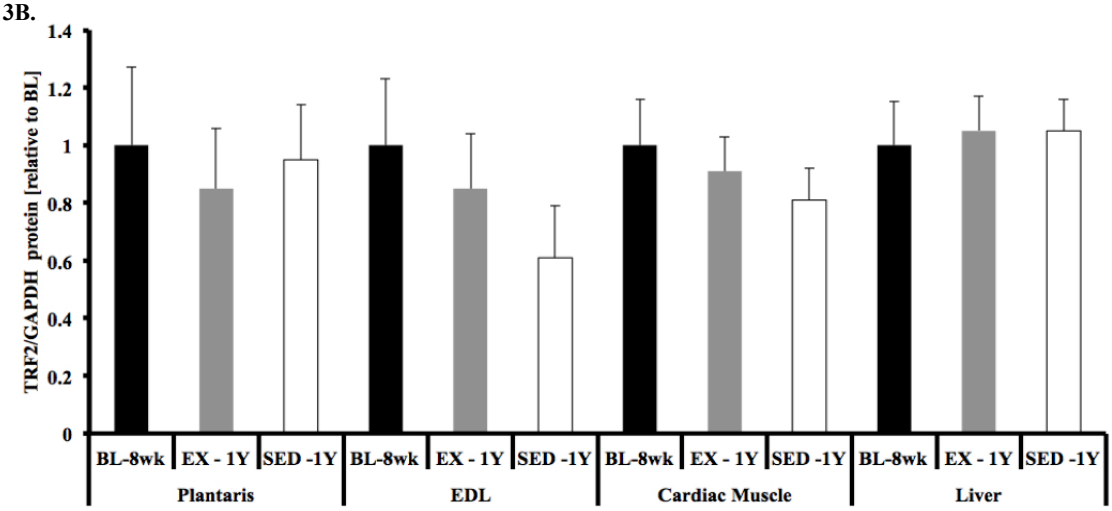


Figure 3.2: *Trf1* gene expression and protein content. A. *Trf1* gene expression was significantly lower in EDL (overall $p < 0.01$; age $p < 0.01$; exercise $p < 0.01$) and cardiac muscle (overall $p < 0.01$; age $p < 0.01$; exercise $p < 0.01$) of SED-1Y animals. *Trf1* gene expression was not different in plantaris or liver tissues. ** significantly different from baseline and exercise. **B.** TRF1 protein content was not different in EDL or cardiac muscle. There was a significant increase in TRF1 protein content in plantaris of SED-1Y animals compared to BL-8wk animals (overall $p = 0.06$; age $p = 0.02$; exercise $p = 0.15$). In liver TRF1 protein content tended to be greater in SED-1Y compared to EX-1Y animals (overall $p = 0.09$; age $p = 0.33$; exercise $p = 0.03$) **C.** Representative immunoblot images of TRF1 and GAPDH. * significantly different between EX-1Y and BL–8wk. # different from EX-1Y. BL-8wk – baseline eight weeks of age; EX = exercise; SED = sedentary; 1Y = one year of age.

3A.


Trf2/Gapdh mRNA [relative to BL]
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
BL-8wk
EX - 1Y
SED -1Y
Plantaris
EDL
Cardiac Muscle
Liver

3B.


TRF2/GAPDH protein [relative to BL]
1.4
1.2
1
0.8
0.6
0.4
0.2
0
BL-8wk
EX - 1Y
SED -1Y
Plantaris
EDL
Cardiac Muscle
Liver

3C.

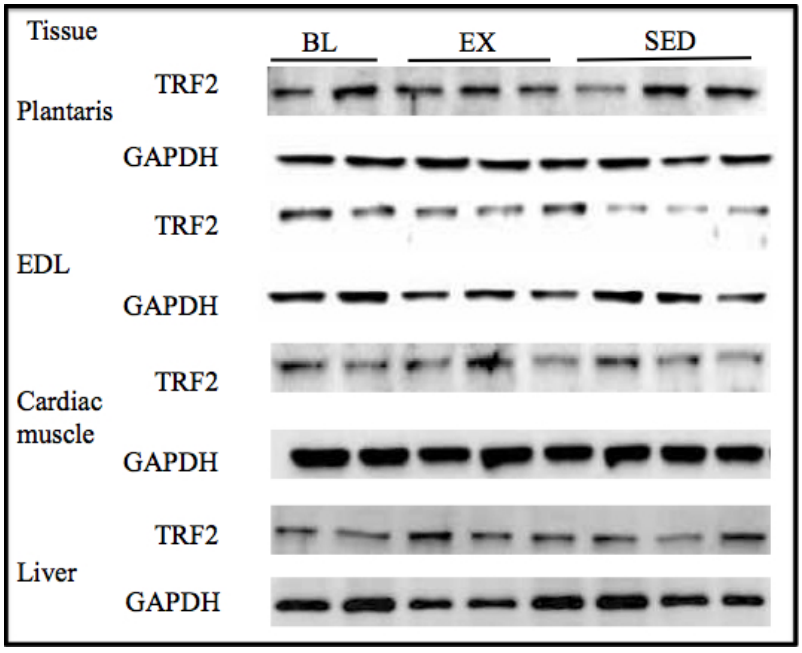


Figure 3.3: *Trf2* gene expression and protein content. A. *Trf2* gene expression was significantly lower in EDL (overall $p = 0.01$; age $p < 0.01$; exercise $p = 0.07$) and cardiac muscle (overall $p < 0.01$; age $p < 0.01$; exercise $p < 0.01$) of SED-1Y animals. *Trf2* gene expression was not different in plantaris or liver tissues. **B.** TRF2 protein content was not significantly different in any of the tissues analyzed. EDL TRF2 protein content in SED-1Y animals tended to be lower compared to BL-8wk young animals (overall $p = 0.36$; age $p = 0.17$; exercise $p = 0.33$). **C.** Representative immunoblot images of TRF2 and GAPDH. ** significantly different from baseline and exercise. BL-8wk – baseline eight weeks of age; EX = exercise; SED = sedentary; 1Y = one year of age.

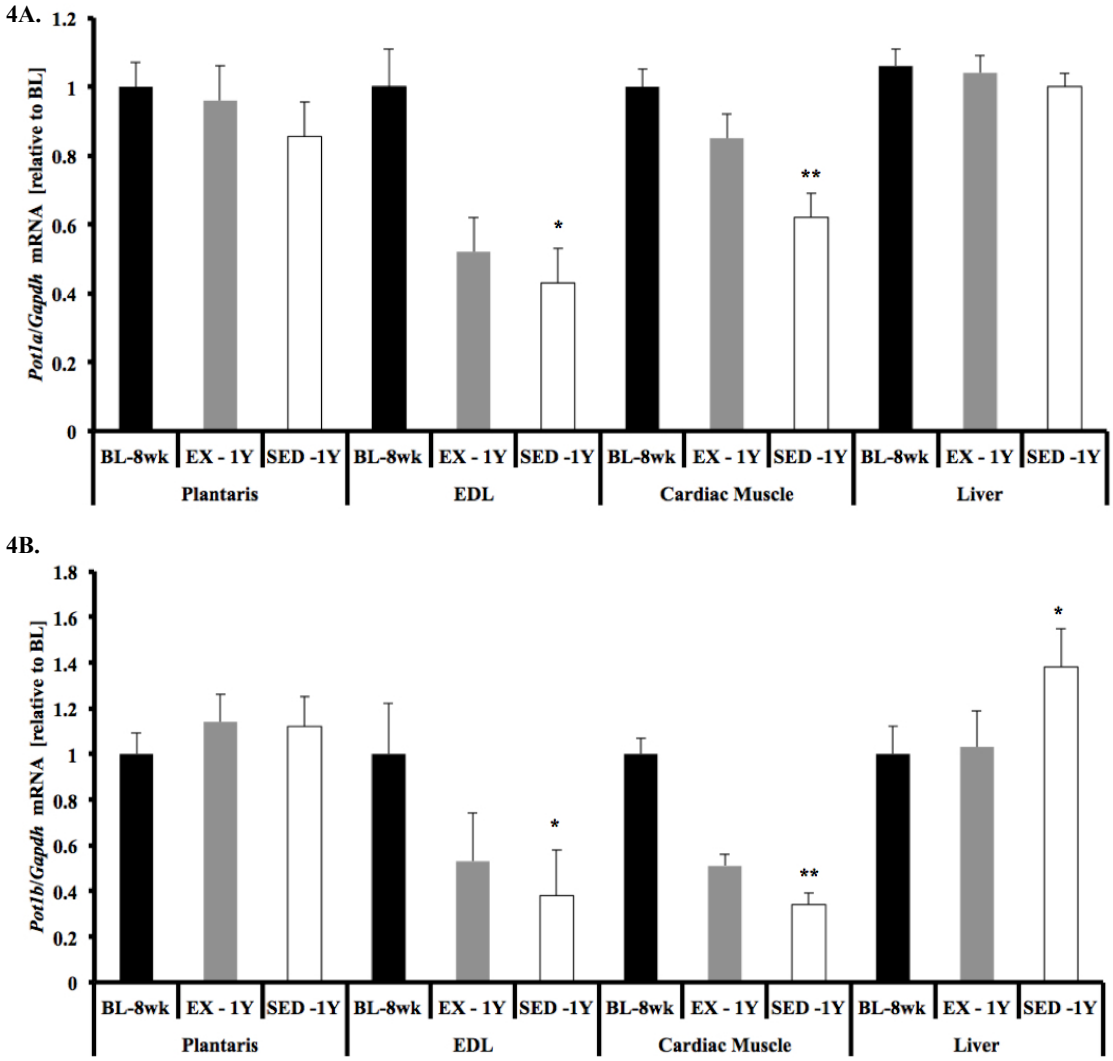


Figure 3.4: *Pot1a* and Pot1b gene expression. A. *Pot1a* gene expression was significantly lower in EDL (overall $p < 0.01$; age $p < 0.01$; exercise $p = 0.50$) and cardiac muscle (overall $p < 0.01$; age $p < 0.01$; exercise $p < 0.01$) of SED-1Y animals. *Pot1a* gene expression was not different in plantaris or liver tissues. **B.** *Pot1b* gene expression was significantly lower in EDL (overall $p = 0.14$, age $p = 0.05$, exercise $p = 0.62$) and cardiac muscle (overall $p < 0.01$, age $p < 0.01$, exercise $p = 0.02$) of SED-1Y animals. *Pot1b* gene expression tended to be lower in liver of BL-8wk compared to SED-1Y (overall $p = 0.06$; age $p = 0.03$; exercise $p = 0.05$; Figure 8) but were not different in plantaris tissue. ** significantly different from BL-8wk and EX-1Y; * significantly different from BL-8wk. BL-8wk – baseline eight weeks of age; EX = exercise; SED = sedentary; 1Y = one year of age.

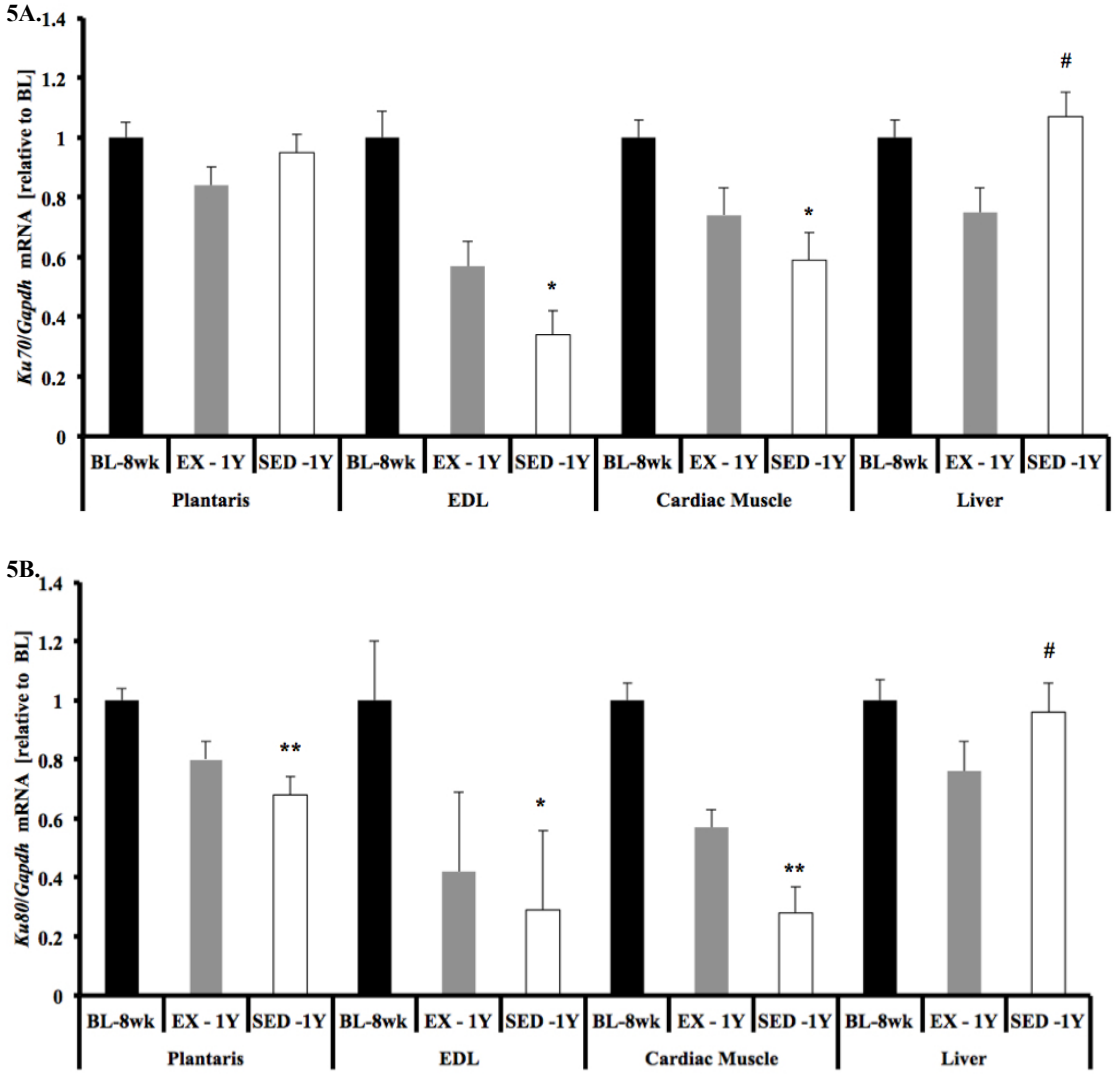


Figure 3.5: *Ku70* and *Ku80* gene expression. A. *Ku70* gene expression was significantly lower in EDL (overall $p < 0.01$; age $p < 0.01$; exercise $p = 0.07$) and cardiac muscle (overall $p < 0.01$; age $p < 0.01$; exercise $p = 0.09$) of SED-1Y animals. *Ku70* gene expression was higher in liver of SED-1Y compared to EX-1Y but was similar to BL-8wk (overall $p < 0.01$; age $p = 0.35$; exercise $p < 0.01$). No differences were observed in plantaris. **B.** *Ku80* gene expression was significantly lower in EDL (overall $p = 0.05$; age $p = 0.02$; exercise $p = 0.63$) and cardiac muscle (overall $p < 0.01$; age $p < 0.01$; exercise $p < 0.01$) of SED-1Y animals. *Ku80* gene expression tended to be higher in liver of SED-1Y (overall $p = 0.07$; age $p = 0.70$; exercise $p = 0.07$) compared to EX-1Y. In plantaris tissue *Ku80* gene expression was significantly lower in SED-1Y (overall $p < 0.01$; age $p < 0.01$; exercise $p = 0.06$). ** significantly different from BL-8wk and EX-1Y; * significantly different from BL-8wk. # significantly different from EX-1Y. BL-8wk – baseline eight weeks of age; EX = exercise; SED = sedentary; 1Y = one year of age.

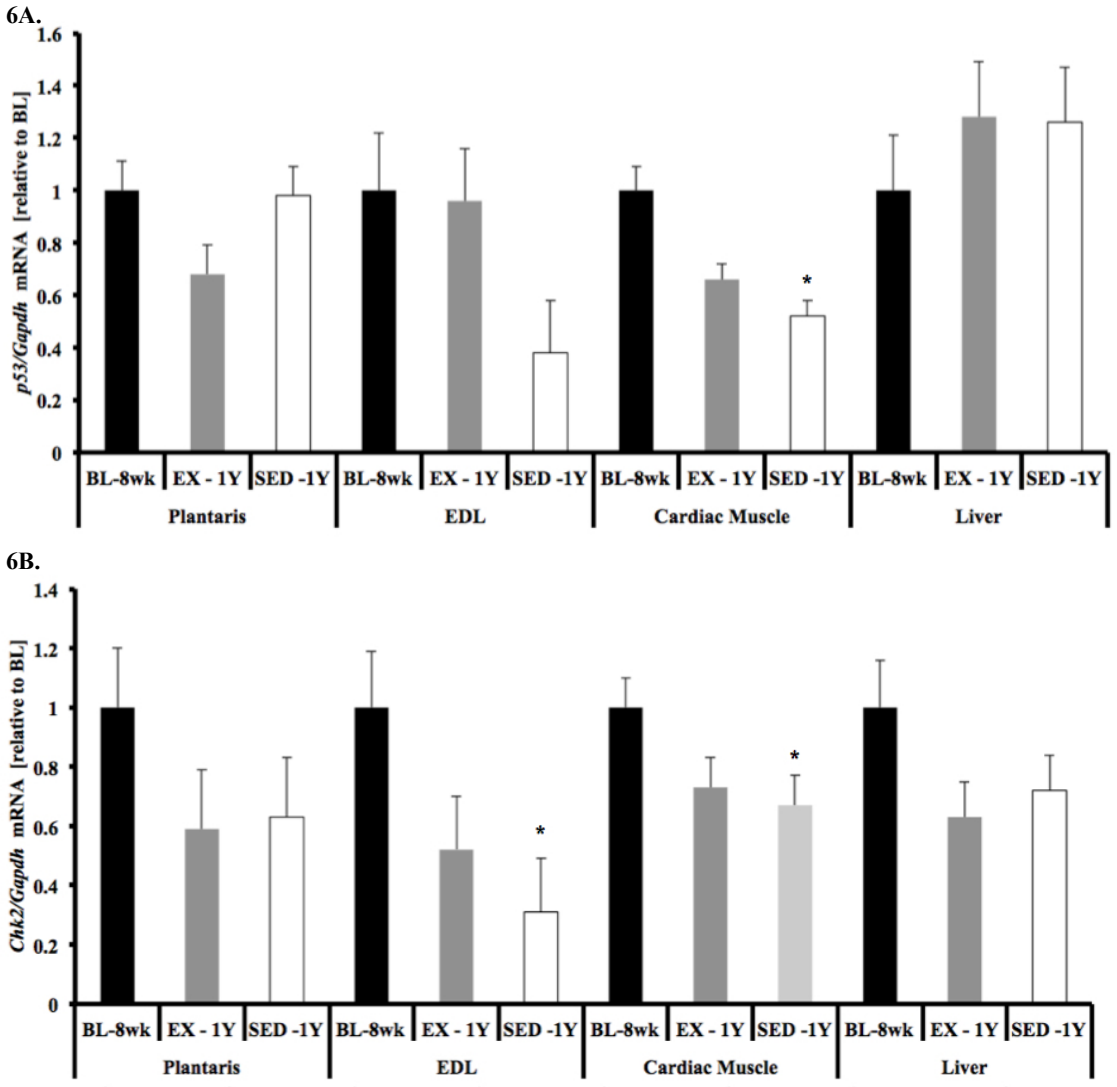
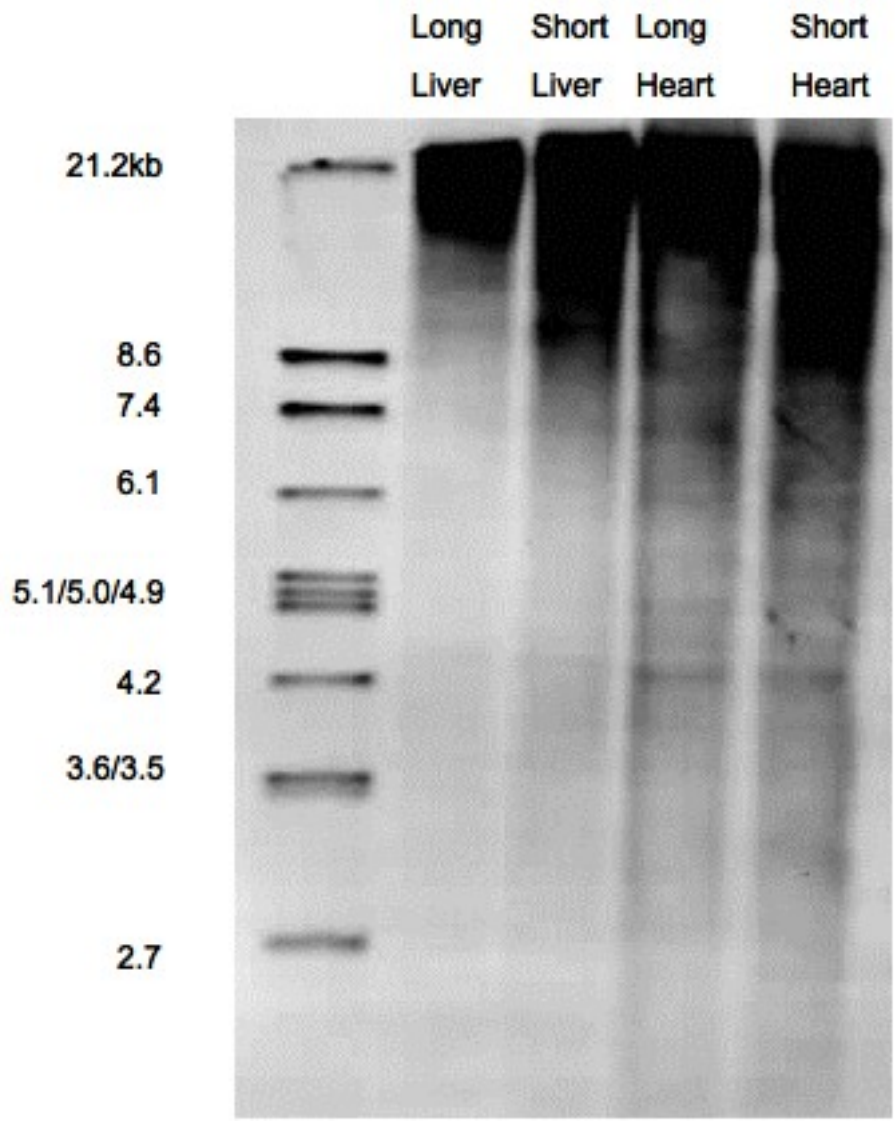


Figure 3.6: *p53* and *Chk2* gene expression. A. *p53* gene expression tended to be lower in EDL (overall $p = 0.08$; age $p = 0.06$; exercise $p = 0.06$) and cardiac muscle (overall $p < 0.01$; age $p < 0.01$; exercise $p = 0.12$) of SED-1Y animals. *p53* gene expression was not different in plantaris or liver tissues. **B.** *Chk2* gene expression tended to be significantly lower in EDL (overall $p = 0.06$; age $p < 0.01$; exercise $p = 0.01$) and cardiac muscle (overall $p = 0.07$; age $p = 0.03$; exercise $p = 0.67$) of SED-1Y animals. *Chk2* gene expression was not different in plantaris or liver tissues. * significantly different from BL-8wk. BL-8wk – baseline eight weeks of age; EX = exercise; SED = sedentary; 1Y = one year of age.

