ABSTRACT

Title of Document: THE EFFECTS OF DIET AND PHYSICAL ACTIVITY ON TELOMERE LENGTH AND TELOMERE-RELATED GENES IN MICE BRED FOR HIGH VOLUNTARY WHEEL RUNNING

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The purpose of this study was to determine the effects of diet and physical activity on skeletal muscle telomere length and mRNA levels of components of shelterin, a six-protein complex that protects telomeres, in mice bred over 52 generations for high wheel running activity compared to control mice. Mice were fed either a regular or high-fat diet and were provided wheel access or kept in cages without wheels for 8 weeks. Telomere length was significantly longer in mice fed a high-fat diet compared to those on a regular diet, but no other differences were observed. There were no differences in mRNA levels of the telomere-protecting shelterin components Trf1, Trf2, Pot1a, or Pot1b for diet, wheel access, or selection. High-fat diet may result in telomere dysfunction in these young mice, but we were unable to support our hypothesis that exercise would modify telomere length or shelterin mRNA levels in these mice.

THE EFFECTS OF DIET AND PHYSICAL ACTIVITY ON TELOMERE LENGTH AND TELOMERE-RELATED GENES IN MICE BRED FOR HIGH VOLUNTARY WHEEL RUNNING

By

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Chapter 1: Introduction

A growing area of research in the fields of aging and age-related disease is the study of telomeres, which are repeat sequences at the ends of linear DNA chromosomes that, in the absence of the enzyme telomerase, have been found to shorten with each cell division (1, 6). Essentially, telomeres act as a biological clock, marking time until cell death or senescence (103). Telomere length, then, is considered to be a potential biomarker of aging (10), and shortened telomeres have also been associated with a number of chronic diseases including heart disease (165), atherosclerosis (142), and obesity (82). Adverse lifestyle factors including smoking (156) and stress level (51, 121) have also been shown to correlate with shorter telomere length in leukocytes. Telomere length, however, is highly heritable (77) and since both genetic and environmental factors are involved in telomere biology, discerning the degree of contribution from each can be very difficult.

Telomere length is maintained by telomerase, a ribonucleoprotein reverse transcriptase that is highly expressed in germ cells but not in somatic cells (63). Telomerase expression is a hallmark of most cancers (15) and is studied extensively in the cancer research field. Telomere shortening, while associated with human aging, is an important process in the prevention of unregulated growth, like that seen in tumor cells (44). Replicative senescence (the state in which a cell can no longer divide) occurs when telomeres become too short for further replication and telomerase does not lengthen the telomere (17). Telomerase-activated cancer cells, then, are able to replicate themselves far beyond normal limits (17), and the result is tumor growth. Therefore, even though introduction of telomerase activity might increase cellular lifespan and thus prevent or

even reverse aging, this could be contraindicated by risk of tumor development and associated health concerns.

The ends of mammalian chromosomes are associated with a six-protein complex called shelterin, which is a complex of proteins that provide a marker for telomerase to distinguish chromosome ends from DNA breaks requiring repair; the amount of shelterin is proportional to the length of the telomere (43). The 3' end of the chromosome is a single stranded overhang that forms a structure called the t-loop (66, 138). The current thinking is that the 3' overhang invades double-stranded telomeric DNA and complementary base pairs bind, forming a lariat-like structure (43). Shelterin specifically binds to telomeric DNA through the actions of telomeric repeat binding factor one and two (Trf1 and Trf2); Trf1 has an affinity for the telomere's double-stranded TTAGGG repeats (37, 172) and Trf2 is able to form t-loop-like structures when an appropriate substrate is provided (149). Similar to Trf1 and Trf2, Pot1 binds TTAGGG sequences, but in single-strand form, and two other proteins, Tin1 and Tpp hold the Trf1, Trf2, and Pot1 together (42). Shelterin is required for recruitment of telomerase (150), and is responsible for enabling the cell to differentiate between its natural chromosome end and DNA breaks as well as repressing DNA repair at chromosome ends (120).

The pro-apoptotic transcription factor p53 has been shown to be down-regulated in exercising mice compared to sedentary mice in myocardium (162) and in the thoracic aorta (161). P53 is a marker of apoptosis and when the telomere becomes critically short, DNA damage checkpoints, including a cell cycle arrest checkpoint signaled by p53, are activated (158). Depending on the type of cell, activation of this p53/p21 pathway results in cell cycle arrest (38) or apoptosis (89). In the present study, p53 was measured to give

insight into the stressors associated with diet and wheel running in this population of mice.

Recently, physical activity has been studied as an environmental factor that may affect telomere length and telomerase activity. Physical activity is known to reduce various markers of stress (124) as well as reduce mortality in humans (14) and extend lifespan in animals (90), all of which provide a rationale for examining the potential role for physical activity in modifying telomere shortening with advancing age. A recent study from our lab showed that telomeres are shorter in humans who do not exercise regularly as compared to the telomeres of people who exercise with moderate exercise energy expenditure (97). The results of the study also showed shorter telomeres for highly active subjects as compared to those who exercise at moderate intensities (97). Similarly, Collins et al. showed that muscle telomere length is shorter in athletes with fatigued athlete myopathic syndrome (FAMS) compared to age and training-matched controls (39). A moderate level of physical activity, then, may be protective against telomere shortening. Another study found telomere length is shorter in inactive people as compared to the physically active, with the most active people having the longest telomere length (34). Cherkas et al. concluded that a sedentary lifestyle may be a risk factor for accelerated aging (34). Werner and colleagues recently showed no difference in leukocyte telomere length between young, highly active athletes and sedentary controls of similar age, but older sedentary controls had shorter telomeres than older athletes and both young groups (161). In the same study, telomerase activity was higher in both athlete groups compared to their age-matched control group.