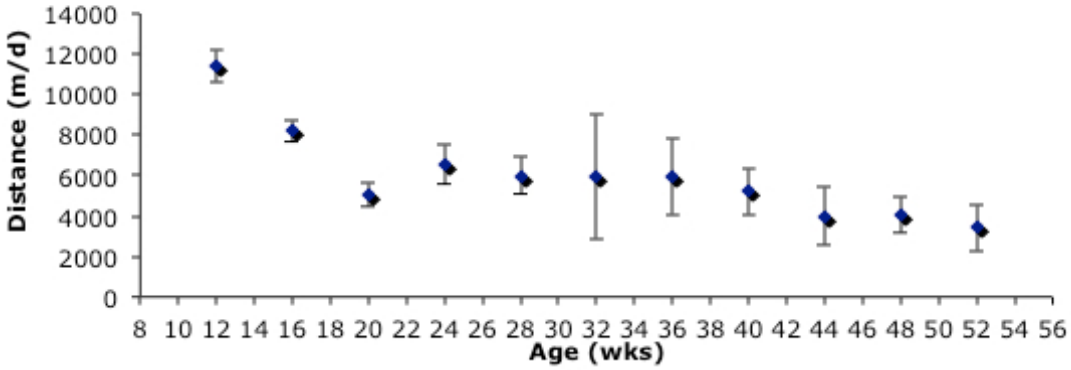
Supplemental Information

Supplemental Table 3.1. Gene Expression Primer Sequences.

Primer Target Gene name	Sequence
Trf1 (9)	F - 5'-CAT GGA CTA CAC AGA CTT AC R - 5'-ATC TGG CCT ATC CTT AGA CG
Trf2 (9)	F - 5'-TGT CTG TCG CGC ATT GAA GA R - 5'-GCT GGA AGA CCT CAA TAG GAA
Pot1a	F - 5'-CCC TGA ATC TAC TCA AGG AAG R - 5'-GAA GCG AAC AAT GTC TCC AA
Pot1b	F - 5'- CTT TAA GCC TCC GGC CTT AAG CAA AG R - 5'- CTT GGA CAT GAT TAT CAG CAA CGA CA
Ku70 (9)	F - 5'- GAG CAT CCA GTG TAT CCA GA R - 5'- CAG CAT GAT CCT CTT GTG AC
Ku80 (9)	F - 5'- TCA CAG TGT GCA GAC CAC CTG R - 5'- AAC TGC AGA GAG ATG CCA GA
p53 (9)	F - 5'- GGG ACA GCC AAC TCT GTT ATG TGC R - 5'- CTG TCT TCC AGA TAC TCG GGA TAC
Chk2 (9)	F - 5'- GCT GTG CTC TGA AGT AAC AAC R - 5'- GAA GTA GAG CTT ACA GGT GG
Gapdh	F – 5'- GTG TCC GTC GTG GAT CTG R - 5'- CCT GCT TCA CCA CCT TCT TG



Supplemental Figure 3.1. Representative telomere restriction fragment southern blot. To confirm our qRT-PCR telomere length assay two of the longest and two of the shortest telomere length samples from heart and liver tissues were digested and blotted as described in the methods section. Long Liver = 20.2kb; Short Liver = 8.8kb; Long Heart = 14.5; Short Heart = 8.5kb.



Supplemental Figure 3.2. Monthly running distance in the exercised animals. Data are presented as mean ± standard error.

Chapter 4: p38 MAPK mediates mRNA expression of telomere binding proteins in skeletal muscle.

The following is a manuscript that is in preparation. The work was performed at the University of Maryland in collaboration with a visiting Ph.D. student from Catholic University of Brasilia, Brazil.

p38 MAPK mediates mRNA expression of telomere binding proteins in skeletal muscle.

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[2]Kinesiology Graduate Program, Catholic University of Brasilia, Brazil

Running Head: Activation of p38 MAPK mediates decreases in *Trf1* mRNA expression in muscle.

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Author Contributions:

Andrew T. Ludlow: Designed and executed the animal study. Designed and executed the i*n vitro* study. Performed laboratory measures, collected and analyzed the data. Drafted the manuscript.

Laila C. J. Lima: Designed and executed the animal study. Designed and executed the i*n vitro* study. Performed laboratory measures, collected and analyzed the data. Edited the manuscript.

Jenny Wang: Performed laboratory measures.

Erik. D. Hanson: Performed laboratory measures. Edited the manuscript.

Espen E. Spangenburg: Designed the animal study. Designed the *in vitro* study. Edited the manuscript.

Stephen M. Roth: Designed the animal study. Designed the *in vitro* study. Drafted and edited the manuscript.

Abstract –

Telomeres are repetitive DNA elements that protect chromosomes and shorten with age in most tissues. I n skeletal muscle, however, telomere length does not shorten with age but paradoxically shortens in response to long-term aerobic exercise training (in contrast to other tissues). Previous results indicate chronic exercise is associated with altered expression of telomere binding proteins within the shelterin complex in skeletal muscle. Shelterin regulates telomere length and acute changes in expression of shelterin components over time may lead to the exercise-induced telomere shortening in skeletal muscle. Thus, we sought to determine whether acute exercise affects gene expression of specific members of the shelterin complex in mouse skeletal muscle. Three groups of female C57BL/6 mice were studied, one control group and two groups that underwent a 30 min treadmill running bout that were sacrificed either immediately following or 1-hour after the exercise. In response to the exercise bout, mRNA expression of telomere-repeat binding factor one (*Trf1*) was reduced in plantaris muscle, but not in tibialis anterior muscle, and this reduction was paralleled by changes in p38 MAPK phosphorylation. To determine if p38 MAPK mediated the decreases in *Trf1* mRNA expression C2C12 myotubes were treated with calcium ionophore (A21387) and we similarly observed reduced *Trf1* gene expression and phosphorylation of p38 MAPK. When C2C12 myotubes were treated in combination with a p38 MAPK inhibitor (SB202190) and A21387 no decrease in *Trf1* mRNA expression was observed, indicating a link between *Trf1* gene expression and p38 MAPK activation. Thus, acute endurance exercise results in a decrease in *Trf1* gene expression, which may be mediated by p38 MAPK. These data provide important mechanistic insights into the paradoxical telomere shortening that occurs in skeletal muscle in response to chronic exercise.

Keywords: shelterin, telomere-repeat binding factor 1, treadmill exercise, skeletal muscle telomeres

Introduction

Telomeres are specialized repetitive DNA sequences (5'-TTAGGG$_n$ -3') found at the ends of linear chromosomes that play a protective role for the chromosomes (198). Common dogma is that in response to cellular replication telomere DNA is lost and the telomeres shorten, potentially contributing to the induction of cellular senescence (63,199). In this sense telomeres are considered a biomarker of aging; however, in certain cell types the ribonucleoprotein telomerase maintains and lengthens telomeres (200), slowing the loss of telomere repeats and potentially delaying cellular senescence. In addition to maintaining the proliferative capacity of cells, telomeres act as an important contributor to genome stability and transcriptional regulation, thus indicating an important role for telomere length in post-mitotic tissues as well (201).

Telomeres also function to protect the ends of chromosomes from end-to-end fusions and from being recognized as DNA damage (173). To protect chromosome ends, telomeres form a T-loop chromatin structure (73) that is maintained by a six-protein complex termed shelterin (79). Shelterin consists of three telomere-specific binding proteins and three accessory proteins that link the complex together and stabilize the DNA-protein interactions (79). The three DNA binding proteins are: telomere repeat binding factors 1 and 2 (TRF1 and TRF2), and protection of telomeres 1 (POT1; POT1a and b in the mouse) (76). TRF1 is critical in telomere length homeostasis and regulates cell cycle progression, while TRF2 helps form the T-loop structure and has telomere length regulating functions (185). POT1 functions in regulating telomerase action at the telomere and also in maintaining telomere structure (41). Additional linker and bridging proteins tie together the shelterin components and stabilize telomere ends (79).

In skeletal muscle the telomere shortening dogma does not hold true: skeletal muscle telomere length does not change with age, likely stemming from skeletal muscle's post-mitotic and low cellular replication status ((191,192) Ludlow et al. under review). Recent evidence has shown that endurance exercise training may shorten telomere length in skeletal muscle ((7,109) Ludlow et al. under review.), which contrasts significantly from other tissues in which chronic exercise is thought to provide a 'telo-protective' effect and slow age-related telomere shortening (5,8,9,130,160). Collins et al. (109) was the first to show that in age-and training- matched endurance athletes, skeletal muscle telomere length was shorter in those displaying symptoms of fatigued athlete myopathic syndrome (FAMS) compared to healthy athletes. In a follow up study of healthy endurance-trained individuals, those who had trained for the greatest number of years and also had trained the greatest number of hours per week had the shortest skeletal muscle telomere lengths (7). In addition, we have recently in CAST/Ei mice that one year of chronic exercise exposure resulted in shorter skeletal muscle telomere length compared to both young (8wk) and age-matched sedentary mice (Ludlow et al. under review). In addition to telomere shortening in the exercised animals, we observed differences in gene expression and protein content of telomere length–regulating genes (i.e., shelterin components). Together, these data indicate that telomere dynamics in skeletal muscle are unique and do not follow the pattern typical of other tissues in response to chronic exercise.

Since skeletal muscle telomere length decreased in response to exercise training, we wanted to understand the initial signals induced by exercise that could lead to this paradoxical result. Mitogen-activated protein kinases are tightly linked to mitosis and

have been implicated in cellular senescence (202-204). The expression of shelterin components has recently been linked to the mitogen-activated protein kinase (MAPKs) family of signaling molecules, with p38 MAPK and JNK1/2 linked to the expression of *Trf1* and *Trf2* in cardiomyocytes (100). Exercise, specifically skeletal muscle contraction, is known to activate several MAPKs (149), which are involved in regulating cellular responses to stress. The terminal kinases in the MAPK signaling cascade are extracellular related kinase (ERK1/2), p38 MAPK, and c-jun n-terminus kinase/stress-activated protein kinase (JNK1/2, SAPK) (149). These proteins are broadly involved in processes such as cell death, differentiation, apoptosis, and cell cycle regulation (205). Exercise is a potent perturbation to homeostasis and results in a stress response in skeletal muscle that is, in part, mediated by MAPKs.

Given the data demonstrating that chronic exercise results paradoxically in reduced telomere length in skeletal muscle, we sought to determine the effects of an acute bout of exercise on components of the shelterin complex. The purpose of our investigation was 2-fold: 1) to determine the effects of an acute treadmill exercise bout on skeletal muscle gene expression of shelterin components; and 2) to determine if a potential relationship could be established between MAPK signaling and any detected alterations in gene expression of the shelterin components. We hypothesized that acute exercise would result in decreased skeletal muscle shelterin mRNA expression associated with activation of MAPKs.

Methods

Animals – All animal experiments were approved by the University of Maryland Institutional Animal Care and Use Committee and conformed to the National Institutes of Health's Guide for the Use and Care of Laboratory Animals (NIH Pub. No. 85-23, revised 1996). Twenty-two female six-week old C57BL/6 mice were purchased (Jackson Laboratories, Bar Harbor, ME). Animals were acclimated to the animal facility for one-week prior to being randomly assigned to treatment groups. The animals were housed at 25ºC on a 12 hour light-dark cycle. Animals were fed *ad libidum* laboratory mouse chow (Prolab RMH 3000, 5P00, LabDiet by Purina, Nestlé S.A., Vevey, Switzerland) and given free access to water. Animals were separated into three groups for the experiment; BL (baseline, n = 6) animals were exposed to a motionless treadmill for thirty minutes prior to being euthanized. The remaining sixteen animals underwent a 2-week treadmill acclimation protocol, peak speed test and were separated into two groups that underwent a 30min treadmill exercise bout and were euthanized either immediately following (time-point one, TP1, n = 8) or one-hour after (time-point two, TP2, n = 8). Skeletal muscles (plantaris and tibialis anterior) were dissected and frozen until processed and analyzed for gene expression and protein content.

Treadmill acclimation protocol – The treadmill was set at a 7% incline for all treadmill sessions. The animals were acclimated to the treadmill over a period of fourteen days consisting of four different sessions each repeated twice and separated by a day of rest, except for session four which was performed on 4 different days separated by a day of rest (Supplemental Table 4.1).

Incremental treadmill exercise test – Forty-eight hours after the last acclimation session, animals from the exercise groups were subjected to an incremental exercise test for assessment of their peak treadmill running speed. First, the mouse was placed for two-min on an immobile treadmill belt. Then, the test began at a speed of 6m/min and increased 3m/min every 2min until running ability was visually impaired as evidenced by the animal sitting on the shock pad for more than 30sec. The speed of the last stage completed was recorded as the peak treadmill running speed.

Acute treadmill exercise bout – Forty-eight hours after the incremental exercise test, the mice were exposed to 30 min of treadmill running at 65.5% (22.8 (3.6) $m*min^{-1}$; mean (standard deviation)) of their peak speed (34.7 (4.6) $m*min^{-1}$) and were sacrificed immediately (TP1; n = 8) or 1h following (TP2; n = 8) the running bout.

Gene Expression – Total RNA was extracted using Trizol® (Invitrogen, Carlsbad, CA). Briefly, one-μg of total RNA was reverse transcribed (Applied Biosystems, High-capacity cDNA kit, Carlsbad, CA), followed by PCR and gel electrophoresis using previously published primers ((9) and Ludlow et al. under review).

Immunoblotting procedures – Total protein was extracted from skeletal muscle samples with lysis buffer containing protease inhibitor cocktail (50 mM Hepes (pH 7.4), 0.1% Triton X-100, 4mM EGTA, 10mM EDTA, 15 mM $Na_4P_2O_7•H_2O$, 100 mM glycerophosphate with a protease inhibitor cocktail - complete mini EDTA-free (Roche, Indianapolis, IN)). Protein concentration was determined with a commercially available bicinchoninic acid protein assay (Pierce, Rockford, IL) to normalize each sample to 30 μg of total protein. Proteins were separated on 7.5% SDS-PAGE gels, transferred onto PVDF membranes (Immobilon-P, Millipore, Billericka, MA) blocked in 5% NFDM for

30 min and exposed to primary antibodies (all from Cell Signaling, Danvers, MA) overnight at 4ºC as follows: phosphorylated p38 MAPK (Thr180/Tyr182, 9211, 1:500), total p38 MAPK (9212, 1:500), phosphorylated Erk1/2 (p44 ERK1/ p42 ERK2, Thr202/Tyr204, 9101, 1:1000), total Erk1/2 (9102, 1:1000) and GAPDH (14C10 Rabbit mAb # 2118 1: 1000). For total protein isoforms and GAPDH analyses, phosphorylated immunoblots were stripped and re-probed with the above antibodies. Products were visualized using enhanced chemiluminescence (ECL) on the Gene Gnome (Syngene Bio Imaging, Frederick, MD). Band intensities were analyzed by densitometry using Image J software (NIH, Bethesda, MD) (181).

In Vitro study – Myoblasts (C2C12, ATCC CRL-1772, Manassas, VA) were seeded onto 6-well plates (Greiner bio-one 657 160, Frickenhausen, Germany) and cultured in growth media (10% FBS (Gibco 10082-189, Carlsbad, CA), 1% penicillin and streptomycin (Invitrogen 15070-063, 5,000 units penicillin 5,000μg streptomycin, Carlsbad, CA) in DMEM, (ATCC 30-2002, Manassas, VA) for 48hrs before being switched to differentiation media which contained 2% Horse serum (ATCC 30-2040, Manassas, VA) and 1% Penicillin and Streptomycin in DMEM for 72hrs. After cells were confirmed to be differentiated (visual confirmation of myotube formation, data not shown) myotubes were treated for various time periods with calcium ionophore (Sigma-Aldrich, C7522 in ethanol, St. Louis, MO) or in combination with a pharmacological inhibitor of p38 MAPK (this inhibitor binds to the ATP binding pocket and does not directly affect phosphorylation status of p38 MAPK at Thr180-X-Tyr182; SB-202190, Sigma S7067 in DMSO, St. Louis, MO). Gene expression and immunoblotting were performed as above.

Statistics - Gene expression targets were normalized to the reference gene *Gapdh* and then expressed relative to the baseline group. *Gapdh* was not different between groups and thus was a sufficient loading control and normalization gene (data not shown). GAPDH was also used as a loading control for protein analysis. All values are presented as means ± standard error of the mean. One-way analysis of variance (ANOVA) was performed with significance accepted at $p \leq 0.05$.

Results

In vivo gene expression results

Plantaris (PLT) - We measured *Trf1*, *Trf2*, *Pot1a*, and *Pot1b* in PLT at baseline (BL), TP1 and TP2. Compared to BL, *Trf1* gene expression was significantly decreased at TP1 ($p = 0.02$). BL and TP2 *Trf1* gene expression tended to be different ($p = 0.07$), while TP1 and TP2 *Trf1* gene expression was similar ($p = 0.58$; Figure 4.1a). *Trf2* gene expression tended to decrease at TP1 compared to BL ($p = 0.1$) but was not different between any other groups (Figure 4.1b). *Pot1a* and *Po1tb* gene expression were not different from BL at either time-point (Figure 4.1c and 4.1d, respectively).

Tibialis Anterior (TA) – No significant changes in gene expression were observed for shelterin components *Trf1*, *Pot1a*, or *Pot1b* (Figure 4.2a, c, and d, respectively) in the TA. Gene expression of *Trf2* in TP1 animals was significantly higher compared to TP2 animals ($p = 0.03$, Figure 4.2b) and tended to be higher than BL animals ($p = 0.08$) but was similar between TP2 and BL animals ($p = 0.8$).

In vivo MAPK results

Plantaris - Phosphorylated levels of ERK1 and ERK2 were not different between any groups ($p = 0.5$ and $p = 0.8$, Figure 4.3a, respectively). Phosphorylated levels of p38 MAPK were significantly different between BL and TP1 animals (50%, $p = 0.03$) but were not different between BL and TP2 ($p = 0.3$) or TP2 and TP1 ($p = 0.2$; Figure 4.3b).

Tibialis Anterior – Since the response of shelterin gene expression to acute exercise was different between PLT and TA we wanted to investigate if MAPK activation was also different. In the TA, phosphorylated levels of ERK1 were not different between any time-points; however, phosphorylated levels of ERK2 were

significantly higher in TP1 animals compared to TP2 (110% p = 0.04) and tended to be greater than BL animals (100% p = 0.08, Figure 4a). No differences were detected between BL and TP2. No differences were observed in the phosphorylation status of p38 MAPK for any time-point (Figure 4.4b).

In vitro results -

We followed up on the down-regulation of *Trf1* because of its critical importance in telomere length regulation and homeostasis (74,185). Previous experiments have indicated that p38 MAPK activation is induced by increased calcium levels in the skeletal muscle cell (206) and activated p38 MAPK can influence telomere biology by altering the expression of shelterin components *Trf1* and *Trf2* (100). We treated C2C12 myotubes with calcium ionophore (A23187) to induce increases in intracellular calcium levels as previously described (207-211) and then determined *Trf*1 gene expression. Treatment with 1μM A23187 has been related to a ~ 4-fold increase in cytosol calcium levels in skeletal muscle myotubes (212). Treatment with A23187 resulted in a significant decrease in *Trf1* gene expression (p = 0.003, Figure 4.5) and significant increases in p38 MAPK phosphorylation, which returned to near baseline levels 24 hrs post treatment (p = 0.02, Figure 4.6). To determine if p38 MAPK levels were directly related to *Trf*1 gene expression, we inhibited p38 MAPK activity with SB202190 as previously described (32,213). Treatment of the C2C12 myotubes with A23187 resulted in a significant decrease in *Trf*1 gene expression, which was completely prevented by inhibition of p38 MAPK with SB202190 (p < 0.001, Figure 4.7).

Discussion

We demonstrate for the first time that acute endurance exercise alters gene expression of *Trf1* in mouse skeletal muscle and also show potential link *Trf1* expression and p38 MAPK activation. TRF1 plays a critical protective role in the maintenance of telomere length, thus these data provide important insights into a possible mechanism for the paradoxical loss of telomere length induced by chronic exercise in skeletal muscle. Understanding how chronic endurance exercise results in telomere shortening will provide further insights into the complex regulation of telomere dynamics in skeletal muscle and perhaps other post-mitotic tissues.

Several groups, including our own, have recently shown that exercise training slows age-related telomere shortening in several tissues, with the important exception of skeletal muscle ((5,8,9,130) and Ludlow et al. under review). In skeletal muscle, multiple groups have demonstrated a loss of telomere length with chronic exercise ((7,109) and Ludlow et al. under review). In our previous study, we found that chronic voluntary exercise (1 yr) attenuated an aging-induced increase in mouse skeletal muscle *Trf1* protein content suggesting that exercise may negatively regulate *Trf1*. Those results suggested a unique regulation of the telomere complex in skeletal muscle in response to chronic exercise when compared to other tissues (Ludlow et al. under review). For example, in cardiac tissue Werner et al. (9) found that wheel running for 21 days in mice resulted in increased *Trf1* gene expression compared to sedentary mice, which further indicates that regulation of telomere biology is different in skeletal muscle. To the best of our knowledge, only one other study has investigated *Trf1* gene expression in skeletal muscle; in Duchenne's muscular dystrophy patients compared to controls, *Trf1* gene

expression was increased (along with shorter telomeres) (214). These intriguing findings led us to examine the role of acute exercise as a means to identify potential signals that may alter *Trf1* gene expression in skeletal muscle.

Numerous cellular signaling pathways are activated by contraction of skeletal muscle, including the MAPK pathway (171). Due to the unique telomere response observed in exercised skeletal muscle, we hypothesized that this shortening may be a stress response induced by the exercise. Thus, we targeted the MAPK signaling pathway since numerous studies have shown that components of this family are induced by external stresses and exercise in particular. Indeed, we observed that our treadmill paradigm resulted in enhanced phosphorylation of p38 MAPK in the PLT muscle, though we found no response in the TA muscle. The activation of p38 MAPK occurs during muscle contraction and the amount of activation is dependent on the type, time, and intensity of the contractions (149). Treadmill running in rodents recruits the PLT to contract to a greater extent than the TA (182,215), which likely explains the measureable p38 MAPK phosphorylation in the PLT but not the TA. Thus, in the muscles where *Trf1* mRNA expression was reduced we found increased phosphorylation of p38 MAPK.