Experts in the field of human telomere biology recently outlined the necessary requirements for both cross-sectional and longitudinal human studies to have sufficient statistical power to detect meaningful relationships between telomere length and environmental stressors (7). They determined that in a cross-sectional study, an age range of 50 years with more than 233 people per group (cases and controls) would be necessary to detect a physiologically significant difference in telomere length. Studying telomere length longitudinally would allow for much smaller sample sizes; however, such studies would have to last long enough to detect changes in telomere length, which would require years and decades rather than weeks or months (7). Therefore, studying telomere length and long-term physical activity exposure mechanistically in humans is extremely difficult, and animal models may be more appropriate. Since then, Werner et al. (162) published findings indicating that six months of voluntary wheel running induces an increase in telomerase activity and the up-regulation of telomere-related proteins in the mouse myocardium, with no change in telomere length. This was the first study of its kind in the mouse. A second paper by the Werner group followed up these findings in myocardium with a study in mouse aortas; they found that telomerase activity was upregulated in the aorta following three weeks of wheel running but there were no differences in telomere length following training (161). Other studies have found alternative roles of telomerase in the cell; the enzyme has been found in the mitochondria (23, 85), though the function there is not yet certain.

Like physical activity, the relationship of diet to telomere biology is not well characterized. Increased body mass is related to decreased lifespan (8) and shorter white blood cell telomere length in humans (33, 108), while calorie restriction is known to

increase lifespan across species (128). Poor eating behavior is related to increased biochemical stressors (inflammation and oxidative stress), which in turn lead to increased abdominal adiposity (99, 136). The increased fat storage leads to insulin insensitivity, which imparts even greater increases of inflammation and free radical production (50). A vicious cycle ensues, and can lead to premature aging and associated diseases, since oxidative stress and inflammation are associated with shorter telomere length (46). Poor diet, then, may also be a risk factor for accelerated aging and telomere shortening (50).

We have acquired skeletal muscle tissue from the 52nd generation of mice from a selective-breeding experiment for high voluntary wheel-running behavior (151). Four founder populations were used to breed four distinct family lines for selected mice, and four different founder populations were bred to create four control lines of mice. Beginning in generation 3, mice were provided wheels for 6 days and mean wheel revolutions were recorded for days 5 and 6. The highest running males and females from each family were bred for the selected lines, and males and females were chosen randomly for the selected line breeding. Rezende et al. (131) found that these selected mice had higher VO_2max and faster running speeds than control mice during wheel running, and treadmill endurance capacity is similarly elevated in the selected mice (106). Since generation 16, in fact, these mice have run distances 2.5 to 3 times as high as compared to control-bred mice (58, 60, 78).

Though studies using diet and exercise as independent variables have been conducted on these experimental lines before, the mice, until now, were only *selected* for physical activity and never underwent any exercise training (with the exception of one study from generation 16, where mice were trained (19)). However, in this particular

study, animals were fed either a standard (Teklad Rodent Diet (W) 8604, 14% kJ from fat) or "Western" high fat, high sugar diet (Harlan Teklad TD.88137 Western Diet, 42% kJ from fat) and were housed in either standard or voluntary wheel-access cages, providing an exercise training stimulus for those mice housed with wheels. As previously mentioned, telomere length is highly heritable (77), and determining the degree to which various factors contribute to telomere length and related measures is difficult in humans. Thus, for the proposed study, animals in the 52nd generation of mice were divided into one of four groups for an eight week intervention: Western diet/no wheel access; regular diet/no wheel access; Western diet/wheel access; or regular diet/wheel access. Control bred mice were also subjected to one the same four treatment groups.

The purpose of the present thesis proposal is to determine the effects of diet and physical activity, alone or in combination, on telomere length, mRNA levels of shelterin components, and mRNA levels of genes that respond to aerobic exercise training in mice bred for high wheel running behavior compared to control mice. Our hypotheses were as follows:

1. Mice that had wheel access and ate a regular diet would have longer telomeres compared to those that did not have wheel access and were fed Western diet, as both regular exercise and healthy diet are associated with improved cellular health.

2. mRNA levels of shelterin components would be highest in the selected, wheel access, and regular diet group, and that wheel access mice would have higher levels than mice without access, mice on regular diet would have higher levels than those on Western diet, and that selected mice would have higher levels than controls.

3. Wheel access mice would have higher levels of PGC1α mRNA and higher mitochondrial DNA copy number compared to mice without wheel access, as wheel access should result in exercise training and improved mitochondrial function.

Chapter 2: Methods

Research Design

Tissue samples from generation 52 of mice selected for high voluntary wheel running behavior (four founder lines) as well as mice bred from unselected controls (four founder lines) have been acquired from Dr. Ted Garland and colleagues, UC Riverside Dept. of Biology (details of overall study design described in (151)). Beginning at 24 days of age, 196 male mice were housed individually in either a wheel or standard cage, with room temperature maintained at ~73° Fahrenheit and a 12/12 light:dark cycle. Water and food (Harlan Teklad Laboratory Rodent Diet [W]-8604, 14% kJ from fat or Harlan Teklad TD.88137 Western Diet, 42% kJ from fat) were available ad libitum. For the regular diet, 33 percent of calories were from protein, 14 percent from fat, and 53 percent from carbohydrate. The fat content included 10 $g \cdot kg^{-1}$ saturated fat, 11 $g \cdot kg^{-1}$ monounsaturated fat and 21 $g \cdot kg^{-1}$ polyunsaturated fat with a total energy content of 13 $kJ \cdot g^{-1}$. For Western diet, 15 percent of calories came from protein, 42 percent from fat, and 43 percent from carbohydrates while total energy content was 19 $kJ \cdot g^{-1}$. 133 $g \cdot kg^{-1}$ came from saturated fats, 59 $g \cdot kg^{-1}$ came from monounsaturated fats, and 9 $g \cdot kg^{-1}$ came from polyunsaturated fat. Also, the Western diet was high in sugar with 341 $g \cdot kg^{-1}$ added sucrose, some of which replaced complex carbohydrates, compared to no added sucrose for the regular diet.