Previous, *in vitro* studies have found that elevations in intracellular calcium levels can activate p38 MAPK (166,206), which is an effect known to happen in skeletal muscle during and after a bout of exercise (216). Thus, elevations in intracellular calcium may act as an upstream signal resulting in reduced *Trf1* mRNA expression. Using previously described methods (207), we exposed cultured C2C12 myotubes to A21387 to increase intracellular calcium (212), which resulted in significant increases in p38 MAPK phosphorylation. Similar to our *in vivo* results, we found that when p38 MAPK was

phosphorylated there was a subsequent decrease in *Trf1* mRNA content. To determine if p38 MAPK was a critical mediator of the calcium effect, we inhibited p38 MAPK using SB202190 in combination with A21387 and prevented the reduction in *Trf1* gene expression. These data indicate a link between p38 MAPK signaling and *Trf*1 gene expression and support our *in vivo* observations that p38 MAPK activation may be regulating the reduced *Trf1* gene expression. We are aware of few data demonstrating this linkage between p38 MAPK and *Trf1* with the exception of Spallarossa et al. (100) who observed decreased *Trf*1 gene expression when treating cardiomyocytes with doxorubicin, which resulted in a simultaneous increase in phosphorylation of p38 MAPK. Our results provide a rationale for future experiments to test possible mechanisms by which long-term aerobic exercise training results in shortened telomeres in skeletal muscle.

Our data indicate that contraction/exercise-dependent decreases in *Trf1* gene expression may be the result of p38 MAPK activation. We speculate that intracellular elevations in calcium may be an important signal in this activation, though it is equally possible that other signals could also activate p38 MAPK during exercise (e.g., reactive oxygen species). We hypothesize that repeated bouts of unaccustomed exercise could result in transient decreases in mRNA expression of *Trf1,* which ultimately result in reduced TRF1 protein content (Figure 4.8). This situation of reduced TRF1 protein could lead to altered telomere length homeostasis and over time a loss of telomere length in exercise-trained skeletal muscles (i.e., repeated exposure to contractions). With long-term training the response of p38 MAPK would diminish and result in a return of basal expression of *Trf1*, though telomere length loss may have already occurred due to the

initial response of *Trf1* to repeated bouts of exercise. Our previous study indicated that after chronic (1 yr) exercise training *Trf1* expression was similar to that of young animals, but that sedentary animals had elevated *Trf1* (Ludlow et al. under review). In combination with the present findings, these results indicate that repeated bouts of exercise would result in decreased *Trf1*, which over time could lead to telomere shortening in chronically active individuals, suggesting unique regulation of mouse skeletal muscle *Trf1* in response to acute exercise.

In summary, we show that the reduced gene expression of *Trf1* in response to acute exercise is in part regulated by contractile activation of p38 MAPK in skeletal muscle. These results represent a first step in understanding the mechanism behind how skeletal muscle telomere length is reduced in chronically-trained rodents or humans. We provide insight into how unaccustomed exercise may initially result in loss of telomere protection (i.e., down regulation of *Trf*1) and thus lead to unrepaired telomere DNA damage and telomere length loss. Future studies are needed to determine the timeline of reduced *Trf1* gene expression in response to exercise training and also the timing and mechanism of telomere shortening in skeletal muscle in response to chronic (repeated acute bouts) of exercise.

Acknowledgements

This work was supported by the Department of Kinesiology Graduate Research Initiative Fund and NIH predoctoral training grant AG000268 (A.T. Ludlow) and by Brazilian CAPES Foundation PDEE scholarship BEX1714090 (L.C.J. Lima).

Supplemental Table 4.1.

Supp. Table 4.1.	**Treadmill acclimation procedures.**			
Session	**Time (min)**	**Belt speed ($m*min^{-1}$)**	**Shock pad**	**Days**
1	0 - 5	0	No shock	1-2
	5 -15	2	No shock	
2	0 - 2	0	No shock	4-5
	2 - 5	2	No shock	
	5 - 10	5	Light	
	10 - 15	10	Light	
3	0 - 2	0	No shock	7-8
	2 - 5	5	Light	
	5 - 10	15	Light	
	10 - 15	20	Moderate	
4*	0 - 2	0	No shock	10-11
	2 - 5	5	Light	
	5 - 10	15	Moderate	13-14
	10 - 15	20 – 24	High	

Supplemental Table 4.1 shows the treadmill acclimation procedures. Light shock (1.02 mA; 2.7 pulses*sec^{-1}); Moderate shock (2.04 mA; 2.7 pulses*sec^{-1}); High shock (3.06 mA; 2.7 pulses*sec^{-1}). Intensity in milliamperes (0.34 to 3.4mA) and repetition rate at 200msec pulses at a rate of 0.3 to 3 pulses per second. * Session 4 was repeated twice.

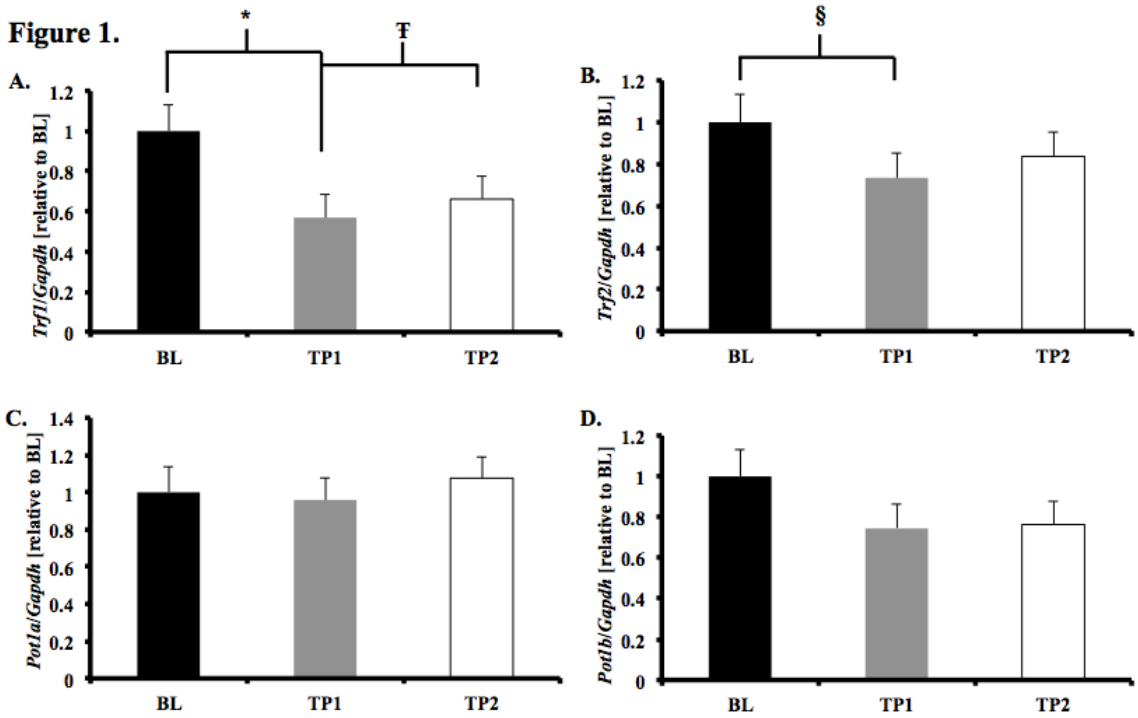

Figure 4.1. Acute exercise reduced shelterin component gene expression in the plantaris. *Trf1* gene expression was significantly reduced in TP1 animals compared to BL animals ($p = 0.02$), tended to remain reduced at TP2 ($p = 0.07$) and was not different between TP1 and TP2 ($p = 0.58$). *Trf2* mRNA expression tended to decrease in TP1 animals compared to BL ($p = 0.1$) but was not different between BL and TP2 ($p = 0.3$, Figure 1b) or between TP1 and TP2 ($p = 0.5$). *Pot1a* and *Po1tb* mRNA expression was not different between any groups ($p = 0.3$ and $p = 0.4$, Figure 1c and 1d, respectively). Data are presented as means ± S.E.M. mRNA abundance was assessed with RT–PCR, corrected for *Gapdh* and expressed relative to the BL group. BL = baseline (n = 6). TP1 = time-point 1 (n = 8) animals sacrificed immediately following the exercise bout. TP2 = time-point 2 (n = 8) animals sacrificed 1hr following the exercise bout. *TP1 significantly different than BL ($p < 0.05$). Ŧtendency for TP2 to be different than BL ($p < 0.1$). §tendency for TP1 to be different than BL ($p < 0.1$).

Figure 2.

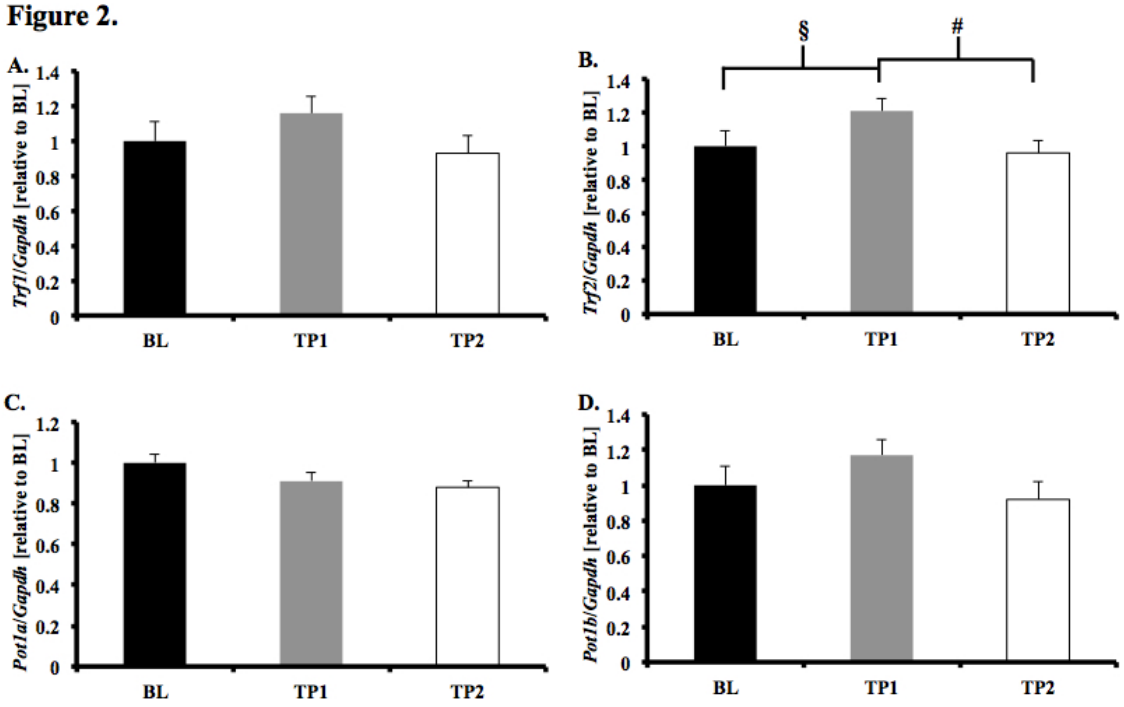

Figure 4.2. Gene expression of shelterin components in tibialis anterior muscle following acute exercise. *Trf1* gene expression was not different in TP1 or TP2 animals compared to BL animals ($p = 0.3$, $p = 0.7$, respectively) and were not different between TP1 and TP2 ($p = 0.1$, Figure 2a). *Trf2* gene expression was higher in TP1 compared to TP2 animals and tended to be higher than BL ($p = 0.03$, $p = 0.08$, respectively) but were similar between BL and TP2 ($p = 0.8$, Figure 2b). *Pot1a* and *Po1tb* mRNA expression were not different between any groups ($p = 0.2$ and $p = 0.2$, Figure 2c and 2d, respectively). Data are presented as means ± S.E.M. mRNA abundance was assessed with RT-PCR, corrected for *Gapdh* and expressed relative to the BL group. BL = baseline ($n = 6$). TP1 = time-point 1 ($n = 8$) animals sacrificed immediately following the exercise bout. TP2 = time-point 2 ($n = 8$) animals sacrificed 1hr following the exercise bout. $^{\#}$TP 1 significantly different than TP2 ($p < 0.05$). §tendency for TP1 to be different than BL ($p < 0.1$).

Figure 3.

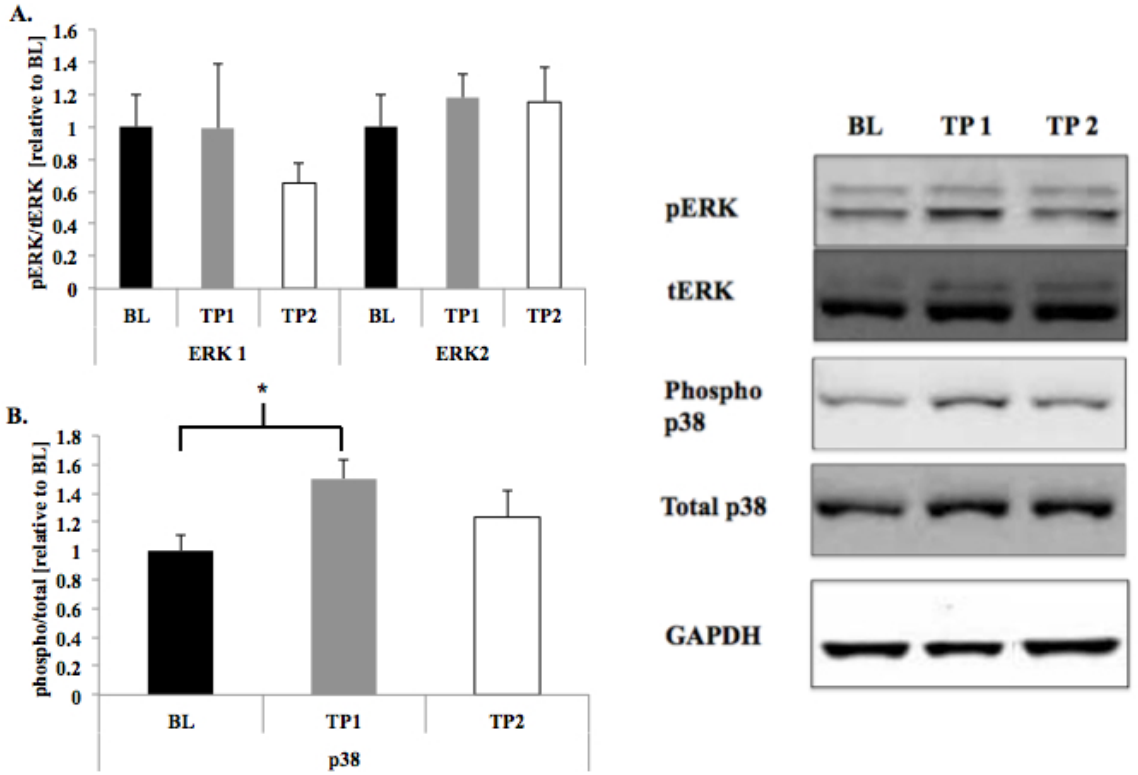

Figure 4.3. p38 MAPK is activated following acute treadmill exercise in the plantaris. We measured the phosphorylation status of the MAPKs ERK1/2 and p38 following acute treadmill exercise. ERK1/2 was not different among any groups (ERK1 p44 p = 0.6) and (ERK2 p 42 p = 0.8). p38 MAPK had significantly increased phosphorylation at TP1 compared to BL (p = 0.03), TP1 and TP2 were similar (p = 0.2) as were BL and TP2 (p = 0.3). Densitometric analysis and representative immunoblot images are shown. Data are presented as means ± S.E.M. Phosphorylated to total protein content ratios were derived and then expressed relative to the BL group for comparisons. GAPDH was a loading reference. BL = baseline (n = 6). TP1 = time-point 1 (n = 8) animals sacrificed immediately following the exercise bout. TP2 = time-point 2 (n = 8) animals sacrificed 1hr following the exercise bout. *TP1 significantly different than BL ($p < 0.05$).

Figure 4.

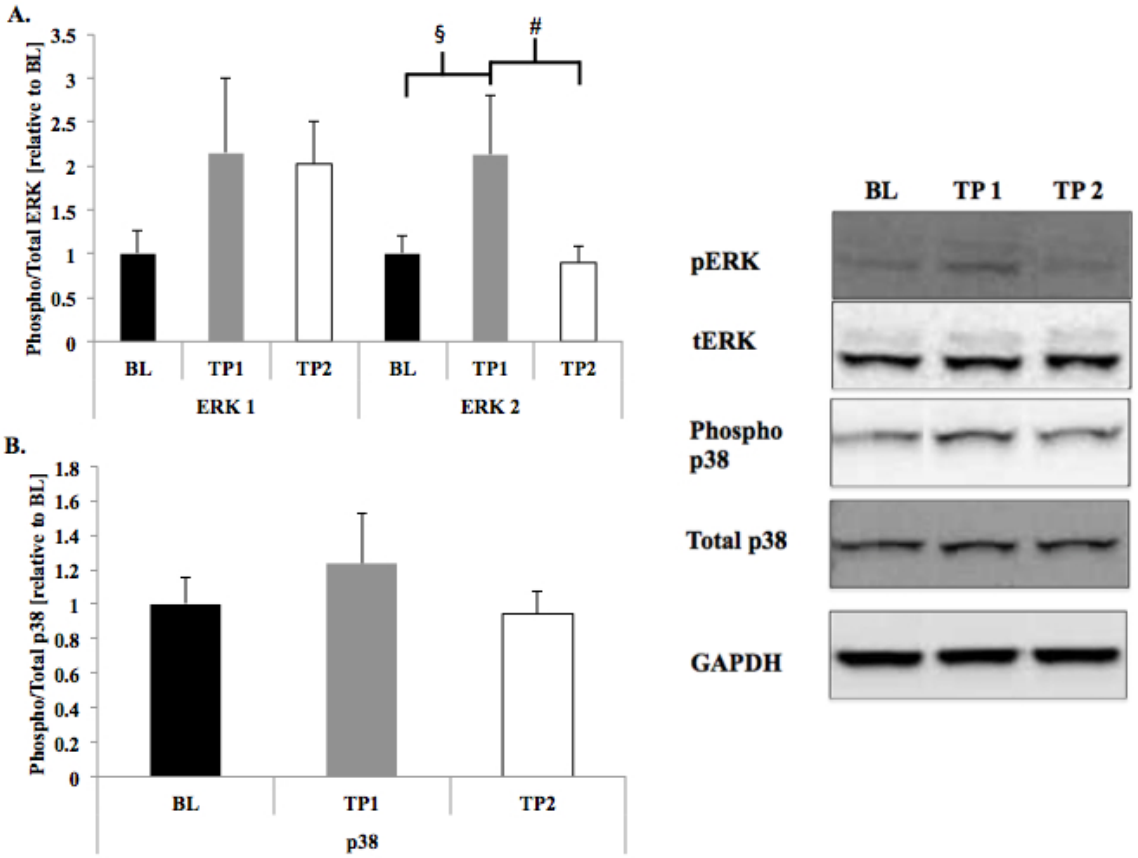

Figure 4.4. p38 MAPK is not activated following acute treadmill exercise in the tibialis anterior. ERK1 (p44) phosphorylation was not significantly altered in the TA due to treadmill exercise ($p = 0.4$). ERK2 (p42) phosphorylation was greater at TP1 compared to TP2 ($p = 0.04$), and tended to be greater than BL ($p = 0.08$), but BL and TP2 were similar ($p = 0.8$). p38 MAPK phosphorylation was not significantly altered by treadmill exercise ($p = 0.6$). Densitomeric analysis and representative immunoblot images are shown. Data are presented as means ± S.E.M. Phosphorylated to total protein content ratios were derived and then expressed relative to the BL group for comparisons. GAPDH was a loading reference. BL = baseline ($n = 6$). TP1 = time-point 1 ($n = 8$) animals sacrificed immediately following the exercise bout. TP2 = time-point 2 ($n = 8$) animals sacrificed 1hr following the exercise bout. $^{\#}$significantly different than TP2 ($p < 0.05$). §tendency for TP1 to be different than BL ($p < 0.1$).

Figure 5.

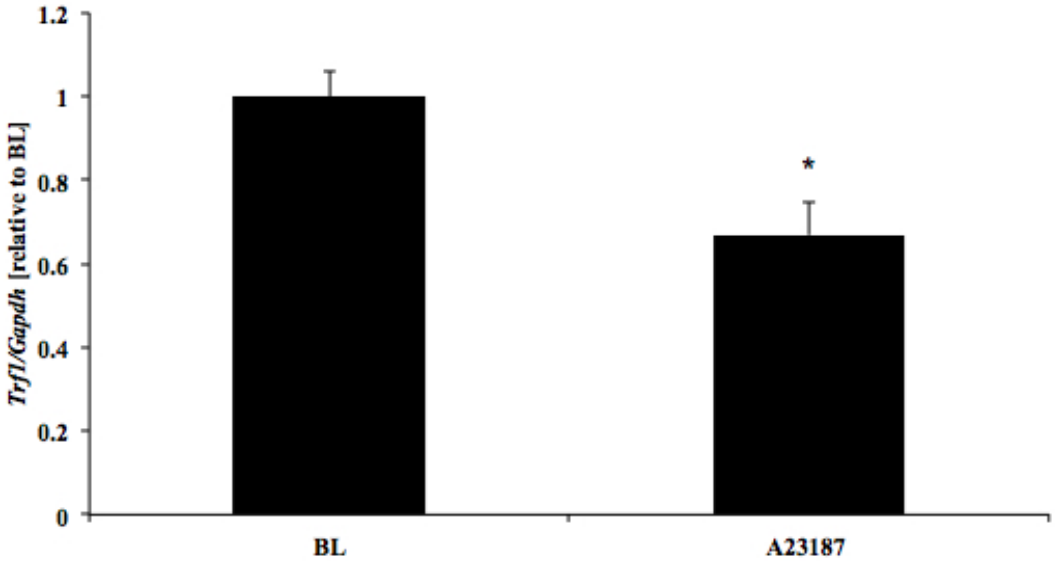

Figure 4.5. Calcium ionophore stimulation in C2C12 myotubes results in down-regulation of *Trf1* gene expression. Myotubes after 72hrs of differentiation were treated with calcium ionophore (A23187, 1μM) for 24hrs to induce a similar reduction in *Trf1* gene expression as observed *in vivo* following treadmill running. All experiments were performed in duplicate on at least 3 separate occasions, in serial passage. Data are presented as means ± S.E.M. mRNA abundance of *Trf1* and *Gapdh* were assessed with RT-PCR, corrected for *Gapdh* and expressed relative to the BL group. *A23187 treated significantly different than BL ($p < 0.05$). BL = vehicle (ethanol) treated.