Each selected mouse was assigned to one of four treatment groups following weaning: high-fat diet/no wheel access; regular diet/no wheel access; high-fat diet/wheel access; and regular diet/wheel access. Each control mouse was assigned to one of the

same four treatment groups, and all mice were maintained in their respective conditions for eight weeks.

For mice in wheel cages, total wheel running (revolutions) was recorded for approximately 23 hours each day, and weekly or bi-weekly averages were calculated for wheel running distance (meters/day).

Tissue Preparation

Following the eight week intervention period, wheels were locked for 4 to 6 hours and mice were fasted for the same amount of time prior to sacrifice. Muscles were removed and immediately flash frozen in liquid nitrogen; samples were then stored at -80 degrees Celsius until powdering.

The left triceps surae muscle group was powdered with mortar and pestle while frozen in a bath of liquid nitrogen to assure that fast and slow twitch fibers from the gastrocnemius and soleus muscles were evenly distributed in each sample of muscle homogenate. The powdered tissue was then divided into three equal portions and stored at -80 degrees Celsius until assays were performed.

Telomere length

DNA was isolated from powdered triceps surae muscle using a PureGene DNA isolation system (Gentra Systems, Minneapolis) for measurement of telomere length. Relative telomere length (Telomere PCR to Single-copy gene PCR, or T/S ratio) was measured via a real-time PCR assay previously described by Cawthon and colleagues (29) and Callicott & Womack (26) but modified slightly for our particular samples. All telomere length assays were performed on an Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Foster City, CA). The forward and reverse primers for the

T-PCR were, respectively [5'-GGT TTT TGA GGG TGA GGG TGA GGG T-3'] and [5'-TCC CGA CTA TCC CTA TCC CTA TCC CT-3']. For the S-PCR, forward and reverse primers were [5'-ACT GGT CTA GGA CCC GAG AAG-3'] and [5'-TCA ATG GTG CCT CTG GAG ATT-3']. For both T and S PCRs, 5.83 uL of 10 ng/uL DNA was added to a mastermix of SYBR green dye, primers, and distilled water. Cycling conditions for the T-PCR were 95 degrees for 10 min followed by 40 cycles of 95 degrees for 15 sec, 52 degrees for 27 sec, 72 degrees for 30 sec, and a dissociation step. Conditions for the S-PCR were 95 degrees for 10 min followed by 40 cycles of 95 degrees for 15 sec, 56 degrees for 60 sec, and a dissociation step. T/S ratios are proportional to average telomere length (29) and were derived from the critical threshold (Ct) values generated by fluorescence of the SYBR green dye that reflects the level of expression of the gene of interest.

Though T/S ratios are not used for absolute quantification of telomere length, we did validate this assay with a Southern blot method. To confirm the real-time PCR method, mean telomere length was measured by terminal restriction fragment (TRF) length analysis. We used samples from liver that represented the two longest and two shortest from the PCR T/S ratio method. Briefly, 2 ug of genomic DNA was digested overnight with Rsa1 and HpaIII (Roche) and electrophoresed on 0.7% agarose gels for 2 hrs and visualized via ethidium bromide staining. Then the gel was electrophoresed for 16 hrs at 60 v to ensure adequate separation of DNA fragments. The DNA was then blotted onto positively charged Nylon membranes (Roche), 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. Mean,

median, minimum and maximum TRFs were determined according to (125). This assay validated the real-time PCR method and confirmed that the T/S ratio assay works within our own laboratory (see Appendix A).

mRNA Levels

RNA was extracted with Trizol Reagent (Invitrogen, Carlsbad, CA) and quantified by spectrophotometry. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was performed (Applied Biosystems, Carlsbad, CA) and primers were designed to evaluate the levels of mRNA of telomere-related genes Trf1, Trf2, Pot1a, Pot1b, p53 (as a marker of apoptosis), and the mitochondrial gene PGC1α, with mRNA levels of Gapdh measured as a positive control. PCR was performed on an MJ thermal cycler and products were run on two percent agarose gels stained with ethidium bromide. Finally, gels were analyzed by semi-quantitative densitometry and assessed using ImageJ software (nih.gov). Appendix B demonstrates the ability of our primers to pick up linear differences in amount of cDNA used in a PCR reaction.

Trf1 forward and reverse primers were, respectively, [5'-CAT GGA CTA CAC AGA CTT AC-3'] and [5'-ATC TGG CTT ATC CTT AGA CG-3'] and cycling conditions were 95 degrees for 5 min, 29 cycles of 95 degrees for 30 sec, 53 degrees for 60 sec, and 72 degrees for 30 sec, all followed by a step of 72 degrees for 5 min. The primers used for Trf2 expression were forward [5'-TGT CTG TCG CGC ATT GAA GA-3'] and reverse [5'-GCT GGA AGA CCT CAA TAG GAA-3'] and cycling conditions were 95 degrees for 5 min, 29 cycles of 95 degrees for 30 sec, 55 degrees for 60 sec, 72 degrees for 30 sec, and a final step of 72 degrees for 5 min. Pot1a primers were forward [5'-CCC TGA ATC TAC TCA AGG AAG G-3'] and reverse [5'-GAA GCG AAC AAT

GTC TCC AA-3']. Cycling conditions were 95 degrees for 5 min, 30 cycles of 50 degrees for 30 sec, 72 degrees of 30 sec, and the final step was 72 degrees for 5 min. Pot1b primers were forward [5'-CTT TAA GCC TCC GGC CTT AAG CAA AG-3'] and reverse [5'-GGA CAT GAT TAT CAG CAA CGA CA-3'] and cycling conditions were 95 degrees for 5 min, 32 cycles of 95 degrees for 30 sec, 55 degrees for 60 sec, and 72 degrees for 30 sec. The final step was 72 degrees for 5 min.

Forward and reverse primers for p53 were, respectively, [5'-GGG ACA GCC AAC TCT GTT ATG TGC-3'] and [5'-CTG TCT TCC AGA TAC TCG GGA TAC-3'] and cycling conditions were 95 degrees for 5 min, 29 cycles of 95 degrees for 30 sec, 62 degrees for 60 sec, and 72 degrees for 30 sec, with a 72 degree 5 min step at the end. PGC1α primers were forward [5'- TGA CAT AGA GTG TGC CCT G-3'] and reverse [5'-TGG TTC TGA GTG CTA AGA CCG CTG-3'] and cycling conditions were 94 degrees for 3 min, 29 cycles of 94 degrees for 30 sec, 58 degrees for 60 sec, and 72 degrees for 60 sec followed by a 10 min step of 72 degrees.

Mitochondrial DNA Copy Number

DNA was isolated as described above and diluted to a concentration of 10 ng/uL. The copy number of the mitochondrial gene Cytochrome Oxidase-1 (Cox1) was measured by PCR and normalized to the copy number of the nuclear-encoded control gene 18S. Cox1 primers were forward [5'-AGC CCA TGC AGG AGC ATC AGT-3'] and reverse [5'-TTG GGT CCC CTC CTC CAG CG-3'] while cycling conditions were 95 degrees for 5 min, 26 cycles of 95 degrees for 30 sec, 65 degrees for 60 sec, and 72 degrees for 30 sec. The final step was 5 min at 72 degrees. PCR products were run on two percent agarose gels stained with ethidium bromide. UV light was used to visualize the

bands and an image was obtained that allowed quantification with ImageJ software (nih.gov).

Statistical Power

Sample sizes for such a new area of research are notoriously hard to estimate, especially given that the bulk of our primary preliminary data are from humans. Sample size estimates were determined for telomere length (T/S ratios) and telomerase enzyme activity. The effect size ratio used for telomere length was a difference of 0.2 units, a difference which has been significantly associated with perceived psychological stress (52) as well as poor survival rate and increased mortality from heart and infectious disease in humans (30). To obtain appropriate statistical power (beta = 0.8; alpha $\leq$ 0.05), 50 total mice, or 7 per group, would be required. Calculations were based on an average T/S ratio of 1.37 with a standard deviation of 0.14 based on preliminary studies from our laboratory. We have been provided with tissues from 196 male mice from eight treatment or control groups, and thus we will have approximately 24 mice per group, which is more than adequate for appropriate statistical power.

Statistical Analysis

For telomere length, gene expression, and DNA copy number assays, cross-sectional statistical analysis consisted of Analysis of variance (ANOVA) was used to test for effects of diet (normal vs. high fat), physical activity (wheel access vs. no wheel access), and line (selected vs. control) among the 8 groups. ANOVA models were also performed to determine differences between the eight treatment groups. In addition, exploratory analyses were performed when scientifically relevant across specific family lines, which could be differentiated into 32 groups of approximately 8 mice per group.

Where appropriate, covariates (e.g., body mass) were used in the models, including the inclusion of gel or assay as a random factor to account for internal assay variation. Multiple comparison procedures were used to determine specific group differences when an omnibus alpha <0.05 was observed. Statistical significance was accepted at an alpha level of less than 0.05.

Chapter 3: Results

Animals:

A total of 196 male mice were sacrificed for tissue extraction. Due to extraction failures, only 184 mice were analyzed for gene expression data and 181 were analyzed for telomere length and mtDNA measures. Table 1 shows wheel running data for mice that were provided wheel access (Exercise groups). Complete animal characteristics are described in (105).

	Selected/ Western Diet	**Selected/ Regular Diet**	**Control/ Western Diet**	**Control/ Regular Diet**
Week 2	8737	4704	2352	2230
Week 4	13440	9184	3360	3360
Week 6	12320	9856	2688	2912
Week 8	10304	8736	2800	3360

Table 1: Wheel running in meters run per day for mice bred for high voluntary wheel running (Selected) and control-bred (Control) mice, and mice fed Regular versus Western diet. Data were collected across the 8 weeks of the intervention.

Telomere length

A general linear model analysis revealed that mice fed a Western diet had significantly longer telomeres (measured by T/S ratio) compared to those on the regular diet ($p=0.001$; Figure 1A). There was no effect of wheel access ($p=0.470$), though selection approached significance ($p=0.064$, Figures 1B, 1C). No interactions among any of the variables were statistically significant. In Figure 1D, telomere length across the eight treatment groups is compared. Mice that were selected, had no wheel access, and

ate a Western diet had significantly longer telomeres than the control/wheel/regular diet group ($p=0.003$), the control/no wheel/regular diet group ($p=0.001$), the selected/wheel/regular diet group ($p=0.004$), and the selected/no wheel/regular diet group ($p=0.018$).