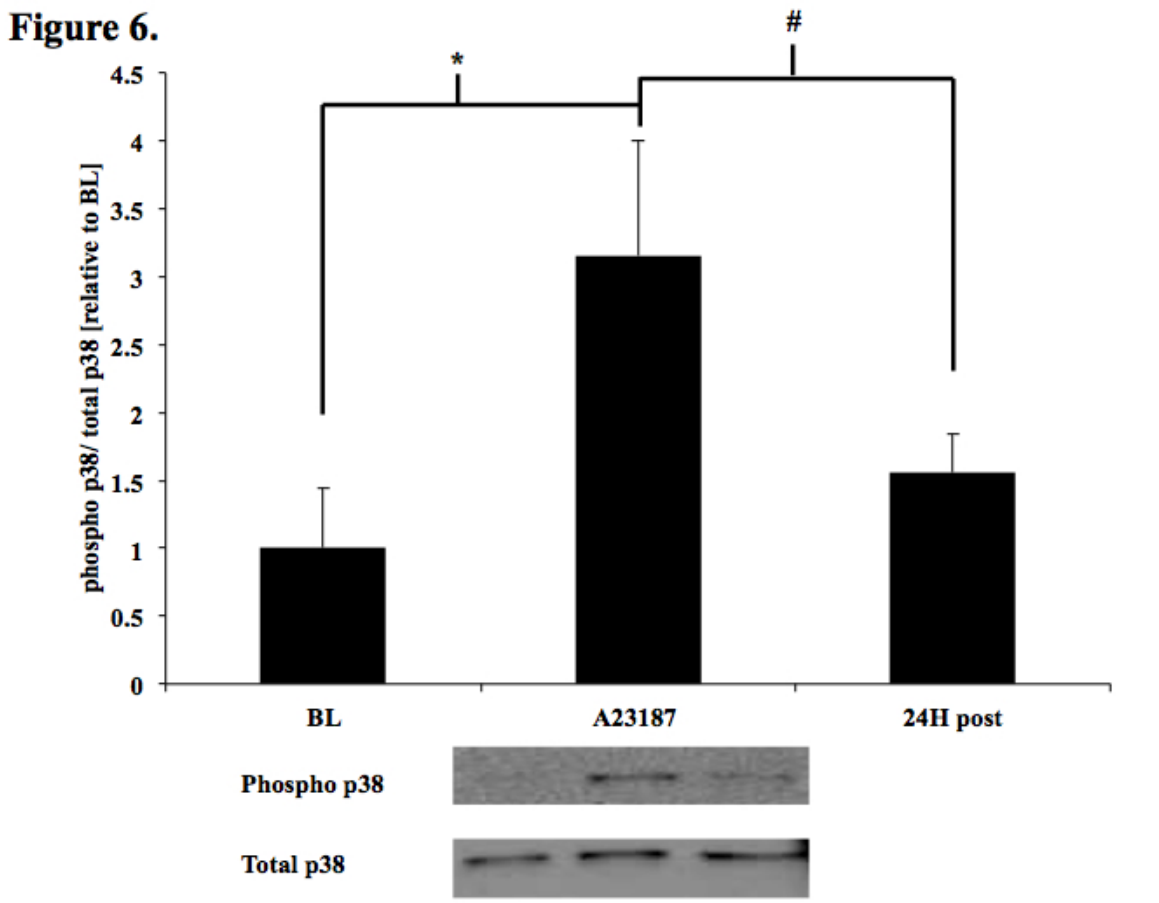

Figure 4.6. Calcium ionophore stimulation results in p38 MAPK phosphorylation in C2C12 myotubes. To determine if calcium ionophore treatment resulted in activation/phosphorylation of p38 MAPK myotubes were treated for 3hrs with 1μM A23187 or control and protein was isolated 30min and 24hrs following treatment. Calcium ionophore treatment resulted in significant phosphorylation of p38 MAPK compared to BL. Densitometric analysis and representative immunoblot images are shown. Data are presented as means ± S.E.M. Phosphorylated to total protein content ratios were derived and then expressed relative to the BL group for comparisons. GAPDH was a loading reference. BL = vehicle treated. A23187 = treated for 3hrs, and protein isolated 30min post treatment. 24H post = myotubes treated for 3hrs, and protein isolated 24hrs post-treatment. All experiments were performed in duplicate on at least 3 separate occasions, in serial passage. *A23187 significantly different than BL ($p < 0.05$). $^{\#}$A23187 significantly different than 24H post ($p < 0.05$).

Figure 7.

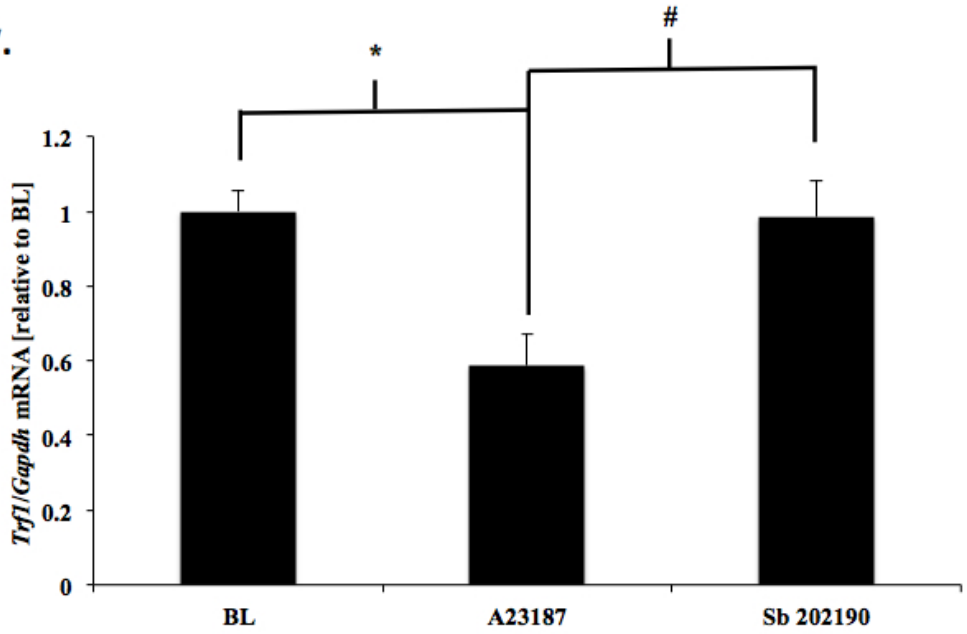

Figure 4.7. Inhibition of p38 MAPK prevents reduction of *Trf1* gene expression. To determine if p38 MAPK activation was directly related to *Trf1* gene expression, we treated myotubes with calcium ionophore and in combination with p38 MAPK inhibitor SB 202190 (note – this inhibitor does not affect phosphorylation at Thr180-X-Tyr182 but inhibits p38 activity by competitively binding to the ATP binding site). Myotubes were treated with control (vehicle), calcium ionophore (A23871, 1μM) or pretreated with SB – 202190 p38 MAPK inhibitor (10μM) for 30min prior to 24 hrs of treatment with A23187. Treatment with p38 MAPK inhibitor prevented the calcium ionophore-induced decrease in *Trf1* gene expression. All experiments were performed in duplicate on at least 3 separate occasions, in serial passage. Data are presented as means ± S.E.M. mRNA abundance of *Trf1* and *Gapdh* were assessed with RT-PCR, corrected for *Gapdh* and expressed relative to the BL group. BL = vehicle treated (DMSO and EtOH). A23187 = ionophore treated. Sb 202190 = myotubes treated in combination with A23187 and p 38 MAPK inhibitor. *A23187 significantly different than BL. $^{\#}$A23187 significantly different than Sb 202190.

Figure 8.

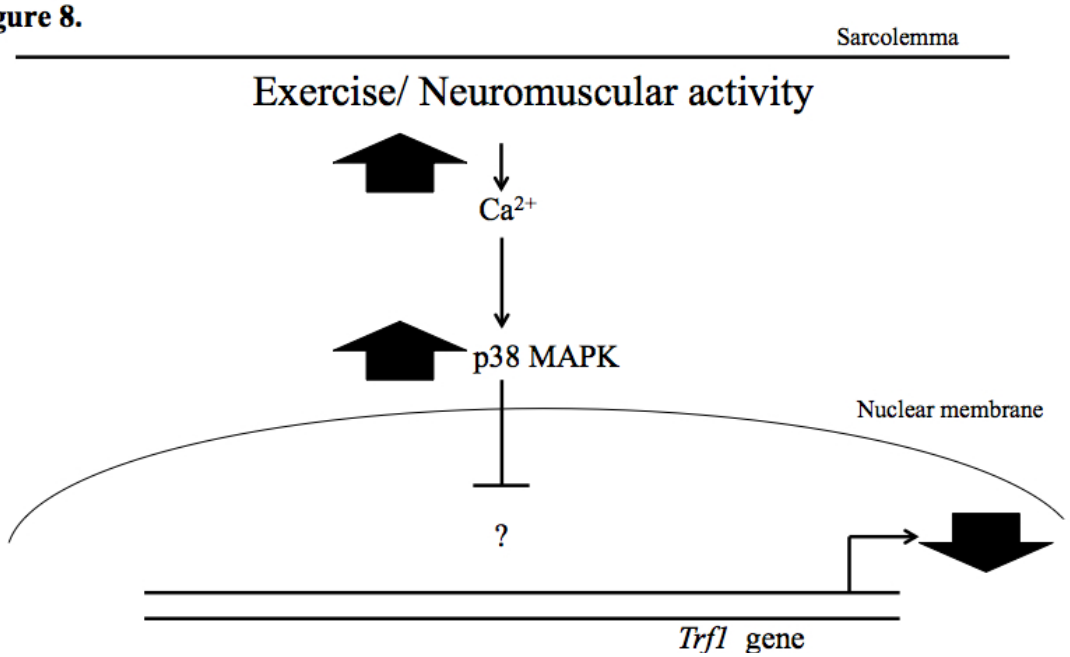

Figure 4.8. Proposed molecular mechanism of altered skeletal muscle *Trf1* gene expression following acute exercise. Our data indicate that *Trf1* gene expression in skeletal muscle is activity dependent and mediated by p38 MAPK. We propose that skeletal muscle contraction-induced calcium transients activate kinases (such as CAMK II) that phosphorylate and activate p38 MAPK resulting in altered activity of unidentifed transcription factors that control *Trf1* gene expression, leading to reduced levels of *Trf1* expression. Repeated acute exercise bouts would then be expected be related to telomere shortening as a result of chronic reductions in TRF1 protein.

Chapter 5: Adult skeletal muscle fiber telomere dynamics following oxidative stress treatment: A comparison between short (CAST) and long (C57BL/6) telomere strains of mice.

The following is a manuscript in preparation based on my final dissertation work.

Adult skeletal muscle fiber telomere dynamics following oxidative stress treatment: A comparison between short (CAST/Ei) and long (C57BL/6) telomere strains of mice.

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Running Head Title – Adult skeletal muscle fiber telomeres shorten in response to oxidative stress.

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Abstract

In most cells telomeres shorten over time due to a combination of incomplete replication, end processing, and unrepaired damage, thus making telomere length a biomarker of aging. Skeletal muscle telomere length, however, does not change with age due to its post-mitotic state. Paradoxically, chronic endurance exercise training, which slows telomere attrition in most tissues, is associated with shorter skeletal muscle telomere length. Since telomeres shorten due to unrepaired oxidative damage in other tissues, we sought to determine if oxidative stress shortens telomeres in isolated adult skeletal muscle fibers. Flexor digitorum brevis muscles were dissected from 15 male C57BL/6 (long telomere) and 20 male CAST/Ei (wild-derived, short telomere) mice and dissociated into single fibers. Fibers were cultured for 5 days in control, hydrogen peroxide (oxidant), or a combination of *N*–acetylcysteine (antioxidant) and hydrogen peroxide. Telomere length, telomerase enzyme activity, and protein content of TRF1 and TRF2 (important for telomere protection) were determined. In both strains, oxidative stress resulted in significant telomere shortening in isolated skeletal muscle fibers. Telomerase enzyme activity was not altered by oxidative stress treatment but was different between strains, with greater telomerase activity in long-telomere C57BL/6 mice ($p < 0.01$). TRF1 protein content was increased in CAST/Ei fibers that were exposed to the combination of oxidant and antioxidant. In oxidant-treated C57BL/6 fibers TRF2 protein content was increased compared to control fibers but this was attenuated in the presence of antioxidant. The response of TRF1 and TRF2 to oxidative stress exposure seems to be dependent on basal telomere length, long-telomere mice responding by expressing TRF2 and short telomere mice by expressing TRF1. These results provide important insights

into a mechanism by which chronic endurance exercise could shorten skeletal muscle telomeres.

Keywords: telomere length, telomere binding proteins, wild-derived mice, DNA damage

Introduction

Telomeres are repetitive DNA sequences (5'-TTAGGG$_N$-3') at the ends of linear chromosomes (61). In most tissues telomeres shorten over time due to a combination of incomplete replication, chromosome end processing, and unrepaired telomere DNA damage (103,197). Adequate telomere length is important in maintaining genome stability and regulating transcription (201,217). The enzyme telomerase can maintain and lengthen telomeres in germline, stem, and other highly proliferative cells (63,188,200,218). Telomere shortening has thus been studied as a biomarker of aging and is implicated in cancers and other age-related diseases such as cardiovascular disease (160,219). A variety of environmental factors important to age-related disease have been studied as modifiers of age-related telomere shortening. Among these, chronic exercise has emerged as a protective stimulus for a variety of cell types, attenuating age-related telomere shortening in heart, vascular tissue and lymphocytes (5,8,9,130).

Contrary to most tissues, telomere length does not change with age in skeletal muscle, likely stemming from its post-mitotic status and low cell turnover (Ludlow et al. under review, (107,191,192,220,221)). Paradoxically, skeletal muscle telomere length from long-term exercise trained individuals is shorter than telomere length of sedentary or young individuals (Ludlow et al. under review, (7,109)). These findings indicate a contradictory situation where shortened telomere length, typically considered a negative cellular phenotype, is occurring concurrently with the many positive health benefits of chronic exercise in skeletal muscle, such as increased insulin sensitivity, improved mitochondrial content, and increased antioxidant enzyme capacity (196).

Literature about the mechanism(s) of how skeletal muscle telomeres respond to chronic exercise is lacking. Skeletal muscle is extremely sensitive to oxidative DNA damage because of low antioxidant capacity compared to other tissues and deficiency in DNA repair enzymes (44). Skeletal muscle-derived reactive oxygen species (ROS) arising during contractions (162) may be a mechanism of damage to telomeres. Telomeres, particularly the 5'-GGG triplet, are extremely sensitive to oxidative DNA damage (45,193) and are also refractory to repair (47). Unrepaired DNA damage in the telomere can result in non-replication-based telomere shortening (193).

In addition to telomerase, a six-protein complex termed shelterin regulates telomere length (79,173,222) by acting to protect chromosome ends from being recognized by DNA damage machinery, maintaining telomere loop (T-loop) structure, and regulating telomerase action at the telomere (73,223). Core shelterin components telomere-repeat binding factor 1 and 2 (TRF1 and TRF2) are critical regulators of telomere length homeostasis and end-protection (74,173,185). TRF1 is a regulator of telomere length and important in cell cycle progression (224), while TRF2 is critical in DNA damage response, telomere length regulation and prevention of end-to-end fusions (173). These proteins are thus critical in the response to telomere DNA damage agents (i.e., oxidative stress) and may play an important role in telomere length regulation in skeletal muscle in response to acute exercise.

In the present study we sought to determine a possible mechanism whereby chronic exercise could result in telomere shortening in post-mitotic skeletal muscle. To do this we cultured isolated adult skeletal muscle fibers to eliminate contaminating cell types (e.g., satellite cells, vascular tissue, etc.), which allowed us to isolate the effect of

oxidative damage directly on skeletal muscle telomeres. In addition, we performed these experiments in two strains of mice with naturally differing telomere lengths in order to complete the following three aims: 1) to determine how telomere length in skeletal muscle is affected by oxidant exposure; 2) to determine the effect of oxidative stress on telomerase enzyme activity; and 3) to determine the effect of oxidative stress on telomere binding protein content (i.e., TRF1 and TRF2). We hypothesized that oxidative stress would decrease skeletal muscle telomere length and increase the protein content of telomere maintaining proteins in both strains of mice.

Methods

Animals - All animal experiments were approved by the University of Maryland Institutional Animal Care and Use Committee and conformed to the National Institutes of Health's Guide for the Use and Care of Laboratory Animals (NIH Pub. No. 85-23, revised 1996). Twenty male CAST/Ei (*Mus Musculus Castaneus*, 7-9 wk old) and fifteen male C57BL/6 (*Mus Musculus*, 8-10wk old) mice were purchased (Jackson Laboratory, Bar Harbor, ME). Animals were acclimated to the animal facility for at least 2 days prior to being randomly assigned to treatment groups. The animals were housed at 25ºC on a 12 hour light-dark cycle. Animals were fed *ad libitum* laboratory mouse chow (Prolab RMH 3000, 5P00, LabDiet, Nestle Purina, Vevey, Switzerland) and given free access to water. The animals were anesthetized (3% isoflurane) and euthanized by heart excision.

Single muscle fiber isolation procedure – Flexor digitorum brevis (FDB) skeletal muscles were dissected with a stereoscope (AmScope, Trinocular Stereo 3.5X-90X model SM-2TZ, Irvine, CA) in a sterile phosphate buffered saline solution (pH. 7.4, room temperature). Following dissection and removal of excess connective tissue, muscles were placed in 2mL of dissociation media (10% Fetal bovine serum (FBS, Gibco 10082-189, Carlsbad, CA), 1% Pennstrep (PS, Invitrogen 15070-063, 5,000 units penicillin 5,000µg streptomycin, Carlsbad, CA), (MEM, Gibco, 11095-080, Carlsbad, CA) with Liberase TM®, (13 Wunsch Units/mL, a blend of type I and II collagenases, Roche, 05 401 119 001, Indianapolis, IN) in a 35mm culture dish and placed in a cell culture incubator for 90 min (37ºC, 5% CO_2, ambient air). After the 90 min incubation, FDB muscles were rinsed of excess collagenase by a series of three one-minute washes in 35mm dishes containing pre-warmed MEM supplemented with 1% PS. The muscle was

then placed into a single well of a six-well culture plate (Greiner bio-one Cell Star®, 657 160, Frickenhausen, Germany) with 2 mL of MEM supplemented with 1% PS and gently titrated with 10-15 strokes with a 1 mL pipette or until the muscle was visibly dissociated (10x inverted microscope inspection). To reduce the incidence of muscle fiber dedifferentiation and myoblast/fibroblast proliferation, FBS was not used in the final culture media (225).

Cell culture and fiber treatments - After fibers from all animals were completely dissociated, 6-well plates were placed into an airtight cell culture chamber (Supplemental Figure 5.1). The chamber was then flushed with a gas mixture for 5 min at 2 p.s.i. (2% oxygen, 5% carbon dioxide and 93% nitrogen) designed to replace the ambient oxygen level (21%) with a physiological oxygen level (~2-5% as previously described (226)) and allowed to rest overnight in the 37ºC cell culture incubator. The following day the fibers were inspected and treated with randomly assigned culture conditions (control = MEM, 1% PS; H_2O_2 = 1μM H_2O_2 (maximum dose tolerable for duration of treatment, see Supplement Figure 5.2, (165)), Sigma A8199; NAC+ = 1μM H_2O_2 plus 100μM *N*-acetylcysteine, St. Louis, MO) for five days. Treatment with 1μM H_2O_2 has been shown to induce intracellular concentrations of ~0.1μM in skeletal muscle fibers which is similar to the amount of ROS produced by electrically-stimulated contraction of FDB fibers (165). Thus this dose of hydrogen peroxide treatment likely represents a physiologically relevant dose of ROS in skeletal muscle fibers. Previous observations (Ludlow et al. unpublished obs.; Study #3; (227)) indicated that telomeric responses may accumulate over time (i.e., repeat exposure effect) and lead to telomere shortening, thus we treated our fibers for 5 days rather than with a single dose.

The two FDBs from each animal were placed into separate wells of a 6-well plate and treated identically, thus each treatment group consisted of fibers from at least 5 animals (exact sample sizes reported in results). Treatments were replaced and the chamber re-flushed with low oxygen gas mixture every 24hrs. After five days of treatment ~800 fibers per animal were removed via inspection with a stereoscope and 200μL pipette and placed into four individual microcentrifuge tubes (~200 fibers/tube) for processing. Data were collected from C57BL/6: ~200 fibers from different five animals per treatment. CAST/Ei: ~200 fibers from 6-7 animals per treatment. Collected skeletal muscle fibers from C57BL/6 or CAST/Ei mice were centrifuged (17,000 x G, 25ºC, 5min) to pellet the fibers. Following centrifugation the supernatant was removed and lysis buffers for extraction of proteins, DNA or RNA were added to the pelleted fibers and frozen at -80ºC until further analysis (details below). These procedures were repeated for each strain of mice.

Telomere length – DNA was isolated from ~200 fibers and quantified as previously described ((130), Ludlow et al. under review). Between 12.5 and 20 ng of total genomic DNA was added to a reaction mixture as previously described (Ludlow et al. under review). All samples were run in triplicate, with a standard curve and reference samples were run on both plates to ensure linearity. The reference samples were from a previous study of CAST / Ei mice that had telomere length determined via telomere restriction fragment analysis (TRF southern blotting technique) (Ludlow et al. under review) to ensure accuracy of the assay.

Telomerase - Fibers were thawed at 4ºC, briefly centrifuged in a bench-top centrifuge, supernatant removed and 200μL of quantitative telomerase lysis buffer

(Quantitative Telomerase Detection Kit; US Biomax, Rockville, MD) was added to the pelleted fibers. Samples were then placed on ice with intermittent vortex mixing (every 15min) for 1hr followed by centrifugation (12,000 x g, 4ºC for 30min). Following centrifugation, supernatants were removed and assayed for protein content using a bicinchoninic acid protein assay (Pierce, Rockford, IL). In addition, cells from a telomerase positive human cancer cell line were treated as above and included as a positive control (HeLa, ATCC CCL-2, Manassas, VA). For unknowns and HeLa control samples 1μg of extracted protein was added to the reaction mixture according to the recommendations of the manufacturer and as previously performed in our lab (Ludlow et al. 2008). Telomerase activity was determined using a commercially available kit utilizing the telomere repeat amplification protocol (TRAP; Quantitative Telomerase Detection Kit; US Biomax, Rockville, MD). Beyond the kit-provided standards, heat-treated samples were assayed as negative controls. Heat-treated samples were concluded to be telomerase negative if the mean of the critical threshold (Ct) for the heat-treated sample duplicates was three standard deviations above that of the telomerase positive sample. Telomerase analysis was done as previously described by our lab ((130), Ludlow et al. under review).

Immunoblotting – Prior to freezing, fibers were lysed in 300μL of lysis buffer (100 mmol/ l Tris [pH 6.8], 4% sodium dodecyl sulfate [SDS], 20% glycerol and protease inhibitor cocktail (complete mini EDTA-free, Roche, Indianapolis, IN). Fibers were thawed on ice for 1hr and assayed for protein content as above. Fifty (TRF2 and GAPDH) and 90μg (TRF1 and GAPDH) samples were prepared for immunoblotting. Proteins were resolved on 10% SDS-PAGE gels, transferred to PVDF membranes and

blocked for 30min in 5% NFDM and exposed to primary antibodies overnight at 4ºC as follows: TRF1 (C-19, SC 1977, 1:200, Santa Cruz Biotechnologies, Santa Cruz, CA), TRF2 (H-300, SC 9143, 1:200, Santa Cruz Biotechnologies, Santa Cruz, CA) and GAPDH (Cell signaling, 14C10 Rabbit mAb # 2118 1: 1000, Beverly, MA). All products were visualized with horseradish peroxidase-linked secondary antibodies using high sensitivity enhanced chemiluminescence (SuperSignal West Dura Chemiluminescent Substrate, Pierce, Rockford, IL) on the Gene Gnome (Syngene Bio Imaging, Fredrick, MD). Band intensities were analyzed by densitometry using Image J software (NIH, Bethesda, MD) (181).