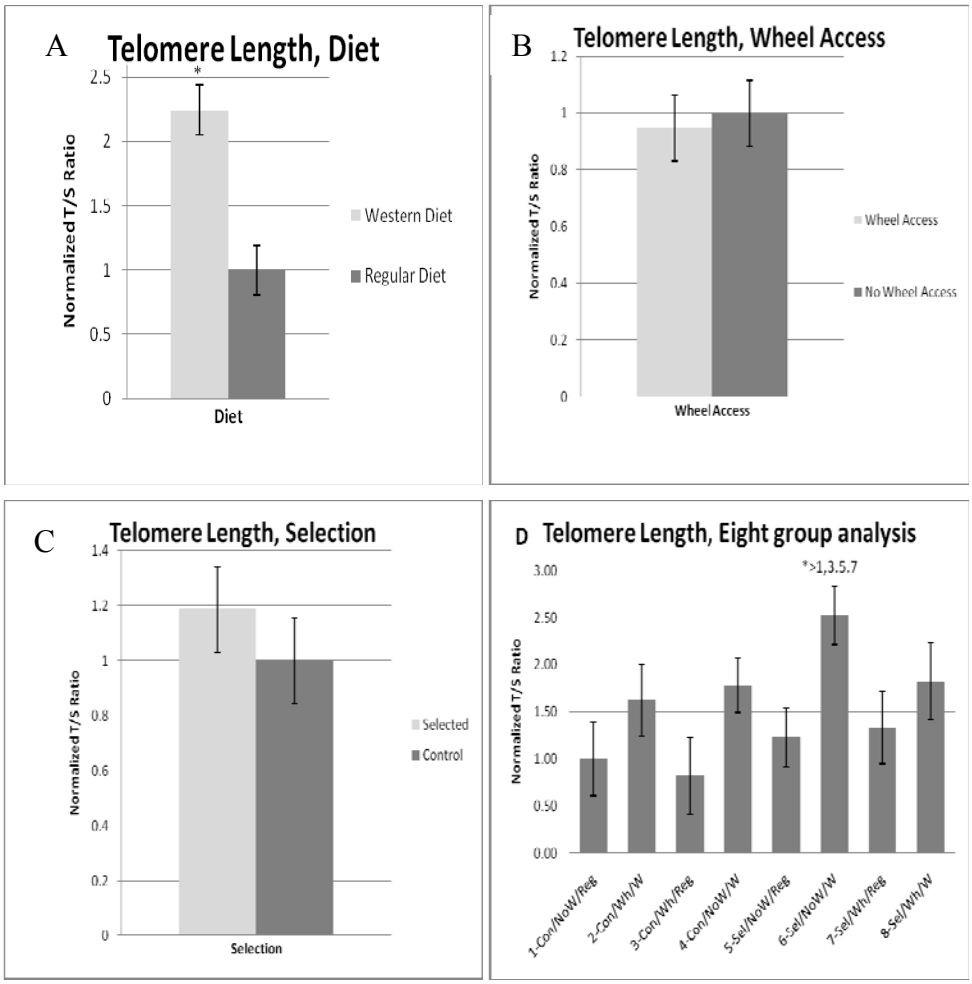


Figure 1: T/S ratio for the main effects of diet (A), wheel access (B), selection (C), and an eight group analysis (D). Mice fed a Western diet had significant longer telomere lengths (0.922±0.080) compared to mice fed regular diet (0.411±0.078; p=0.001). Mice with wheel access (0.648±0.080) did not have different telomere length compared to those without (0.685±0.079; p=0.470), and selection for voluntary wheel running over 52 generations (0.723±0.094) did not alter telomere length compared to control bred mice (0.610±0.095; p=0.064). All figures show values normalized to the control condition (regular diet, no wheel, and control-bred). Figure 1D shows the comparison between the eight different treatment groups. Abbreviations include Con for Control-bred, Sel for selected, NoW for no wheel access, W for wheel access, Reg for regular diet, and W for Western diet. Group 6 (selected, no wheel, Western diet) had significantly higher T/S ratios compared to groups 1, 3, 5, and 7 (p=0.003, 0.001, 0,004, 0.018, respectively).

mRNA Levels of Shelterin Components

mRNA levels of Trf1, Trf2, Pot1a, and Pot1b were measured by RT-PCR and quantified with NIH ImageJ software. The control gene Gapdh was not significantly different for any main effects including diet (p=0.656), wheel access (p=0.413), or selection (p=0.317), or any interactions (data not shown). Since Gapdh was consistently expressed across our groups and due to the variability in semi-quantitative PCR and the large number of samples (n=184), we used a general linear model (ANOVA) to explore differences in these shelterin component mRNA levels without normalization to Gapdh in addition to normalizing to Gapdh. mRNA levels for Trf1 were not significantly different between diets (p=0.196), wheel access (p=0.205), or selection compared to controls (p=0.141). However, Trf1 mRNA levels were higher in group 1 (control/no wheel/regular diet) compared to all other groups (Figure 2A). Trf2 mRNA levels were not altered by diet (p=0.216), wheel access (p=0.225), or selection (p=0.945), though group 8 (selected/wheel/Western diet) had lower mRNA levels than groups 6 or 7 (Figure 2B) rather than groups 3 and 4 in the normalized results. Finally, there were no significant differences for diet (p=0.753, 0.319), wheel access (p=0.962, 0.952), or selection (p=0.359, 0.261) for Pot1a or Pot1b mRNA levels, respectively, or any differences in the eight group analyses (data not shown).