Statistical analysis - All values, unless otherwise stated, are presented as means ± standard error of the mean. Generalized linear models were used to analyze the data with SAS version 9.2. We collapsed all treatment groups and compared absolute values of each strain to assess strain differences. Treatment effects for each strain were determined with one-way ANOVA. *A priori* contrasts were analyzed with one-tailed t-tests. Differences were considered significant at $p < 0.05$.

Results

Single muscle fiber morphology is not altered at physiological oxygen tension. In an attempt to culture skeletal muscle fibers at or near physiological conditions (PO_2 in resting skeletal muscle is ~6-7 Torr or ~1%), we reduced the oxygen levels to 2-5% in an airtight cell culture chamber (Supplemental Figure 5.1) (228,229). Since oxygen levels of cultured murine cells are related to increased DNA damage, reducing the exposure of the skeletal muscle fibers to oxygen allowed us to more accurately assess the effects of our oxidant exposure (230). No noticeable differences in single muscle fiber morphology between strains or among treatment groups were observed in the cultures (Figure 5.1). In addition, we did not observe any fiber dedifferentiation (i.e., nuclei blebbing) or myoblast/fibroblast integration into single muscle fibers in any strain or treatment group.

Telomere Length is reduced in H_2O_2 treated adult single muscle fibers. Isolated single fibers from both strains were treated with control (CONT; C57BL/6 n = 5, CAST/Ei n = 7), hydrogen peroxide (H_2O_2; C57BL/6 n = 5, CAST/Ei n = 6) or a combination of H_2O_2 and the antioxidant *N*-acetylcysteine (NAC, combination treatment NAC+; C57BL/6 n = 5, CAST/Ei n = 6), as previously described (165). Hydrogen peroxide treated fibers from C57BL/6 mice tended to have shorter telomeres compared to control treated fibers ($p = 0.08$), shortening that was significantly attenuated by treatment with the antioxidant NAC (H_2O_2 vs. NAC+, $p = 0.05$; Figure 5.2), indicating a causal role of oxidative stress in telomere shortening. In CAST/Ei mice, H_2O_2 resulted in significant telomere shortening compared to control ($p = 0.04$; Figure 5.2); however, antioxidant

treatment did not result in attenuation of telomere shortening (H_2O_2 vs. NAC+, $p = 0.4$; Figure 5.2) and tended to be shorter than control ($p = 0.07$; Figure 5.2).

Telomerase enzyme activity was not altered by oxidant treatment in muscle fibers. As shown in Figure 5.3, H_2O_2 treatment did not result in any significant change in telomerase enzyme activity in either strain of mice (C57BL/6 $p = 0.8$; CAST/Ei $p = 0.6$), nor did the addition of NAC impact telomerase activity in either strain (C57BL/6 $p = 0.8$; CAST/Ei $p = 0.6$).

Oxidative stress treatment resulted in altered protein content of TRF1 and TRF2.

TRF1: In C57BL/6 fibers, no differences in protein content of TRF1 between H_2O_2 or NAC+ treatments were observed ($p = 0.9$; Figure 5.4). In the CAST/Ei fibers, no differences in TRF1 protein content were observed between control and H_2O_2 ($p = 0.5$; Figure 5.4); however, CAST/Ei fibers treated with a combination of NAC and H_2O_2 had significantly greater TRF1 protein content than both control ($p = 0.04$) and H_2O_2-treated fibers ($p = 0.01$; Figure 5.4).

TRF2: In C57BL/6 fibers, H_2O_2 treatment resulted in increased protein content of TRF2 compared to control ($p = 0.1$; Figure 5.5) that was attenuated in NAC+ treated fibers ($p = 0.05$; Figure 5.5), indicating a causal role of ROS in the TRF2 response. In CAST/Ei fibers, no significant alterations in TRF2 protein content were observed in either treatment group (Figure 5.5).

Strain comparisons reveal differences in telomere length and telomerase activity but not shelterin protein content. To determine strain differences we compared C57BL/6 and CAST/Ei mice regardless of treatment. As shown in Table 5.1, typical laboratory mice (C57BL/6), mice had longer telomeres compared to wild-derived CAST/Ei mice, confirming earlier reports ($p < 0.01$; Figure 5.2 and Table 5.1) (60). The C57BL/6 telomere length was reduced 4.1 fold (76% of control) in response to H_2O_2 treatment while the CAST/Ei telomere length was reduced 2.9 fold (67% of control). C57BL/6 mice had significantly greater telomerase enzyme activity compared to CAST/Ei ($p < 0.01$; Figure 5.3 and Table 5.1), similar to previous findings (231). For both TRF1 and TRF2 protein content, no differences were observed between strains (TRF1 $p = 0.4$; TRF2 $p = 0.7$; Figure 5.4 and Figure 5.5, respectively and Table 5.1).

Discussion

We demonstrate for the first time in isolated adult mouse skeletal muscle fibers that oxidative stress is a causative factor in telomere shortening. Oxidant treatment resulted in dramatic telomere shortening that was prevented with antioxidant treatment, most significantly in the long-telomere C57BL/6 strain. Telomerase activity was not impacted by the treatments, but we describe differences in activity between typical laboratory (long telomere) and wild-derived (short telomere) strains of mice that impact interpretation. Moreover, both TRF1 and TRF2 showed differential, strain-specific responses to oxidant and antioxidant treatment indicating strain-specific telomere dynamics in skeletal muscle. Our results indicate in C57BL/6 mice that TRF2 protein content is increase with ROS, possibly to prevent further telomere shortening. In CAST/Ei mice neither TRF1 nor TRF2 responded to ROS treatment independently, indicating important strain differences in the response to ROS that may be dictated by differences in basal telomere length. These results provide insights into the complex regulation of telomeres in post-mitotic tissues and clarify how factors (i.e., ROS) associated with muscle contraction and chronic endurance exercise may result in shortened telomeres in skeletal muscle.

Telomere dogma states that telomeres shorten with replication or exposure to DNA damage signals (e.g., ROS) and over time may reach a critical length causing telomere and tissue dysfunction (31). While this dogma holds true for aging of most human somatic cells, skeletal muscle cells do not follow this pattern of age-related telomere attrition. Our results and those of others have shown little to no age-related telomere shortening in skeletal muscle of healthy older mammals, both human and rodent

(Ludlow et al. under review, (191)). Recent reports and data from our lab have, however, shown skeletal muscle telomere shortening in skeletal muscle homogenates from mice and humans that have undergone chronic exercise training (Ludlow et al. under review, (7,197)). In addition, we have recently observed that a single bout of treadmill exercise produced a stress response that resulted in *Trf1* down-regulation in skeletal muscle (Ludlow et al. unpublished obs. Study #3). These data indicate that the initial response to acute exercise in skeletal muscle could predict long-term telomere length loss and that repeated exercise bouts could result in accumulated damage to the telomere.

Telomere length shortens in skeletal muscle under oxidative stress. *In vivo* it is thought that telomere length in skeletal muscle only shortens when the satellite cells are induced to divide and incorporate into existing fibers (106), such that shorter telomere nuclei accumulate in the tissue over time. Endurance exercise is not associated with enhanced satellite cell proliferation or incorporation into muscle fibers (106) making this an unlikely cause of such telomere shortening. Instead, we show for the first time, similar to other tissues (85,99), that oxidative stress can cause telomere shortening in skeletal muscle fibers devoid of satellite cells. Interestingly, treatment with the antioxidant NAC attenuated telomere shortening in skeletal muscle fibers of C57BL/6 mice but not in CAST/Ei fibers. This could indicate that skeletal muscle fibers from CAST/Ei mice have inherently higher basal oxidative stress thus requiring a greater dose of antioxidant to overcome the additional oxidative stress burden of hydrogen peroxide treatment. Alternatively, intrinsic differences in telomere length and telomere dynamics between the strains could underlie this differential response. Overall, we report that ROS is indeed a possible mechanism for telomere shortening in adult skeletal muscle fibers of

both typical and wild-derived strains of mice, though the dynamics of this shortening are strain-specific.

Telomerase enzyme activity is not increased in single muscle fibers exposed to oxidative stress. Previous studies have reported ambiguous results regarding telomerase enzyme activity following oxidative stress treatments across a range of tissues (67,144,232,233). Interestingly, we report no effect of oxidant challenge on telomerase activity, but that telomerase activity is greater in muscle fibers of C57BL/6 compared to short-telomere CAST/Ei mice, supporting previous findings (231). Our results confirm those of Cattan et al. (64) who showed that telomerase enzyme activity was not increased in skeletal muscle of CAST/Ei animals exposed to oxidative stress (long-term glutathione depletion). This could indicate that telomerase activity in skeletal muscle of mice is detectable, but extremely low compared to other tissues and therefore plays a minimal role in skeletal muscle fiber telomere dynamics.

TRF protein content is altered in response to oxidative challenge but differs by strain. Several recent studies have observed altered expression of TRF1 and TRF2 induced by multiple stimuli (8,9,100,234). We have previously shown that *Trf1* gene expression was reduced with acute exercise in skeletal muscle (Ludlow et al. unpublished obs.). In chronically exercised animals, exercise prevented an age-associated increase in TRF1 protein content and an age-associated decrease in TRF2 gene expression and protein content in skeletal muscle (Ludlow et al. under review). Contrary to other tissues that undergo age-related telomere shortening, skeletal muscle telomere length does not change. The increase TRF1 protein content observed in skeletal muscles older sedentary

animals may have protected/stabilized telomere length from shortening compared to shortened telomeres and lower TRF1 protein content in exercised animals.

In the present study, we show that TRF1 protein content is not influenced by oxidative stress treatment in skeletal muscle fibers of C57BL/6 mice. In the CAST/Ei fibers oxidative challenge alone did not change TRF1 protein content but the combination of antioxidant and oxidative stress increased TRF1 protein content, possibly indicating a bell shaped curve for TRF1 in response to ROS. A moderate increase in ROS (combination treatment) may result in a permissive gene expression environment while a large increase in ROS may repress TRF1 expression (ROS treatment). A study of skeletal muscle from Duchenne's muscular dystrophy patients (a condition associated with increased skeletal muscle ROS) compared to control skeletal muscle showed TRF1 protein was increased along with shortened telomeres (113). We speculate that TRF1 is responding to ROS in a strain-specific fashion based on telomere length and the amount of telomere shortening. We hypothesize that short telomere organisms (humans and CAST/Ei mice) may preferentially up-regulate TRF1 under stress while long telomere animals (C57BL/6) do not up-regulate TRF1 to stabilize telomere length in skeletal muscle. Future antioxidant dose-dependent experiments are needed to clarify these findings.

TRF2 protein content was increased in H_2O_2 treated skeletal muscle fibers of C57BL/6 animals but unchanged in CAST/Ei fibers. Our results in C57BL/6 mice are in line with previous reports indicating that oxidative stress results in increased TRF2 protein content (234). The increase in TRF2 protein content could be a compensatory mechanism of the fibers attempting to prevent further telomere loss, since TRF2 is

important in telomere length homeostasis (185). In addition, TRF2 is known to associate with several DNA damage sensing kinases, such as ATM (235), thus TRF2 could be mediating a DNA damage signal at ROS-induced shortened telomeres leading to telomere protection and repair (236). These data indicate that TRF2 is associated with the response of ROS-induced telomere shortening in C57BL/6 skeletal muscle. TRF2 was not induced in short-telomere mice, possibly related to underlying differences between strains in either telomere biology and/or antioxidant capacity.

The response of TRF1 and TRF2 protein content to oxidant challenge differed strikingly between mouse strains. Long-telomere mice up-regulated TRF2 but not TRF1, while short telomere mice did not alter the expression of either protein with ROS treatment, but increased TRF1 with combined oxidant and antioxidant treatment. The differential results between the CAST/Ei and C57BL/6 strains could be due to genotype or differences in telomere length (197,237). Recent evidence indicates that there may be differences in the response to oxidative stress between species with different telomere lengths, possibly explaining our results (197). The relatively recent inbreeding of the CAST/Ei strain compared to the long-term inbreeding of the C57BL/6 strain could represent evolutionary divergence within the species (197,238), which could also explain the differential results.

The current investigation is not without limitations. Our data provide no insight into a time-course of change of telomere length or expression of telomere length-maintaining proteins. In addition, a greater dose of antioxidant would also provide significant depth to the current investigation given the CAST/Ei results by clarifying the response of TRF1 to ROS and NAC treatment. This information would allow us to

determine if a greater dose of antioxidant would result in prevention of ROS-induced telomere shortening in the CAST/Ei strain.

In summary, we describe that ROS results in telomere shortening in skeletal muscle fibers of mice, but with strain-specific responses of shelterin components. These data indicate important strain differences in the response of telomeres and telomere-related proteins that may be driven by intrinsic differences related to telomere length and/or antioxidant enzymes or other unknown factors. We provide a potential mechanism by which chronic exercise and its related ROS production, especially in the early, unaccustomed stages, could induce telomere shortening in skeletal muscle.

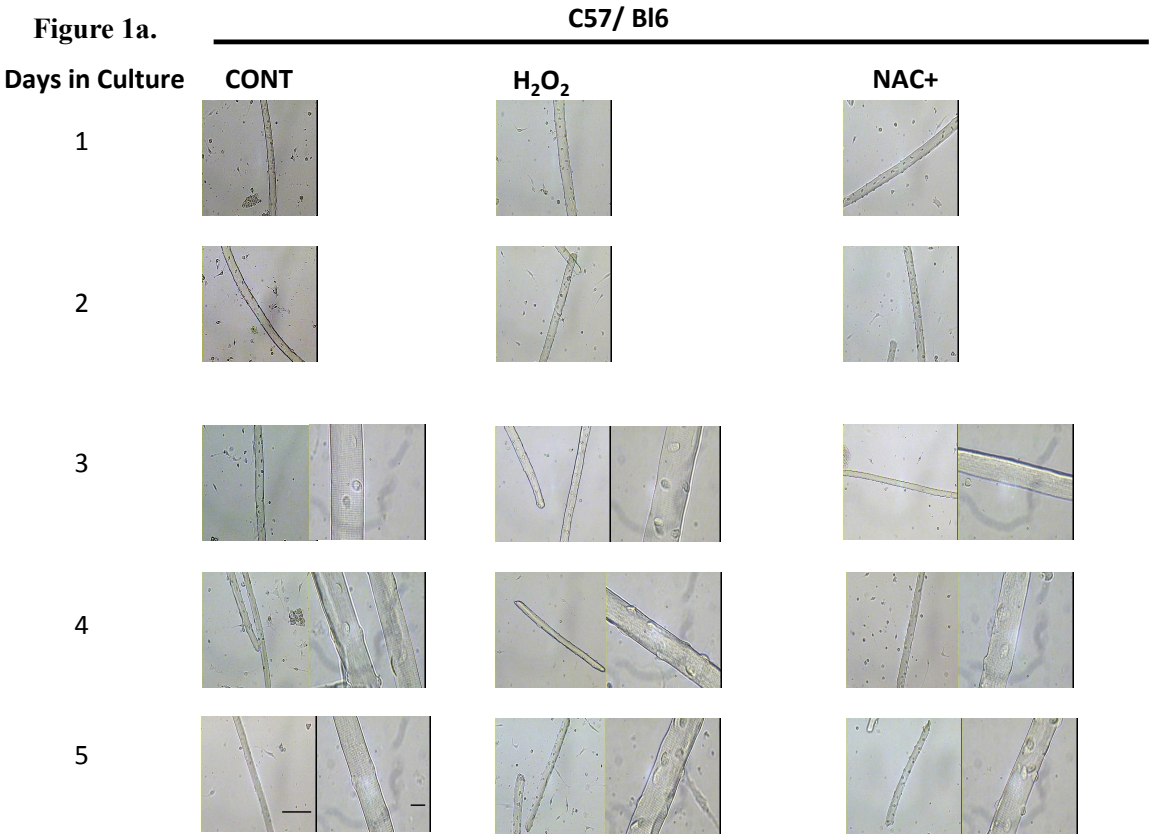
Tables

Table 5.1. Animal characteristics and strain comparisons.

Strain	Age (wks)	Body Mass (g)	Telomere length (T/S ratio)	Telomerase activity (Amoles/μg)	TRF1 (AU)	TRF2 (AU)
C57BL/6	9.5 ± 0.1	23.8 ± 0.3	2.6 ± 0.5	$1 \times 10^{-4} \pm 2 \times 10^{-5}$	0.43 ± 0.2	0.05 ± 0.01
CAST/Ei	7.7 ± 0.1*	13.5 ± 0.2*	0.75 ± 0.5*	$1 \times 10^{-5} \pm 2 \times 10^{-5}$ *	0.76 ± 0.2	0.04 ± 0.01

Data are presented as mean ± S.E.M. * $P < 0.05$. wks = weeks, g = grams, T/S ratio = telomere copy PCR to single copy gene PCR ratio. Amoles = attomoles, AU = arbitrary units. Age was significantly different between strains but not within strain between treatment groups (data not shown). TRF1 and TRF2 protein content was normalized to GAPDH.

Figure 1a.



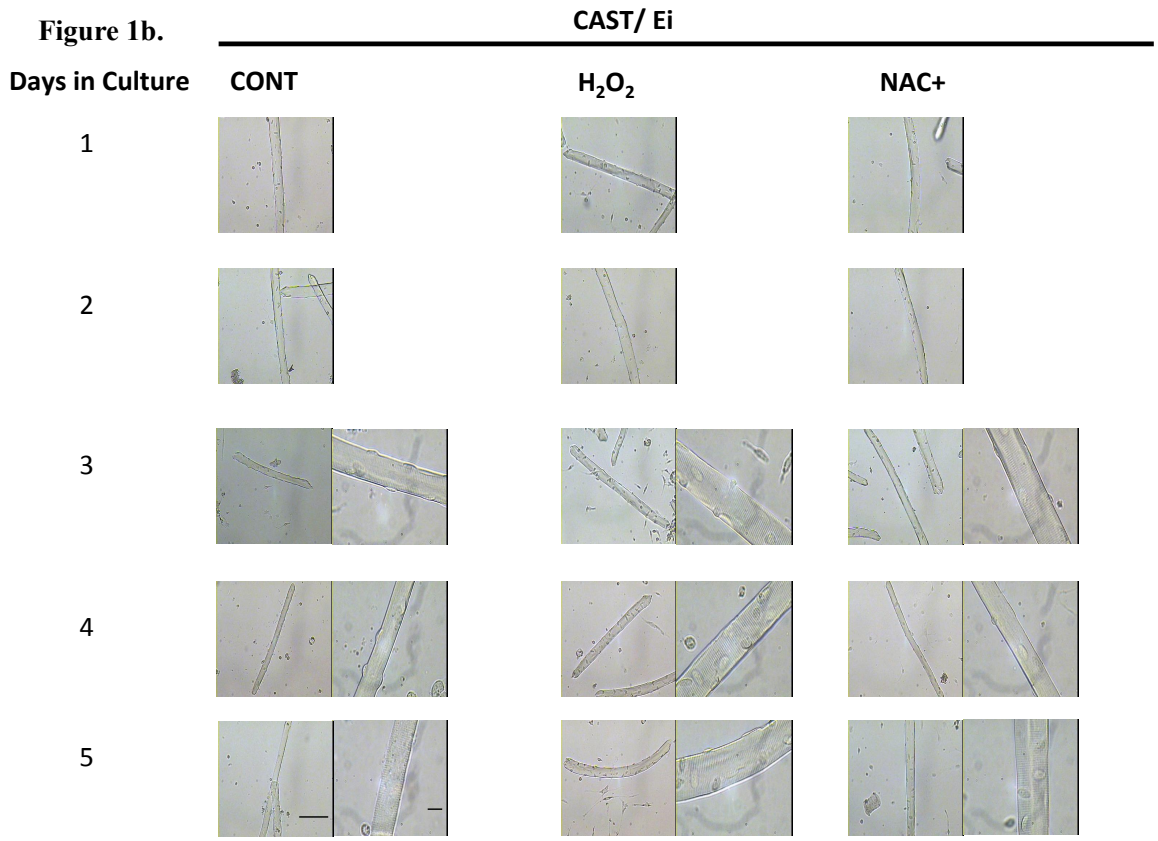


Figure 5.1: Morphology of skeletal muscle fibers cultured at physiologic oxygen and treated with hydrogen peroxide with and without antioxidant (NAC) for 5 days. Skeletal muscle fibers from both strains of mice were cultured in low oxygen conditions and treated for 5 days with H_2O_2 (1μM), H_2O_2 plus *N*-acetylcysteine (NAC, 10μM and 1μM H_2O_2), or control (vehicle, water) treated. Treatments were replaced every 24hrs. **A)** Micrographs of C57BL/6 skeletal muscle fibers cultured over five days at low oxygen. No visible differences were observed among treatment groups. Images were taken at low (10x) and high magnification (40x) from an inverted light microscope. The high magnification images show intact sarcomeric structure. **B)** Micrographs of CAST/Ei fibers over five days at low oxygen. Representative images are presented from each treatment group. CONT = control; H_2O_2 = hydrogen peroxide treated; NAC+ = hydrogen peroxide and NAC treated. Scale bar on low magnification image = 1mm; high magnification = 25μm.

Figure 2a.

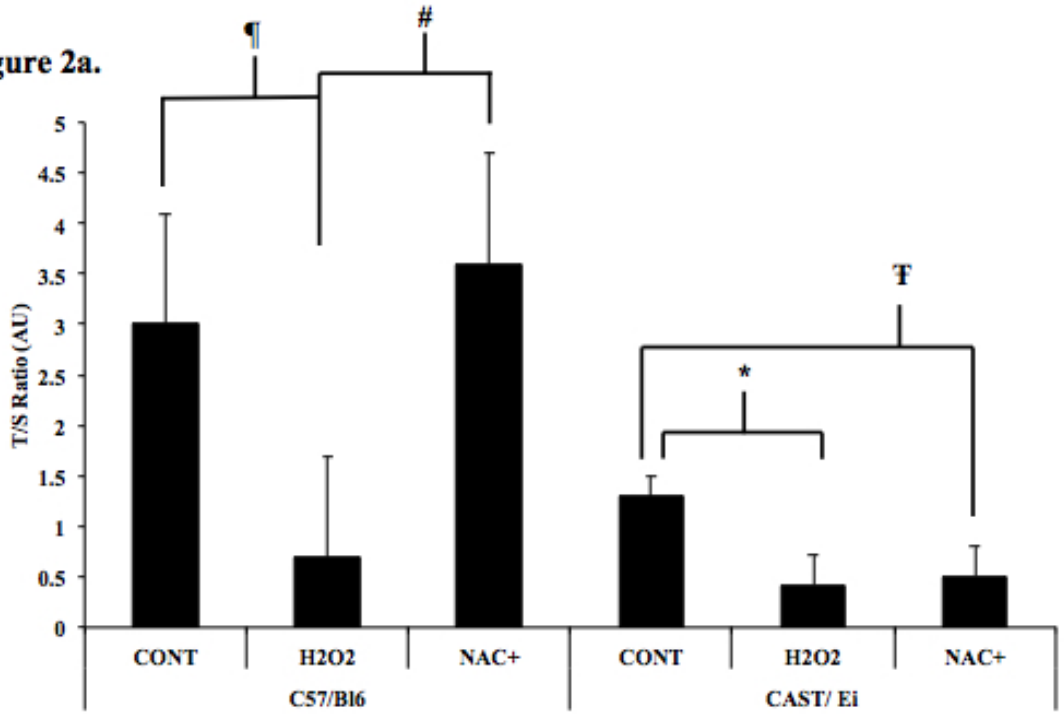


Figure 2b.

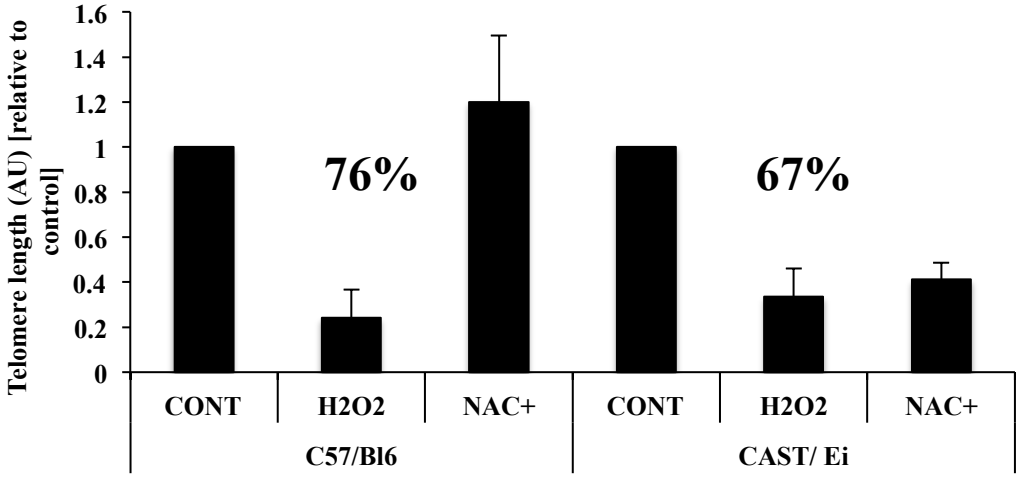


Figure 5.2: Oxidative stress shortens skeletal muscle fiber telomere length. Skeletal muscle fibers from both strains of mice were cultured in low oxygen conditions and treated for 5 days with H_2O_2 (1μM), H_2O_2 plus *N*-acetylcysteine (NAC, 10μM and 1μM H_2O_2), or control (vehicle, water) treated. **A.** Telomere length as measured by T/S ratio. * H_2O_2 significantly different from control ($p = 0.05$). # H_2O_2 significantly different from NAC+ ($p = 0.05$). ¶ tendency for H_2O_2 to be different from control ($p = 0.08$). Ŧ tendency for NAC+ to be different from control ($p = 0.07$). **B.** Telomere length expressed relative to each strain's control condition to show the relative change in telomere length. Data are presented as means ± S.E.M. CONT = control; H_2O_2 = hydrogen peroxide treated; NAC+ = hydrogen peroxide and NAC treated. T/S ratio = Telomere repeat copy to single copy gene ratio. AU = arbitrary units.

Figure 3.

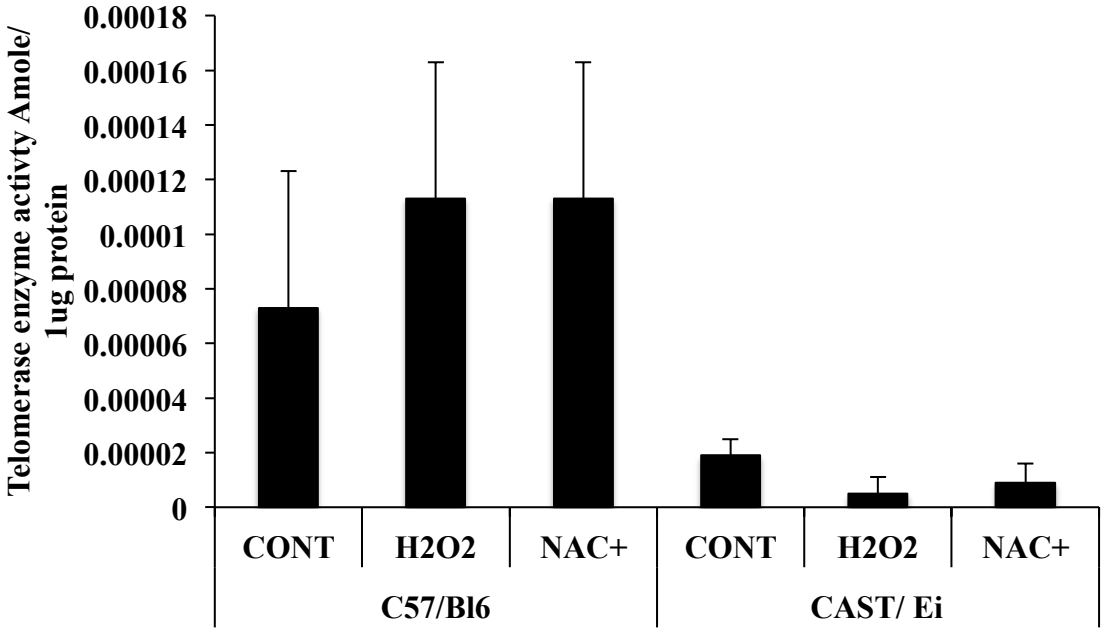


Figure 5.3: Oxidative stress does not alter telomerase enzyme activity in skeletal muscle fibers. Oxidative stress treatment did not result in altered telomerase enzyme activity in either strain of mice (C57BL/6 $p = 0.8$; CAST/Ei $p = 0.6$). Overall comparison between strains revealed greater telomerase enzyme activity in C57BL/6 compared to CAST/Ei animals ($p < 0.01$). Data are presented as means ± S.E.M. CONT = control; H_2O_2 = hydrogen peroxide treated; NAC+ = hydrogen peroxide and NAC treated. Amoles = attomoles.

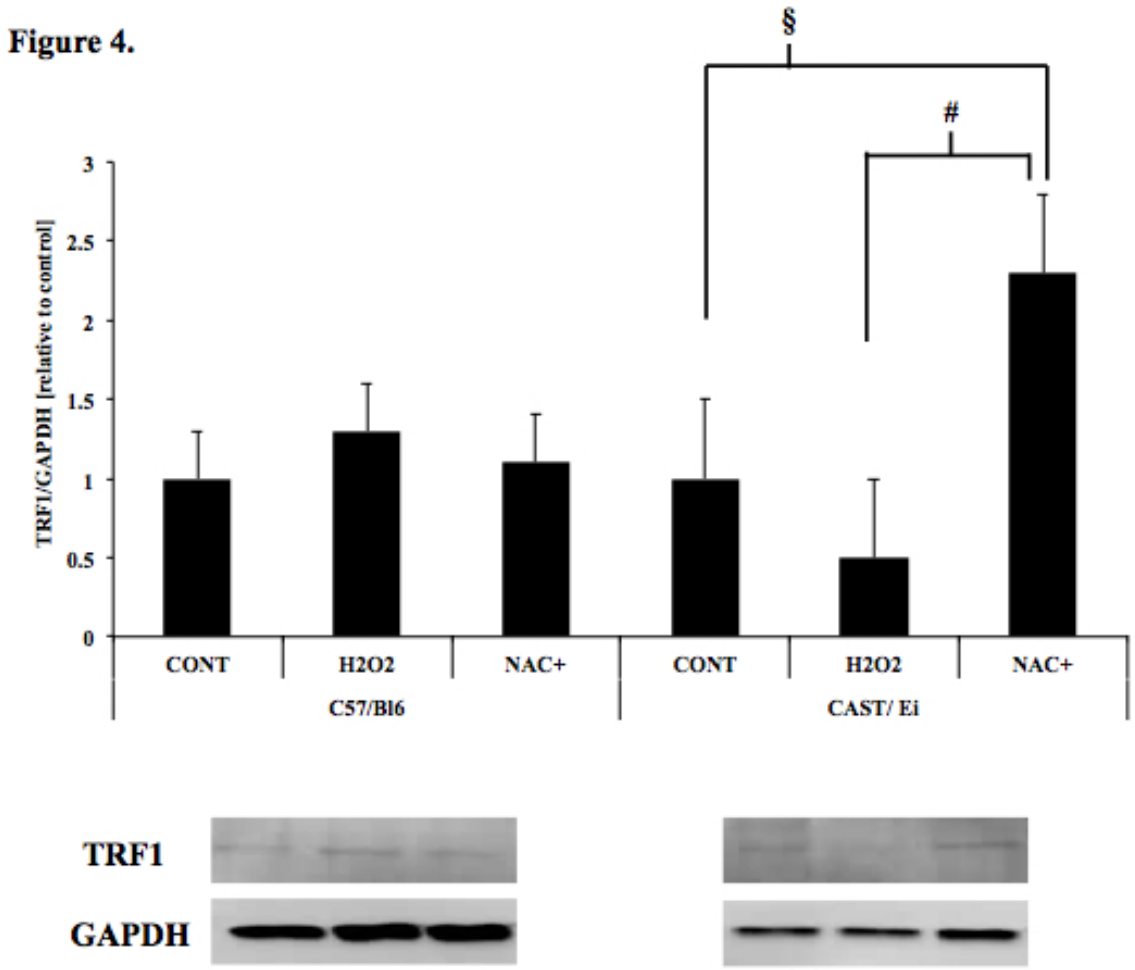


Figure 5.4: TRF1 protein content is not altered by oxidative stress treatment. In C57BL/6 fibers oxidative stress treatment did not alter TRF1 protein content ($p = 0.9$). In the CAST/Ei mice there was no difference between CONT and H_2O_2 treated fibers ($p = 0.5$). NAC+ treated fibers had greater TRF1 protein content compared to CONT ($p = 0.04$) and H_2O_2 ($p = 0.01$) treated fibers. No significant difference was observed between strains for TRF1 normalized to GAPDH protein content (C57BL/6 0.43 ± 0.2 vs. CAST 0.76 ± 0.2 AU; $p = 0.4$). Densitometric analysis and representative immunoblot images of TRF1 and GAPDH are shown. GAPDH was a loading control. Data are presented as means ± S.E.M. CONT = control; H_2O_2 = hydrogen peroxide treated; NAC+ = hydrogen peroxide and NAC treated. AU = arbitrary units. $^{\#}$ H_2O_2 significantly different from NAC+ ($p < 0.05$). § CONT significantly different from NAC+ ($p < 0.05$).

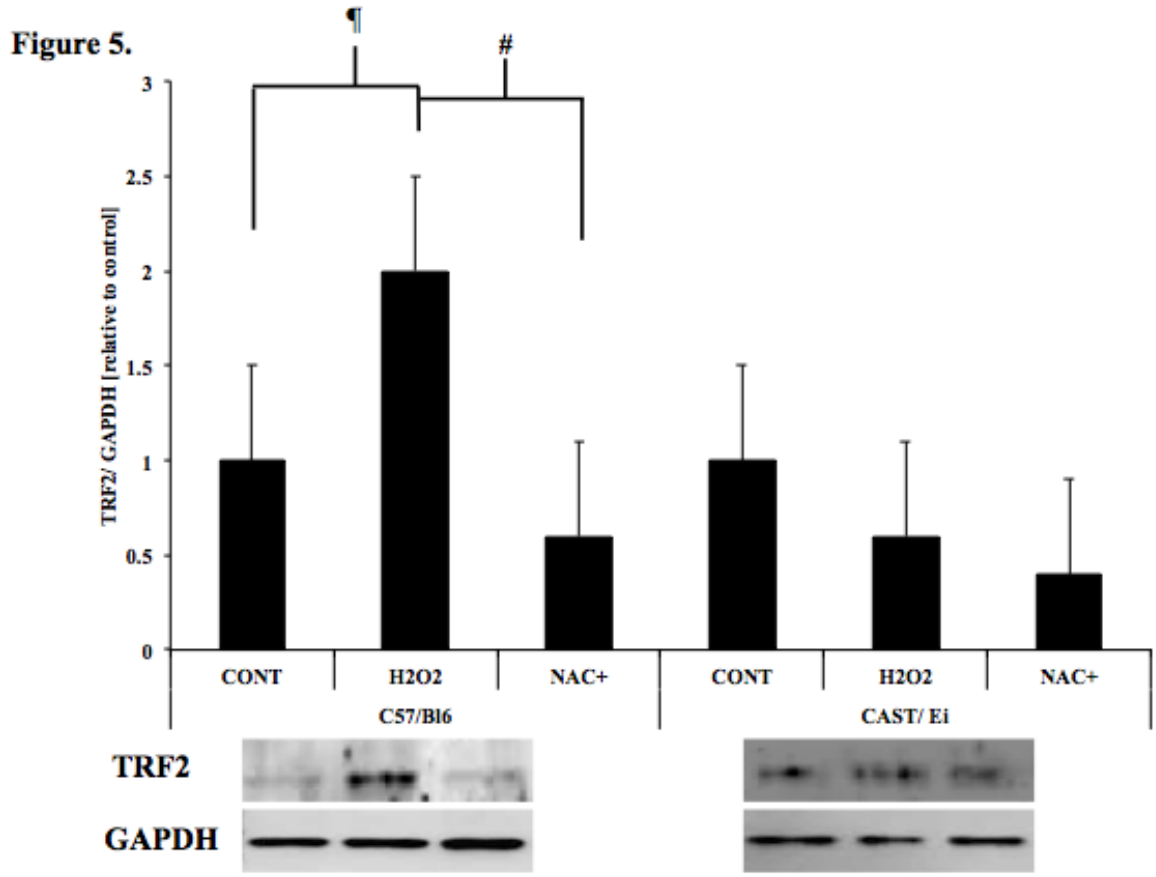


Figure 5.5: TRF2 protein content is increased in response to H_2O_2 in C57BL/6 muscle fibers. C57BL/6 H_2O_2 treated fibers had increased levels of TRF2 protein content compared to NAC+ ($p = 0.05$) and tended to be greater than CONT ($p = 0.1$) fibers. CONT and NAC+ fibers had similar TRF2 protein content ($p = 0.3$). In CAST/Ei animals treatment did not result in altered TRF2 protein content ($p = 0.7$). No significant difference was observed between strains for TRF2 normalized to GAPDH protein content ($p = 0.7$). Densitometric analysis and representative immunoblot images of TRF2 and GAPDH are shown. GAPDH was a loading control. Data are presented as means ± S.E.M. CONT = control; H_2O_2 = hydrogen peroxide treated; NAC+ = hydrogen peroxide and NAC treated. AU = arbitrary units. ¶ tendency for H_2O_2 to be different from control ($p = 0.1$). $^{\#}$ H_2O_2 significantly different from NAC+ ($p = 0.05$).

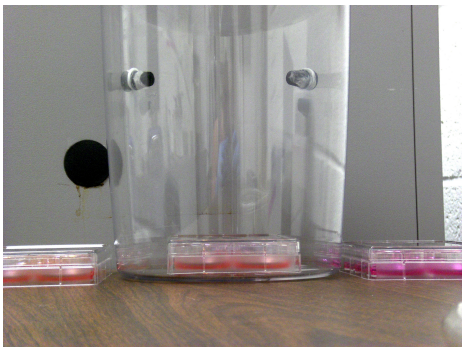
Supp. Figure 1.

Supplement
al Figure 1.

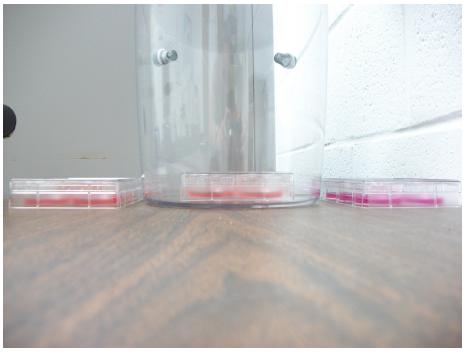
Low O2 incubator leak test

Baseline – l to r – CO2 incubator control, low O2 just after flush remained at ambient for test, ambient

24hrs post same order as BL



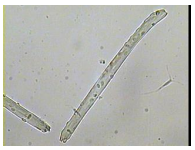
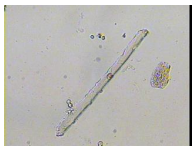
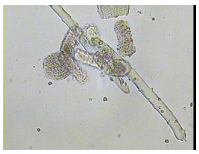
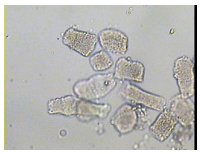
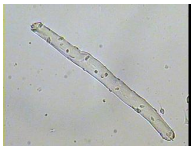
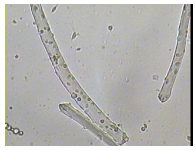
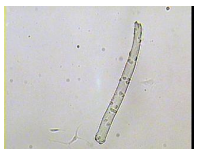
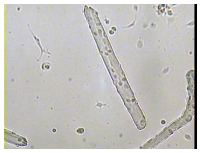
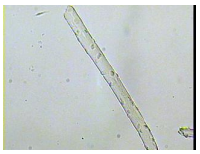
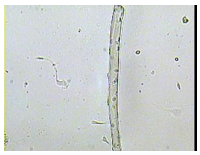
48hrs post same order as BL



Supplemental Figure 5.1. Low oxygen incubator leak test. To ensure that the polystyrene container (equally impermeable to carbon dioxide (CO_2) and oxygen (O_2)) would maintain low O_2, we filled three six-well plates with cell culture media and phenol red indicator dye. Plate 1 was left on the lab bench at room temperature and ambient oxygen levels. Plate 2 was placed in the 5% CO_2, 37ºC and ambient O_2. Plate 3 was placed into the low O_2 container, flushed with the low O_2 gas mixture and left in ambient air and at room temperature. The media will maintain its red-orange color if the container is airtight (i.e., maintaining 5% CO_2 and the pH of the media such that the color does not change). Therefore, if plate 3 was similar in color to plate 2, and different from plate 1 then the container was considered to be air tight and suitable to maintain low O_2 levels. Plates 2 and 3 were similar in red-orange color, while plate 1 turned a purple color, indicating that 5% CO_2 concentration was maintained. This test showed that the incubator was not leaking and thus suitable to maintain 5%CO_2 and ~2-5% O_2 when flushed with the gas mixture.

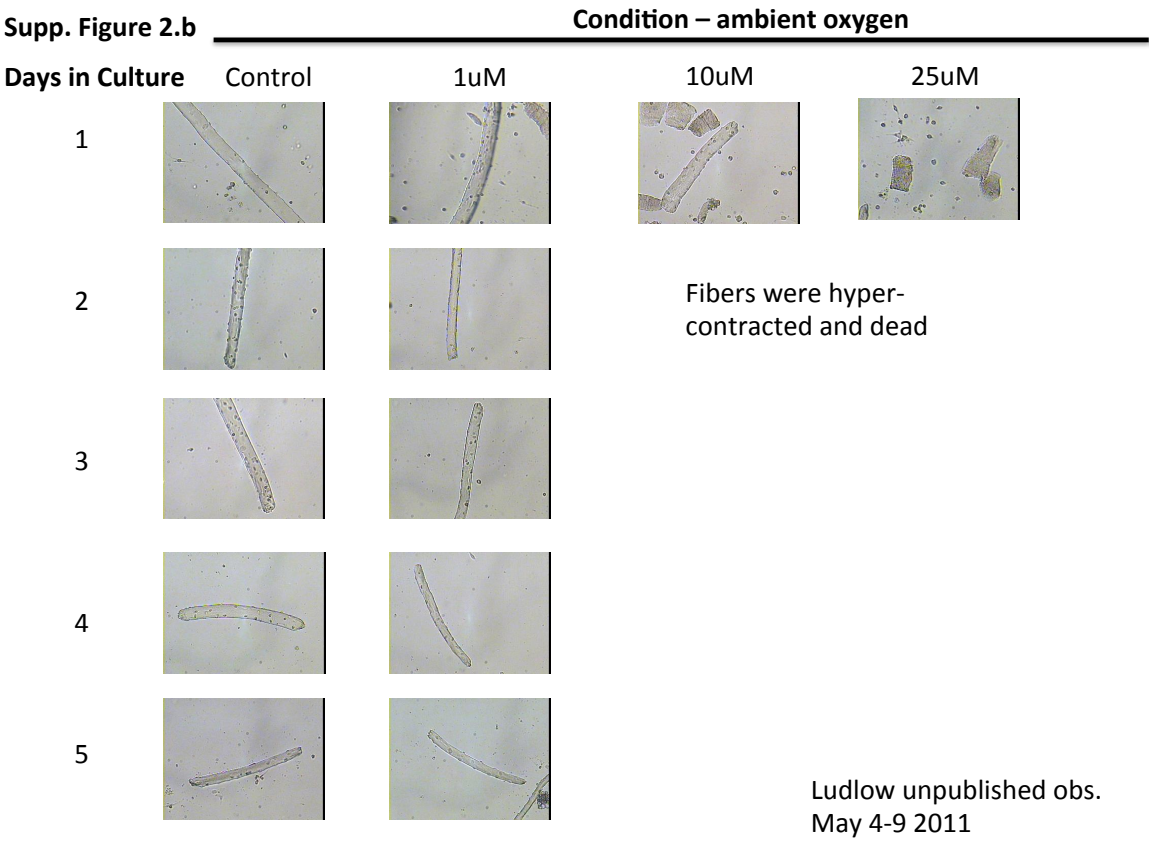
Supp. Figure 2.a

Condition – Low oxygen

Days in Culture	Control	1uM	10uM	25uM
1				
2				
3				
4				

Fibers were hyper-contracted and dead

Ludlow unpublished obs. June 2011



Supplemental Figure 5.2. Hydrogen peroxide dose optimization at low and ambient oxygen levels. C57BL/6 skeletal muscle fibers were isolated (n = 4 animals; 2 per oxygen condition) and allowed to incubate overnight in low oxygen or ambient oxygen in the cell culture incubator prior to hydrogen peroxide treatment. The following day fibers were treated with control, 1μM, 10μM, or 25μM hydrogen peroxide. Fibers were inspected on the following day (day 2 in culture). Fibers treated with greater than 1μM were hyper-contracted and dead. From these data we determined that 1μM hydrogen peroxide was the maximum tolerable dose that skeletal muscle fibers could tolerate for the 5-day treatment period. **A.** Fibers cultured at low oxygen levels (~2-5%). **B.** Fibers cultured at ambient oxygen levels. Representative micrographs at low magnification (10x).

Chapter 6: Summary and Conclusions

Summary

The studies performed for this dissertation have contributed the following new information: (1) long-term voluntary wheel running, a form of exercise training in mice that produces expected health-related benefits, results in telomere protection in heart and liver, but results in telomere shortening in skeletal muscle (Study #1); (2) acute exercise alters the expression of telomere binding proteins, a response that appears to be mediated in part by calcium activation of mitogen-activated protein kinases (Study #2); (3) reactive oxygen species (ROS) exposure results in skeletal muscle fiber telomere shortening (in the absence of satellite cell incorporation), increased telomere binding protein expression, and divergent telomere-related responses in mouse strains of differing telomere length (Study #3). The combined results of these studies have further affirmed that exercise affects telomere biology, but in a tissue-specific and strain-specific manner.