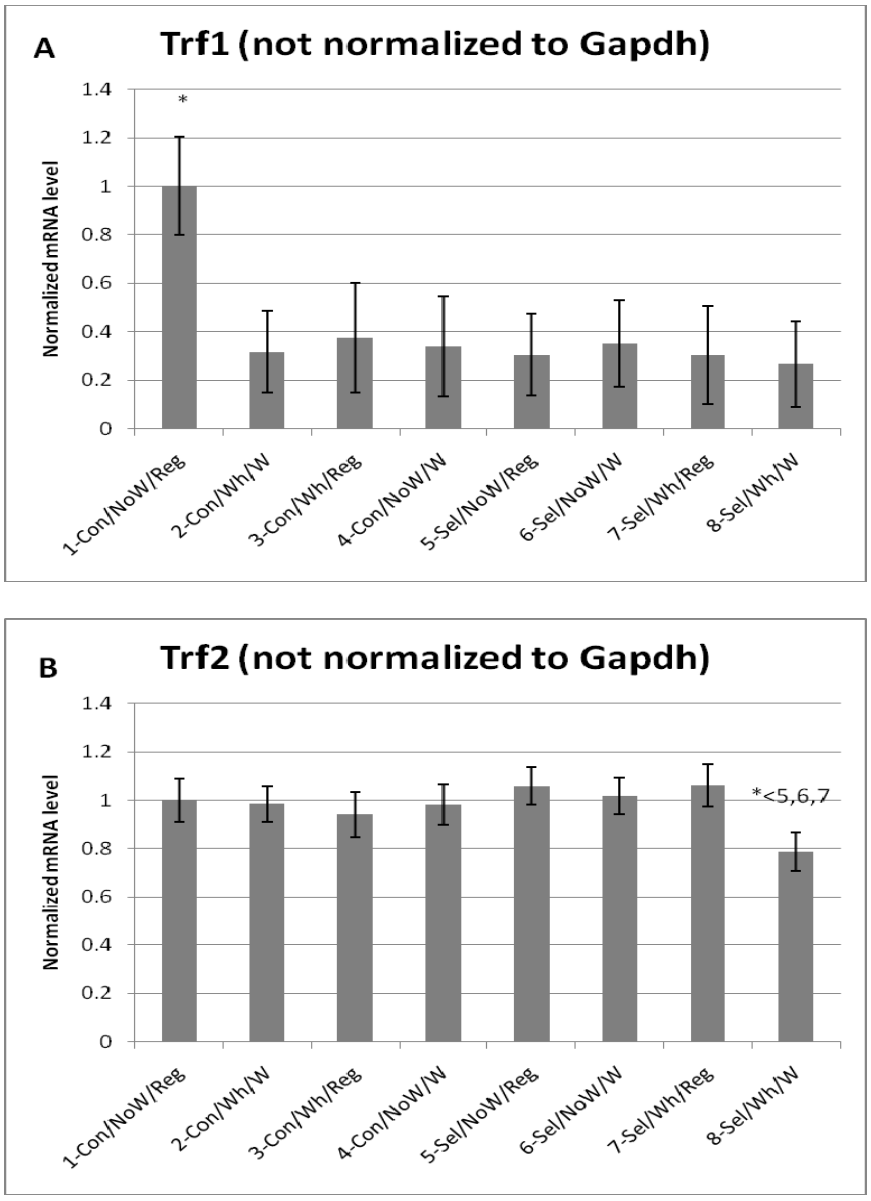


Figure 2: mRNA levels of Trf1 and Trf2 without normalization to Gapdh. In 2A, mRNA levels of Trf1 were significantly higher in mice in the control/no wheel/regular diet group compared to all other groups. In 2B, Trf2 mRNA levels are significantly lower in selected/wheel/Western diet mice compared to the selected/no wheel/regular diet, selected/no wheel/Western diet, and selected/wheel/regular diet groups ($p=0.015$, 0.038, and 0.021, respectively). There were no other significant differences for any comparisons for Trf1 and Trf2.

mRNA levels (normalized to the control gene Gapdh) for Trf1 were not significantly different between diets (p=0.342), wheel access (p=0.525), or selection compared to controls (p=0.293). However, Trf1 mRNA levels were higher in group 1 (control/no wheel/regular diet) compared to all other groups except group 3 (control/wheel/regular diet) (Figure 3A). Trf2 mRNA levels were also not altered by diet (p=0.216), wheel access (p=0.225), or selection (p=0.945) but group 8 (selected/wheel/Western diet) had lower mRNA levels than groups 3 or 4 (Figure 3B). There were no significant differences for diet (p=0.859, 0.280), wheel access (p=0.333, 0.249), or selection (p=0.326, 0.755) for Pot1a or Pot1b mRNA levels, respectively (data not shown), or any differences in the eight group analyses (Figure 4A, 4B). Appendix C shows representative gels for the eight different intervention groups.

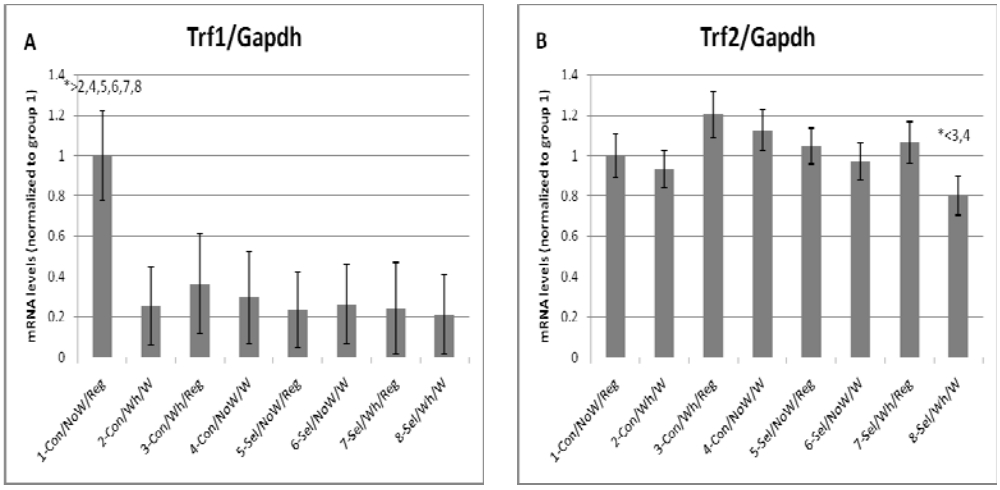
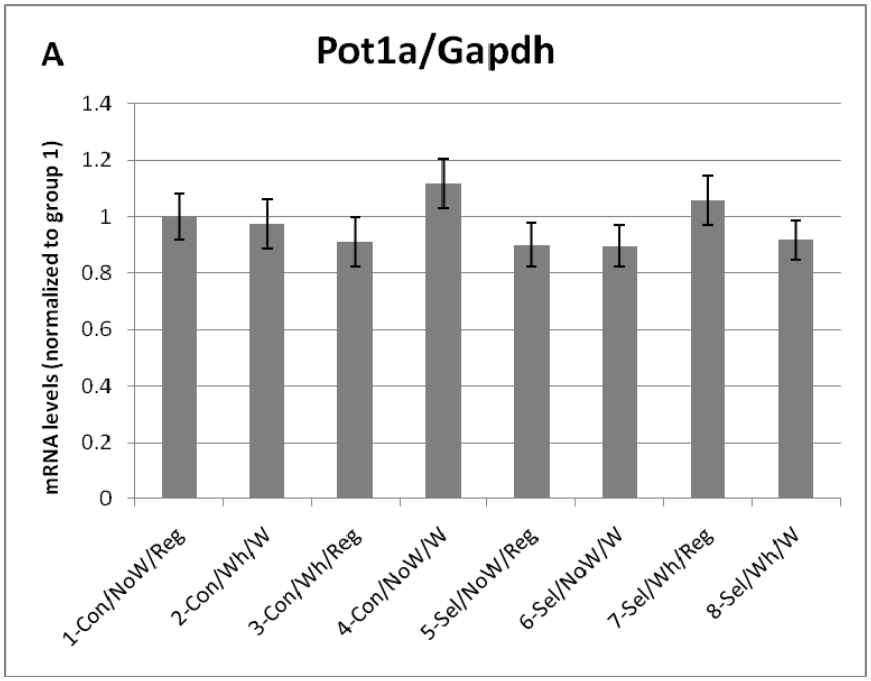