More specifically this dissertation has focused on the role of both acute and chronic endurance exercise and the effects of ROS on telomere biology in skeletal muscle. Studies #2 and #3 attempted to define possible mechanisms for the interesting and paradoxical telomere shortening observed in skeletal muscle of chronically active mice (Study #1). Skeletal muscle contraction activates many pathways during acute exercise, two of the most well characterized being changes in calcium flux and the production of reactive oxygen species. Both calcium and ROS have been linked to changes in gene expression in general and are considered vital second messengers in the adaptive responses in skeletal muscle. Thus when skeletal muscle is induced to contract during an exercise stimulus, we speculate that the following events occur with regard to

telomere biology: increases in intracellular calcium activate p38 MAPK likely via a CAMK II mediated event, which leads to the repression of shelterin gene expression through activation or repression of undefined transcription factors; simultaneously, muscle contractions increase ROS production from NADPH oxidase and mitochondria; the combined effect of acute repression of shelterin components and increased ROS production leads to unrepaired telomere DNA damage; over time (the first several repeated bouts of unaccustomed exercise), reduced shelterin gene expression leads to lower levels of shelterin protein, exposing telomere ends to damaging ROS; skeletal muscle then responds to the chronic stress by increasing the expression of TRF2; as the skeletal muscle adapts to exercise training (i.e., repeated bouts over a sustained period of time) other systems such as antioxidant enzyme defenses are increased, leading to reduced ROS levels and possibly lower MAPK activation, thus leading to restoration of shelterin levels. The end result is telomere length loss that likely occurred prior to the full adaptation of the skeletal muscle to the exercise stimulus. Thus (as shown in Figures 6.1 and 6.2), our data provide evidence for a model whereby calcium and ROS (via MAPKs) act to alter telomere binding protein expression in skeletal muscle that, along with ROS-induced telomere damage, results in reduced telomere length in response to repeated bouts of endurance exercise. With chronic training the effect of ROS will be decreased by increased ROS scavenging by antioxidant enzymes such as glutathione peroxidase and superoxide dismutase, which in turn reduce MAPK signaling and increase telomere protection by TRF2. This model, which is limited to skeletal muscle in mice, will require substantial testing for confirmation but provides a basis for future work.

Summary Figure 6.1.

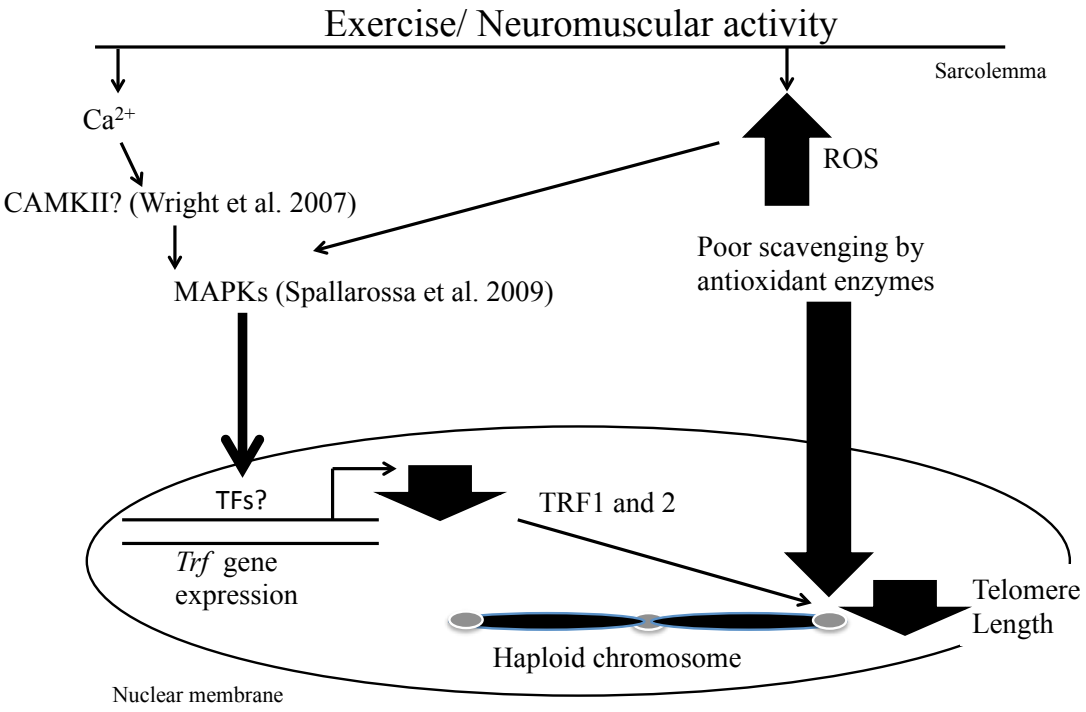


Figure 6.1. Proposed mechanism of telomere shortening in skeletal muscle of animals exposed to acute exercise. This model shows that unaccustomed exercise results in increased calcium and ROS within skeletal muscle cells. Calcium results in activation of MAPKs, possibly via CAMKII, leading to reduced gene expression of TRF1 and TRF2. ROS likely adds to the activation of MAPKs. The reduced expression of TRF1 and TRF2 leads to exposure of the telomere end, which is then more susceptible to the damaging effects of ROS and leads to telomere shortening.

Summary Figure 6.2.

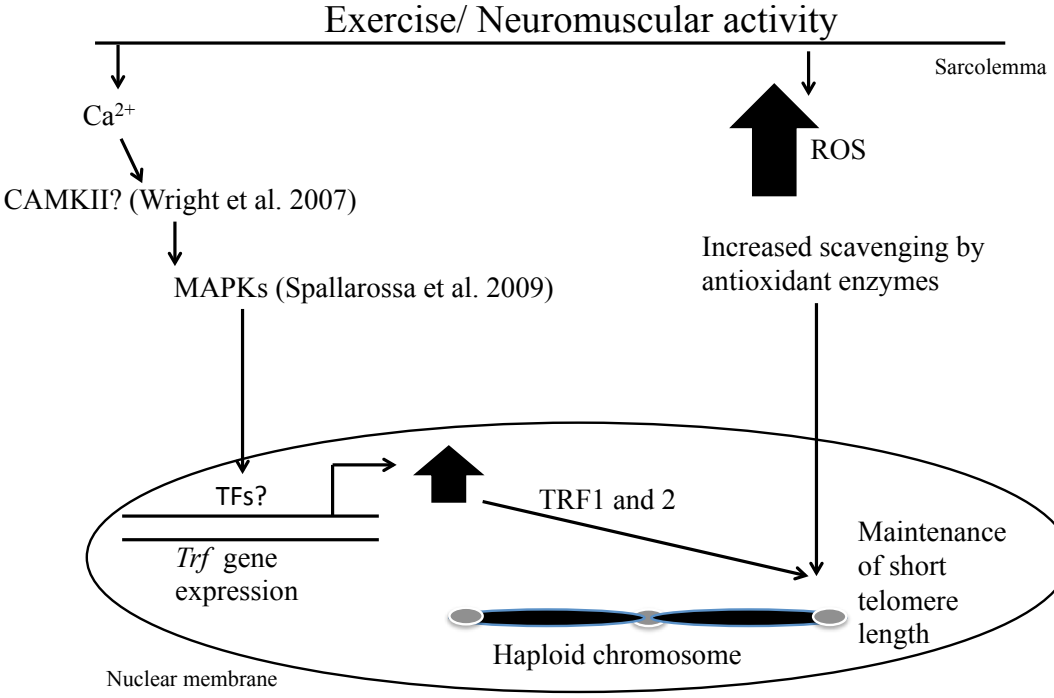


Figure 6.2. Proposed mechanism of telomere shortening in skeletal muscle of animals exposed to chronic exercise. This model shows that over time the skeletal muscle adapts to exercise training by increasing the expression of antioxidant enzymes thus increasing its ability to scavenge ROS. In addition, we have shown that TRF2 expression is increased in muscle of chronically exercised animals, which is expected to improve telomere protection. While calcium transients will still activate MAPKs, the additional activation stimulus from ROS will be diminished via scavenging leading to less of a repression signal for TRF1 and TRF2 gene expression. Thus, the majority of skeletal muscle telomere shortening in response to exercise is speculated to occur in response to the first several bouts of unaccustomed exercise with less shortening over time.

Possible strain and species-specific considerations – The above model does not take into consideration the strain- and possible species-specific differences in telomere biology. Recent reports and our data indicate that telomere length differences between mouse strains and species in general may dictate the response of telomere length to ROS exposure (197). In addition consideration of telomere length may be important when

studying telomere repeat binding factors; for example, short-telomere mammals (mice, Study #3 and humans (113)) may respond to ROS by stabilizing telomere length with TRF1, while long-telomere mammals appear to respond by up-regulating TRF2 (Study #3). In addition the function of TRF1 and TRF2 in skeletal muscle telomere dynamics is unclear and may not follow the negative regulator model proposed by the de Lange group (185). The model put forth by de Lange and colleagues of TRF1 and TRF2 function has been studied almost exclusively in mitotic cell types, however, and thus may not be completely relevant to post-mitotic tissues, such as skeletal muscle. Our data indicate that further study is needed to clarify the function of TRF1 and TRF2 in skeletal muscle in general and in species-specific contexts. These hypotheses require further testing, some of which is outlined below.

Future Perspectives - Though the present studies provide important and novel findings on telomere biology and exercise, a number of important questions remain for future investigation. In general the mechanism of how exercise signals to the genome to produce a telomere length-reducing stimulus in skeletal muscle is largely unknown. We have demonstrated that stress activated kinases (MAPKs) play a role in the response of telomere binding proteins to exercise; however, our data do not provide a mechanistic explanation of if or how exercise is directly reducing telomere length. Studies #1 and 2 point to a repeat-bout effect such that the transient acute effects of exercise may accumulate and result in the shortened telomeres seen after chronic exercise in skeletal muscle. We have also shown that increased ROS exposure is a possible mechanism for telomere shortening in adult skeletal muscle. These data indicate the need for follow-up long-term exercise interventions to study the rate of change in telomere length and

telomere-related components and whether ROS *in vivo* is a causative mechanism of telomere shortening in skeletal muscle.

How is exercise reducing telomere length in skeletal muscle in vivo? Telomeres can shorten due to several factors including mitosis, unrepaired telomere DNA damage, and altered end-processing, the latter being more relevant to skeletal muscle due to its post-mitotic status (173). Our group and other labs have demonstrated that endurance exercise contributes to telomere shortening in skeletal muscle ((7,109) Ludlow et al. under review). We provide evidence that ROS could be a causative mechanism of telomere shortening in adult skeletal muscle fibers; however, these results need to be confirmed *in vivo*. Exercise training enhances the antioxidant enzyme system of skeletal muscle (142), but long term exercise training may lead to the accumulation of ROS damage at the telomere leading to shortening. To clarify these results the following experiments could be performed. First, one would have to consider the model organism of choice, as results may vary depending upon strain of mouse or species chosen. Human tissue experimentation would be preferred but genetic manipulation will be needed to clarify the mechanism of telomere shortening, thus mouse models will have to be used; in particular the CAST/Ei background would be preferred as this strain has telomere lengths most similar to humans (60). To test if ROS produced during acute exercise is related to telomere shortening, genetically altering the antioxidant system specifically in skeletal muscle (e.g., loss of function experiments) and exercise training wild-type and knock-out animals will allow the dissection of whether or not ROS produced during acute and chronic exercise is a telomere shortening stimulus. Since the knock-out animals would lack the ability to increase ROS scavenging with training, telomere shortening should be

greater in these animals compared to wild-type animals, thus providing *in vivo* evidence that ROS shortens telomeres in skeletal muscle. In addition transgenic animals overexpressing antioxidant enzymes in skeletal muscle could be used to show protection against exercise training-induced telomere shortening. Our data also showed that telomere shortening in skeletal muscle may be a function of the initial bouts of unaccustomed exercise, therefore future research is needed to determine the time-course of telomere length change in skeletal muscle fibers under oxidative challenge (i.e., exercise animals for 1 week, 3 months, 6 months, 1 year and longer). Additionally, research in skeletal muscle (or other tissues) should pay particular attention to the frequency, intensity and duration of the exercise stimulus, as information pertaining to these variables is currently lacking. This could be clarified with similar knock-out and transgenic animals as mentioned above.

Common telomere dogma states that telomere length in skeletal muscle only shortens *in vivo* when satellite cells are induced to proliferate, yet endurance exercise is not a satellite cell proliferative stimulus, so this mechanism is unlikely. We showed that ROS is a telomere-shortening stimulus in skeletal muscle fibers devoid of satellite cells *in vitro* (Study #3). An exciting new tool has recently been developed by McCarthy et al. (239) that allows the skeletal muscle-specific depletion of satellite cells by altering the expression of Pax7 in post-natal skeletal muscle. Using the Pax7 satellite cell depletion model would remove the possibility of satellite cell incorporation contributing to telomere shortening in skeletal muscle and the study of other telomere shortening mechanisms (e.g., ROS) could be elucidated in an intact organism. Experiments with

skeletal muscle in these animals could lead to a mechanistic explanation for how exercise training results in telomere shortening in skeletal muscle.

In addition future research is needed to determine if having short telomeres in skeletal muscle results in a dysfunctional phenotype. For example, do shortened telomeres in skeletal muscle reduce the ability of the skeletal muscle cells to contract when stimulated or repair cellular damage? Collins et al. (109) provided some evidence of reduced endurance performance in fatigued athlete myopathic syndrome (in which shortened telomeres are observed) but the performance decrement could be due to factors other than short telomeres. Important functions to test in skeletal muscle fibers with shortened telomeres would be their ability to undergo excitation contraction coupling, apoptosis susceptibility, fatigue resistance, and metabolic functions. To clarify these questions one could perform experiments with late generation (G4-G6 mTERT or mTERC) knock-out animals with short telomeres in their skeletal muscles. Understanding whether or not a dysfunctional phenotype is associated with short telomeres in skeletal muscle results is a critical next step in this line of research. This information is not only relevant to exercise-trained skeletal muscle but also to skeletal muscle conditions associated with short telomeres (e.g., muscular dystrophies), as telomere position effects in these conditions could play a substantial role in affecting gene expression.

How is exercise affecting telomerase expression and activity in skeletal muscle? In our Study #1 of long-term exercised CAST/ Ei mice we observed greater telomerase enzyme activity in skeletal muscle homogenates of one-year old exercised animals compared to age-matched sedentary animals. How exercise training resulted in increased

skeletal muscle telomerase lacks explanation; the expression and activity of telomerase is complex, due to multiple transcripts species-specific enhancers and repressors, and differences in telomere length between species (197). Possible mechanisms of telomerase regulation by exercise include ROS-induced activation of Nfκβ, as TERT expression is linked to Nfκβ signaling (240), but these seem unlikely in skeletal muscle given Study #3's results showing that ROS did not alter telomerase in either mouse strain. However, Study #3 was only five days long, therefore longer interventions with ROS manipulation are needed to fully elucidate the role of ROS in skeletal muscle telomerase activation.

In addition, extra-nuclear actions of telomerase have been proposed (e.g., reduction of mitochondrial ROS), but not investigated with regard to exercise in skeletal muscle. Manipulations such as exercising animals with normal (C57BL/6) length telomeres (e.g., G1-2 mTERT $^{-/-}$) without the presence of telomerase could provide a unique way to investigate the role of telomerase in the adaptation of skeletal muscle telomeres to exercise. A recent mouse model has been developed that allows the precise control of re-activating telomerase on a telomerase null background (101). This animal model could be used to compliment the studies in mTERT $^{-/-}$ mice. Important to all of these questions is the fact that skeletal muscle telomerase activity was very low and may not be important for telomere biology in this tissue, which should be addressed separately using the mTERT knock-out and transgenic (conditional and tissue-specific) mice (241). Understanding the role of telomerase in skeletal muscle will be important for diseases or conditions where the proliferative capacity of skeletal muscle progenitor cells is compromised.

What are the functions of Telomere-repeat binding factors 1 and 2 in post-mitotic skeletal muscle fibers? The functions of TRF1 and TRF2 in skeletal muscle need clarification. Our experiments resulted in conflicting findings in relation to the current knowledge regarding shelterin. The majority of studies concerning TRF1 and TRF2 have been performed in proliferating tissues or cells such as human immune cells, human fibroblasts, human cancer cell lines (immortalized), or mouse embryonic fibroblasts (69). These studies have produced a model for the function of TRF1 and TRF2 in which these two shelterin components act as negative regulators of telomerase-dependent telomere elongation (185). In post-mitotic tissues, however, this model may not hold. Evidence from Werner et al. (8,9) and Zhang et al. (236) show that post-mitotic tissues such as cardiac tissues and neurons respond to external stressors (i.e., exercise and ROS, respectively) by up-regulating shelterin, TRF2 specifically. In all three dissertation studies we similarly observed altered expression of shelterin components in concert with changes in telomere length. These findings require follow-up to clarify the roles that TRF1 and TRF2 are playing in skeletal muscle following acute and chronic exercise. In addition, strain-specific telomere length seems to dictate the response of TRF1 and TRF2 to ROS treatment, with long-telomere mice up-regulating TRF2 and short-telomere mice increasing TRF1 protein content. Thus consideration of strain will be important for translating any findings to the human condition.

To further elucidate the importance of these proteins in skeletal muscle genetic manipulation is required. TRF1 and TRF2 knock-out animals are embryonic lethal; however, the Blasco group has recently made several shelterin component tissue-specific knock-out animals. Tissue-specific and conditional deletion and/or expression tools offer

exciting possibilities to elucidate mechanisms of shelterin gene function in specific tissues. Muscle-specific deletion of TRF1 or TRF2 in combination with exercise stimuli may be used to determine the roles of these proteins in telomere function in skeletal muscle. Moreover, TRF1 and TRF2 have extra-telomeric functions as well and these have yet to be explored in skeletal muscle of exercised animals (224,236). Improving our understanding of the role of these proteins in skeletal muscle may provide insights into skeletal muscle aging and diseases of skeletal muscle.

How is exercise regulating the expression of telomere binding proteins TRF1 and TRF2 in skeletal muscle? We observed significant alterations in TRF1 and TRF2 gene expression and protein content in these studies. We used acute exercise to manipulate the gene expression of these proteins. Since acute exercise is a potent perturbation to homeostasis many signal transduction pathways are activated making *in vivo* identification of causative pathways nearly impossible, therefore *in vitro* analysis is needed. Our *in vitro* results indicate that MAPKs may be involved in repression of shelterin genes. Future studies manipulating the activity of the MAPKs and also identifying the exercise-induced signals (e.g., ROS or calcium) that result in these transcriptional changes are warranted. The important response elements in the promoter regions of the shelterin genes are unknown. Once the response elements in the promoters are identified, determining which transcription factors are responsible for altering the gene expression of shelterin would be possible. Reporter gene assays for the rodent shelterin components would also be very useful in determining regulatory elements and important exercise-activated transcription factors. Unfortunately, the reporter plasmids to perform this work are currently not available and will need to be constructed. This

information will allow the testing of these pathways *in vivo* in conditional knockout, knockdown and transgenic models. These gene regulation studies will compliment the information gained from the functional studies of TRF1 and TRF2 in skeletal muscle by providing potential drug targets aimed at altering the expression and function of these proteins in skeletal muscle.

How can we perform mechanistic studies of telomere biology in humans? As mentioned throughout this document, telomere biology differs in mice and humans. For example, mouse telomeres (most strains) are much longer compared to humans and do not appear to limit cellular replication *in vivo*. Recent work has been aimed at determining the appropriate model organism for human telomere biology, but much work remains in this area. Performance of telomere biology studies in humans is hampered by the long period of time needed to observe a detectable change in telomere length (on the order of decades) and, as with all research in humans, tissue samples are difficult to obtain. With regard to skeletal muscle, recent advances in optimal culture conditions and certain genetic manipulations (hTERT and CDK4 transgene induction to bypass p53 induced senescence and stasis, respectively) have allowed researchers to selectively immortalize skeletal muscle myoblasts from primary cultures of both healthy and diseased patients (242,243). With this system in place clonal skeletal muscle cells can now be studied over long periods to ascertain information concerning cellular phenotypes related to telomeres. Once the phenotypes are identified, biochemical and genetic manipulation can be used to develop treatments and also study environmental influences such as pathways activated in response to exercise or muscle contractile activity. Similar technologies/strategies could be employed in other tissue types that are difficult to obtain

(e.g., cardiomyocytes). In addition the advent of induced pluripotent stem (iPS) cells and directed differentiation of these cells (e.g., into myocytes) may make the study of human samples even more attractive, though not without limitations. Evidence shows that iPS cells are epigenetically reprogrammed, have elongated telomeres compared to the tissue source from which they were derived, and also re-express telomerase at levels similar to native stem cells. These issues provide a roadblock to using iPS cells in telomere related research.

Conclusions

Overall we conclude that chronic exercise is related to reduced skeletal muscle telomere length, and that both acute and chronic exercise alter telomere binding protein expression in mouse skeletal muscle. We provide evidence for a role of the MAPK protein family in the exercise-induced expression of telomere binding proteins and also demonstrate in skeletal muscle that Ca^{2+}-induced signaling and ROS are important mediators of skeletal muscle telomere biology. Calcium appears to be related to activation of signaling pathways associated with expression of shelterin, while ROS appears to be related to both signal transduction and to direct telomere shortening in skeletal muscle. While we provide evidence that telomere shortening occurs in muscle fibers in response to these stimuli, we do not know if this shortening has any functional outcome at the level of the telomere (e.g., telomere position effects) or at the level of the muscle fiber. In the present studies we provide substantial evidence that exercise and exercise-related signaling are associated with altered skeletal muscle telomere biology, which may prove important for understanding post-mitotic tissue aging and disease susceptibility.