Figure 3: mRNA levels of Trf1 (A) and Trf2 (B) normalized to Gapdh. Figure 3A shows that control/no wheel/regular diet mice have higher Trf1 mRNA levels than all other groups except group 3 (control/wheel/regular diet) mice. Figure 3B shows that group 8 (selected/wheel/Western diet) mice had lower Trf2 mRNA levels than groups 3 (control/wheel/regular diet) and 4 (control/no wheel/Western diet). No other differences in these genes, including in analyses of the eight groups, were significant.



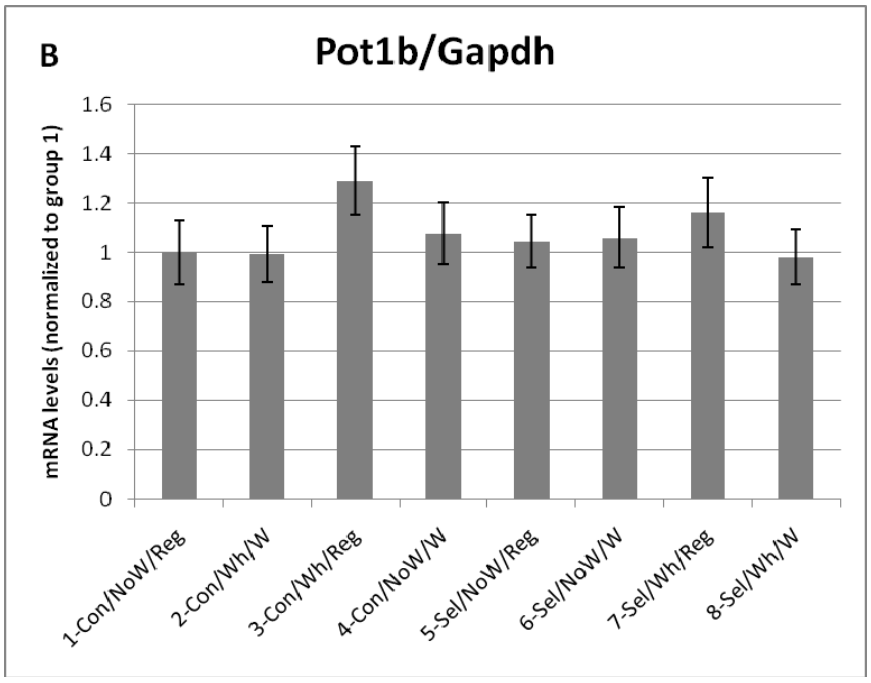


Figure 4: mRNA levels of Pot1a (A) and Pot1b (B) normalized to Gapdh. Among the eight treatment groups, there were no significant differences between any of the groups for Pot1a or Pot1b.

mRNA Levels of p53

There were no differences in p53 mRNA levels with normalization to Gapdh; neither diet (p=0.963), wheel access (p=0.960), nor selection (p=0.293) impacted mRNA levels and there were no differences in the eight group analyses. Without normalization to Gapdh, there were still no significant differences in p53 mRNA level for individual variables [(diet (p=0.983), wheel access (p=0.751), selection (p=0.083)] or in the eight group analyses (see Figure 5).