Appendices

APPENDIX A:

Expanded methods

Supplemental expanded methods

Telomere Length Measurement

Measurement of relative telomere lengths (T/S ratios) was determined by quantitative real time polymerase chain reaction (RT-PCR) as described by Cawthon (176) with the following modifications. The forward primer for the telomere PCR was tel1b [5'-CGG TTT $(GTTTGG)_5$ GTT-3'] used at a final concentration of 125 nM. The reverse primer was tel2b [5'- GGC TTG $(CCTTAC)_5$ CCT-3'] used at a final concentration of 312.5 nM. S PCR (mouse acidic ribosomal phosphatase PO, 36B4; Forward 5'- AAC AAG GCA GGA GTG AGA CTG; Reverse 5'- CCA GGG ATA CGG GAG AAA A). SYBR Green Master mix (Applied Biosystems) was used and added to 8.75µL of sample and standard DNA. Tubes containing 50, 25, 12.5, 6.25 and 3.125 ng/µL of reference DNA were included in each PCR assay so that the quantity of research sample DNA could be determined relative to the reference DNA sample by the standard curve method (Figure A.1.). The same reference DNA was used in all PCR runs. Cycling conditions for the T PCR were 50ºC for 2 min, 95ºC for 10 min followed by 30 cycles of 95ºC for 10 sec and annealing at 56ºC for 1 min with data collection. Conditions for the S PCR were 50ºC for 2 min, 95ºC for 10 min, 35 cycles of 95ºC for 15 sec, annealing at 56ºC for 1 min with data collection, extend 72ºC for 1 min. followed by a dissociation stage of 95°C for 15sec., 60°C for 30 sec., 95°C for 15 sec to ensure specificity of products. The assay was performed using an Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Foster City, CA). A plot of Ct versus log (amount of input reference DNA) is linear and was used to determine the integrity of the assay. The Ct value is the fractional cycle number at which the fluorescence emitted by the sample crosses a set threshold that is above the baseline fluorescence (26). The

Telomere PCR (T-PCR) to Single copy gene PCR (S-PCR) ratio was derived from the Ct values of each unknown sample relative to a reference sample in each assay via the method of Livak (244). The ratio of telomere repeat copy number to a single copy gene copy number allowed the relative quantification of PBMC telomere length. This ratio was proportional to the average telomere length. The T/S ration was determined according to the following formula:

$2^{-\Delta\Delta Ct}$ = 2 ^ - (CT unknown telomere copy PCR- CT reference telomere copy PCR) - (CT unknown single copy PCR - CT reference single copy PCR).

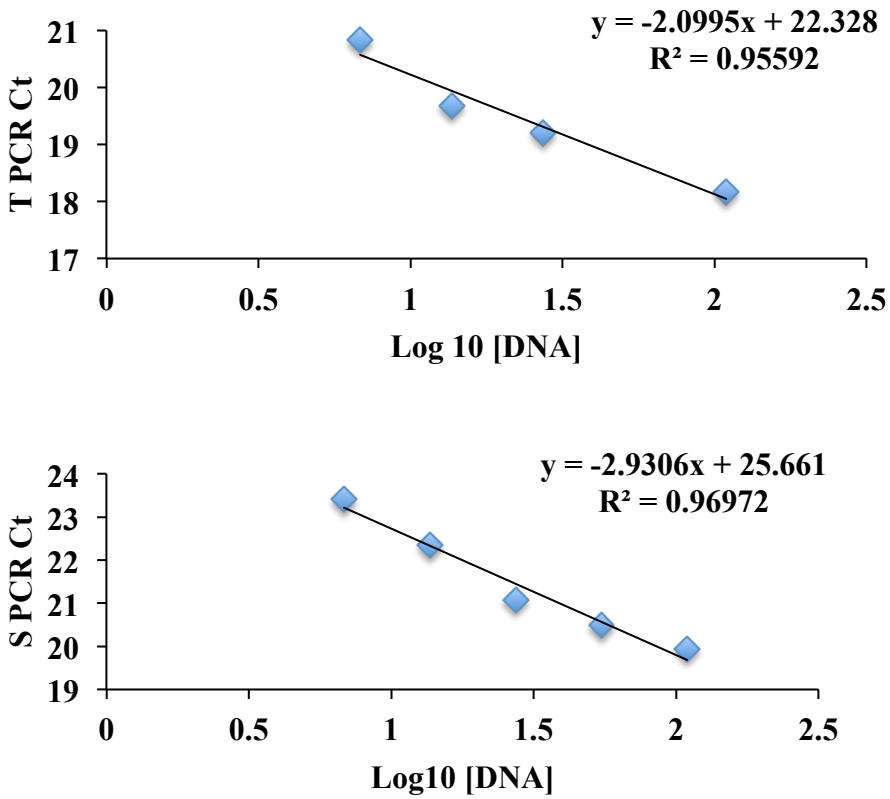


Figure A.1. Telomere PCR and Single-copy gene PCR standard curves. T PCR Ct = telomere PCR critical threshold. S PCR Ct = single cope gene PCR critical threshold. Log10 [DNA] = logarithmic scale base 10 concentration of DNA.

Terminal restriction fragment length analysis of telomere length - Briefly, 2µg of genomic DNA was digested overnight with Rsa1 and HpaIII (Roche, Indianapolis, IN)

and electrophoresed on 0.7% agarose gels for 2hrs and visualized via ethidium bromide staining. The gel was then electrophoresed for 16hrs at 60v to ensure adequate separation of DNA fragments. The DNA was blotted onto positively charged Nylon membranes (Roche, Indianapolis, IN), heat cross-linked and hybridized overnight at 50ºC with the telomeric probe (digoxigenin 3′-end-labeled 5′-$(CCTAAA)_3$) and visualized using a chemiluminescence detection system (Syngene Bio Imaging, Frederick MD; Supporting Figure 1). Mean, median, minimum and maximum TRFs were determined according to previous methods (178). We generated conversion equations from the resulting TRFs to convert T/S ratio data into kilobase data by plotting the T/S ratio of each respective sample versus the corresponding kilobase measurement from the TRF assay. Representative image (Supplemental Figure 3.1).

Telomerase enzyme activity determination

The following section will outline the measurement of telomerase enzyme activity. Measuring telomerase can be done in a few different methods relying on the telomere repeat amplification protocol (TRAP) (179) or an enzyme linked immunosorbant assay (ELISA). For the present studies we chose the TRAP that utilized real-time PCR amplification and emitted fluorescence as the means for quantifying telomerase enzyme activity. The assay uses two fluorometrically labeled primers that when incorporated into the TRAP product emit fluorescence and allow for simple quantification of telomerase activity. This procedure eliminated isotopic, gel- based and ELISA complications and the risk of carryover contamination since the fluorescence are measured directly from the reaction vessel. Briefly, a reaction mixture containing a template stand and primers is added to the PBMCs and PCR is performed. During the PCR the telomerase, if active in the cell sample, will add the fluorescently labeled primers to the template strand. During PCR amplification the

fluorescence reaction will occur and accumulate as more primers are added by telomerase. Figure A.2 below shows a schematic of this process.

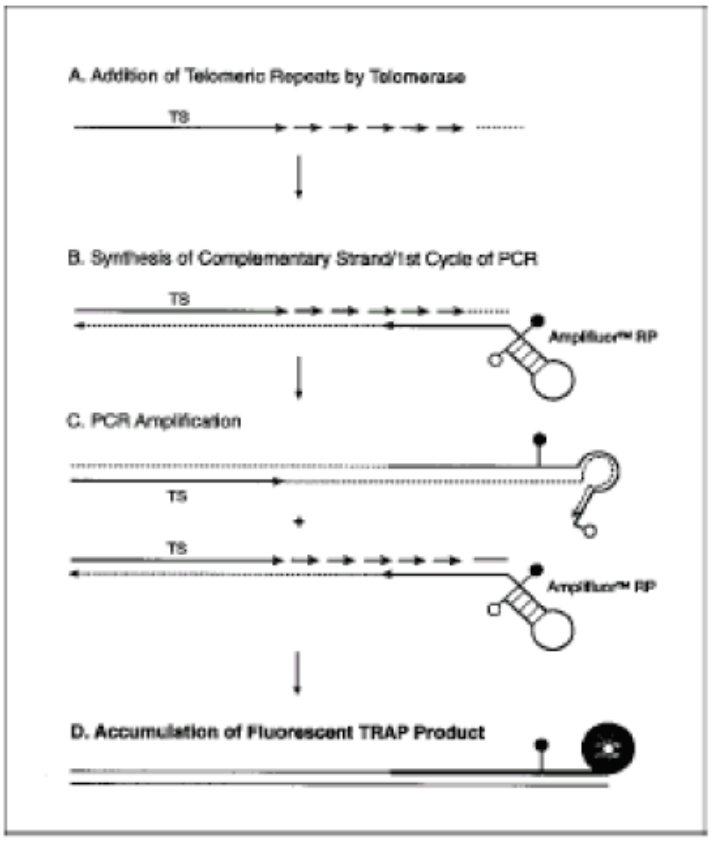


Figure A.2. A. Showing the addition of telomere repeats by telomerase. B. The synthesis of complimentary strand during the first cycle of PCR. C. Amplification of the product and the activated fluorescence. D. Accumulation of the activated product allowing for visualization with the appropriate software. Figure adopted from the TRAPeze kit insert, Chemicon 2000.

The assay procedures followed the recommendations of the manufacturer. Briefly, after the cells/ tissues were prepared at a protein concentration of 1μg, a reaction mixture was made containing master mix, sample and PCR grade water. Samples consisted of unknowns, heat-treated unknowns, HeLa cell positive control, HeLa heat-treated, and standard provided in the kit. The plate was then loaded into the ABI 7300 RT-PCR machine and PCR performed with the following thermocycling profile 30°C for 20 min, 95°C for 2 min followed by 35 cycles of 95°C for 15s, 60°C for 60s, 45°C for 32s. The telomerase PCR extension and

fluorescence took place in the reaction vessel. The fluorescence was detected by the ABI 7300 and quantified by the software provided by ABI and a critical threshold (Ct) value determined. The enzyme activity will be determined following the standard curve method. From a standard sample provided in the kit, seven standards will be diluted with a concentration ranging from 0.5 amoles/μL to 0.0000064 amole/μL. A plot of log(TSR concentration) versus Ct value is linear (Figure A.3.). By inserting each unknown's Ct value into the X value of the linear standard curve and algebraically solving, one can determine each unknown's enzyme activity in amoles.

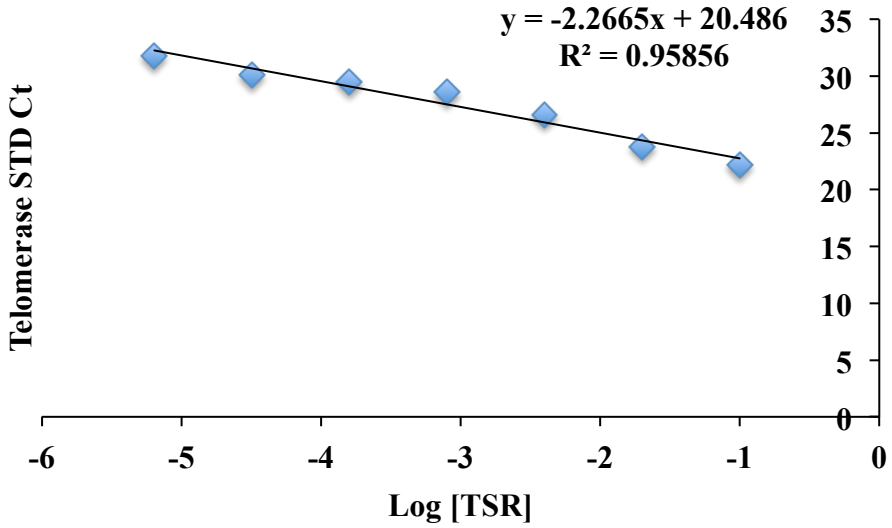


Figure A.3. Telomerase PCR standard curve.

Reverse-transcriptase PCR gel electrophoresis analysis

Gene expression analysis concerns determining the amount of expression of a gene product in the form of messenger RNA (mRNA). To do this total RNA was extracted with a Guanidinium thiocyanate-phenol-chloroform method (245). Following isopropanol precipitation and ethanol wash, total RNA was re-hydrated in tris-EDTA (TE, pH. 8.0) buffer. The concentration and relative purity of the RNA was determined spectrophotometrically use the absorbance at 260 and 280nm in the ultraviolet light spectrum. Using the following formula, 1μg of total RNA was reverse transcribed to complimentary DNA.

Formula: [RNA] in μg/μL = (A_{260} * dilution factor *40)/1000
Where 40 is a conversion factor for single stranded nucleic acids
Dilution factor is the dilution of RNA used in the spectrophotometer.
A_{260} is the absorbance of the nucleic acid solution at 260nm (UV light).

Once the cDNA was attained PCR reactions with gene and protein coding transcript specific primers were performed. Several optimization reactions were undertaken to ensure specific binding and the ability of the primer pairs to detect a difference in the linear range of the PCR reaction. First we determined the appropriate buffer to attain specific binding (i.e., a single amplicon band detected on the agarose gel). Second a PCR cycle analysis was performed to determine the appropriate number of PCR cycles to ensure the amplicon was in the linear range of the PCR reaction (Figure 4.A). Next we tested the ability of the primer pair to detect an increase in cDNA available to bind. To do this we added various amounts of cDNA from the same sample and performed the PCR reaction with the goal of detecting a ~2-fold difference between amount of cDNA added.

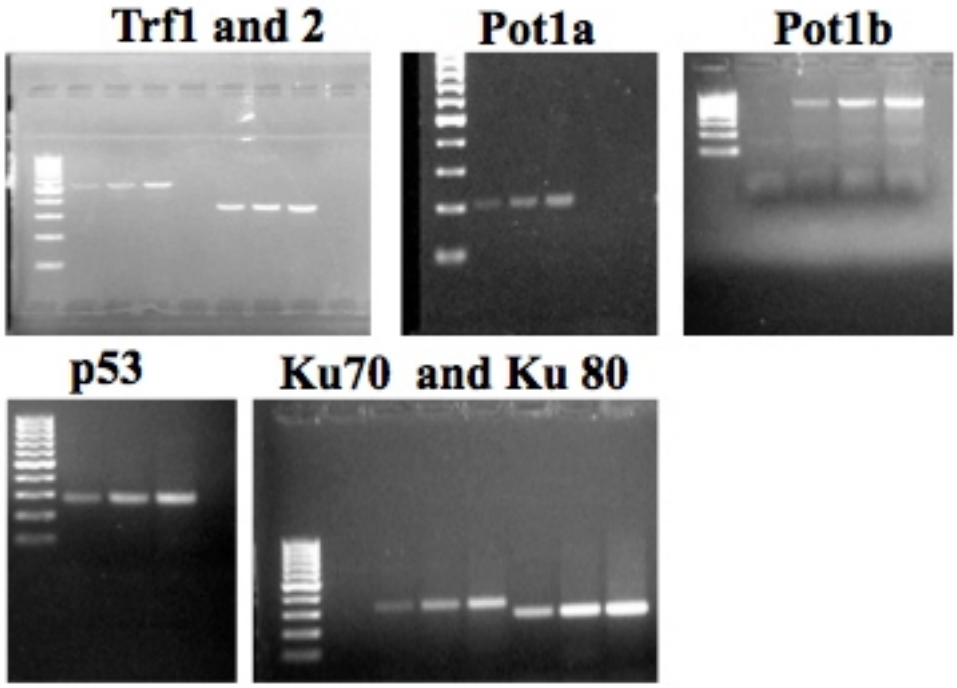


Figure A4. Gene expression primer linearity optimizations. Each gene expression primer pair was optimized for cycle (data not shown) and then to ensure linearity of primers, 0.5μL, 1μL and 2μL of 1μg cDNA was added to each PCR reaction and resolved on a 2% agarose gels.

Immunoblotting

In general proteins were isolated (from tissues or cells), separated on sodium-dodecyl sulfate poly-acrylamide gels by electrophoresis, wet-electrotransferred onto PVDF membranes, probed with primary antibodies directed towards targets of interest, and enzyme linked secondary antibodies directed towards primary antibody species specific immunoglobulins for visualization purposes. Two different total protein lysis buffers were utilized in this dissertation, a general lysis buffer for investigation of total cellular proteins (both nuclear and cytoplasmic) and the second more specific for studying post-translational modifications, such as phosphorylation of proteins. Below are the methods utilized to optimize and validate the immunoblots in this dissertation work.

TRF1 and TRF2– Telomere-repeat binding factor 1 and 2 are chromatin bound proteins that are involved in stabilizing chromosome ends, regulating telomere length, and progression through the cell cycle. To determine the protein content of these proteins the following methods were employed.

Total protein was isolated from skeletal muscle, cardiac muscle or liver tissues. Protein concentration determined via a BCA protein assay. To determine the amount of protein necessary to detect TRF1 and 2 proteins, a range of protein concentrations were loaded onto a SDS-PAGE and immunoblotted as described above. Figure A. 4 shows the detectable range of protein concentrations in liver tissue for TRF1 and TRF2. In addition we determined non-specific bands by probing membranes with only primary or only secondary antibodies (data not shown).

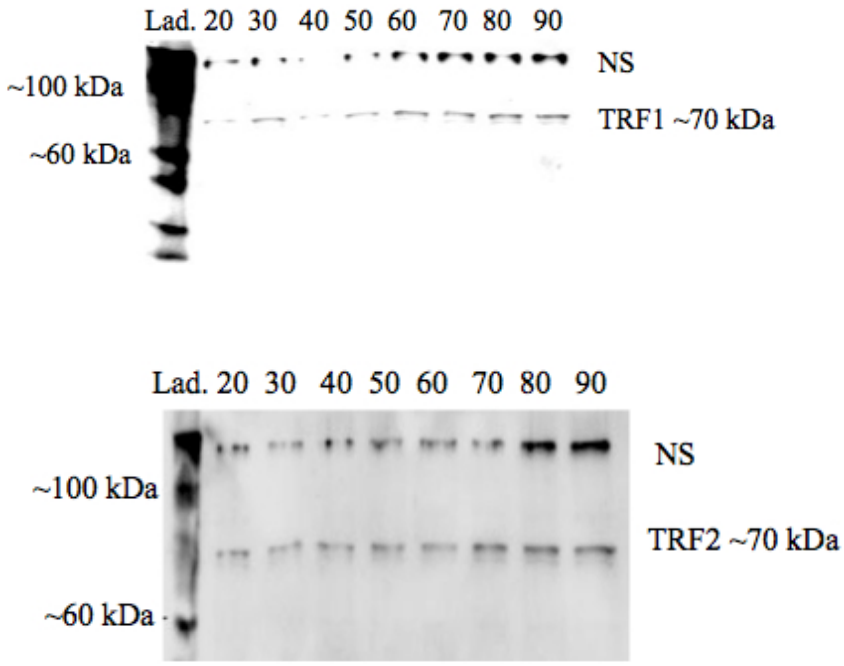


Figure A.5. Linearity confirmation of TRF1 and TRF2. Lad. = molecular weight marker or ladder. NS = non-specific protein band. This was confirmed to be non-specific with a secondary only antibody check. Secondary was not changed due to the large difference in size between the target band and the NS band.

APPENDIX B:

Institutional animal care and use committee approval

Institutional Animal Care and Use Committee Approval

UNIVERSITY OF MARYLAND
DIVISION OF RESEARCH
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

1204 Marie Mount Hall
College Park, Maryland 20742
301.405.5037 TEL 301.314.1475 FAX
W. Ray Stricklin
IACUC Chair
wrstrick@umd.edu
Phone: (301)405-7044

June 28, 2011

Dr. Eva Chin
Department: Kinesiology
University of Maryland
erchin@umd.edu

Dr. Chin,

This letter is to inform you that on **June 23, 2011**, the members of the Institutional Animal Care & Use Committee (IACUC) reviewed your protocol for:

Analysis of skeletal muscle function in transgenic mice and in mouse and rat models of muscle wasting

R-09-20

The IACUC found the animal studies to be acceptable and appropriate. However, the following points must be addressed before the IACUC will approve the protocol.

1. **Number justification** – Please provide additional justification for the additional animals and strains.

Please forward your responses to the above questions to the IACUC Manager, #1204 Marie Mount Building. Upon receipt of appropriate response, I will forward to you the official IACUC approval letter. If you have any questions about the above, please contact Dr. Powell or Amanda Underwood. Thank you for your cooperation during the review process.

Sincerely,

W. Ray Stricklin
Asst. Dean, College of Ag. & Natural Resources
Chair, IACUC

UNIVERSITY OF
MARYLAND
DIVISION OF RESEARCH
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

1204 Marie Mount Hall
College Park, Maryland 20742
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W. Ray Stricklin
IACUC Chair
wrstrick@umd.edu
Phone: (301)405-7044

August 15, 2011

Dr. Eva Chin
Kinesiology
erchin@umd.edu

Dr. Chin,

This letter is to inform you that on **July 1, 2011**, the members of the Institutional Animal Care & Use Committee (IACUC) reviewed and approved the amendment for:

Analysis of Skeletal Muscle Function in Transgenic Mice and In Mouse and Rat Models of Muscle Wasting

R-09-20

Please note that approval of an addendum does not change the original expiration date of your protocol, which is **April 14, 2012**. All work extending beyond the expiration date of the protocol must be submitted to the IACUC as a new protocol.

Sincerely,

W. Ray Stricklin
Asst. Dean, College of Ag. & Natural Resources
Chair, IACUC

CC: Doug Powell, Amanda Underwood

UNIVERSITY OF
MARYLAND
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INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

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November 18, 2010

Dr. Steven M. Roth
Department: Kinesiology
University of Maryland
sroth1@umd.edu

Dr. Roth,

This letter is to inform you that on **November 18, 2010**, the members of the Institutional Animal Care & Use Committee (IACUC) reviewed and approved the protocol for:

Role of Maternal Exercise Environment on Transgenerational Offspring Health
R-10-93

Please note that an approved protocol is valid for three (3) years unless there is a change in the protocol. Thus, this protocol is valid until **November 18, 2013**. Federal laws indicate that protocols must be reviewed yearly. Thus, in order to keep your approved protocol active you **MUST** submit a protocol renewal/update by the first of the month of the anniversary of your approval (November 2011 & November 2012). All work extending beyond the approval date of the protocol must be submitted to the IACUC as a new protocol.

Sincerely,

W. Ray Stricklin
Asst. Dean, College of Ag. & Natural Resources
Chair, IACUC

CC: Doug Powell, Amanda Underwood

UNIVERSITY OF
MARYLAND
GRADUATE STUDIES AND RESEARCH
Institutional Animal Care & Use Committee

Jim Dietz
IACUC Chair
jmdietz@umd.edu
Phone: (301)405-6949

April 1, 2008

Dr. Espen Spangenburg
Department: Kinesiology
University of Maryland
espen@umd.edu
Phone: (301)405-2483

Dr. Spangenburg,

This letter is to inform you that on **April 1, 2008**, the members of the Institutional Animal Care & Use Committee (IACUC) reviewed and approved the addendum for:

Exercise and Gene Expression
R-06-76

Please note that approval of an addendum does not change the original expiration date of your protocol, which is 1 January 2010. All work extending beyond the expiration date of the protocol must be submitted to the IACUC as a new protocol.

Sincerely,

Jim Dietz
Professor, Biology
Chair, IACUC

Dear Dr. Dietz (IACUC Chair):

We request an addendum for IACUC # R-06-76, Exercise & Gene Expression. We would like to test our research questions on additional mice strains than were originally proposed in our IACUC application. We are interested in studying the importance of physical activity on tissue gene expression and have found evidence in our experiments and within the literature that different mice strains have different "inherent" levels of physical activity. It will be important for us to confirm our findings in BalbC mice in other strains to as to improve the generalizability of our results.

As such, we are requesting the addition of 40 animals in each of the following five mice strains to be added to the existing IACUC approval: **CAST/Ei; FVB; C57/Bl6; A/J; SWR/J**. The procedures, justifications for animals and animals numbers, etc., fall under those previously presented in the original application; the goals of the science have not changed. Our most recent examination of the research literature in this area shows that no additional studies have been published that inform our specific questions of interest.

Please contact me with questions.

Sincerely,

Espen E. Spangenburg, Ph.D.
Assistant Professor
Dept. Kinesiology

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