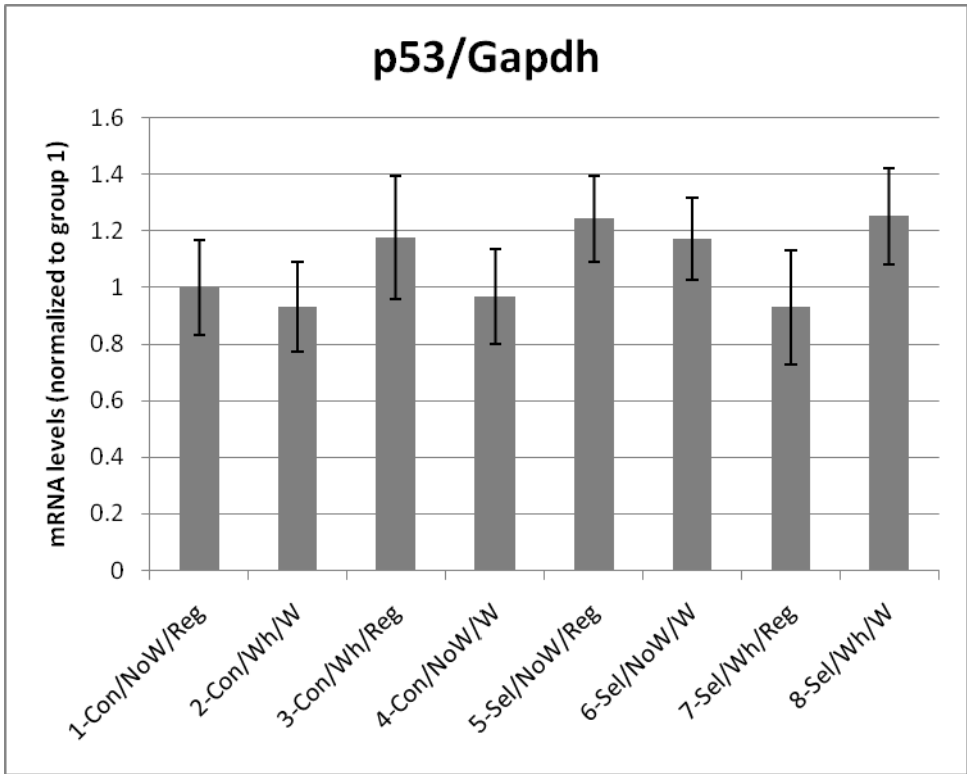
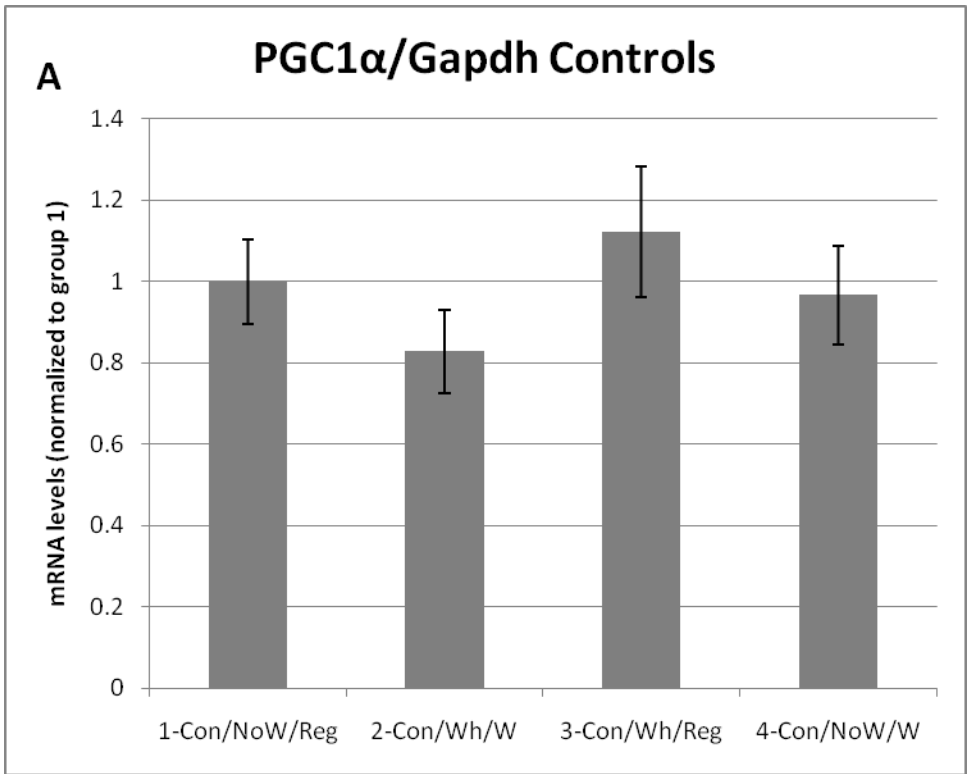


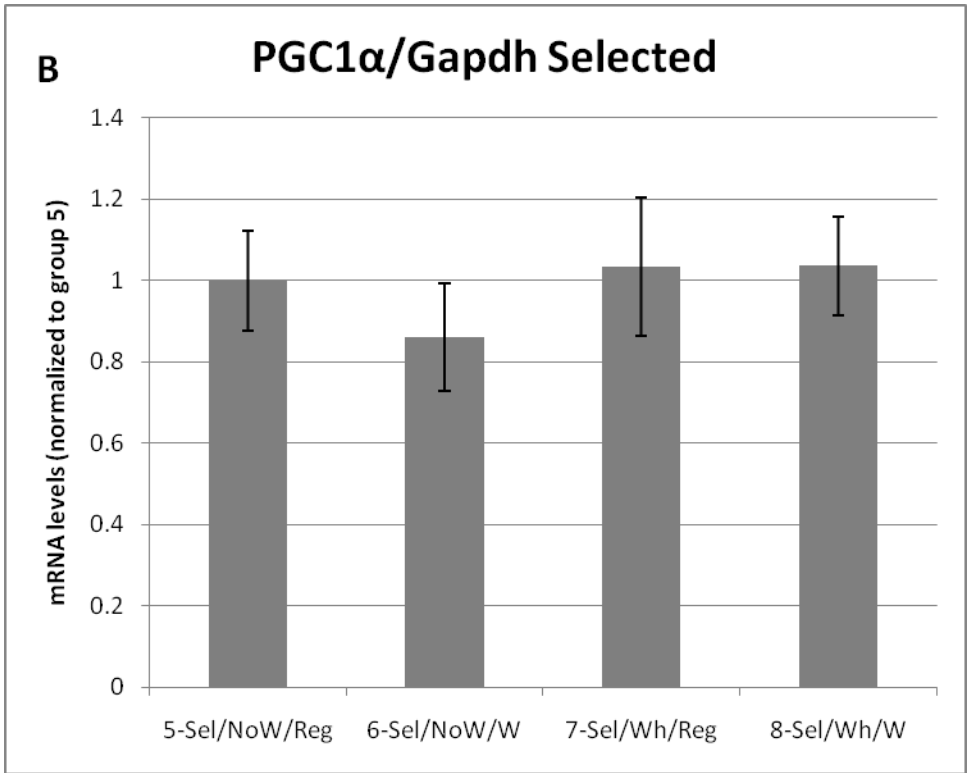
Figure 5: mRNA levels of p53 with normalization to Gapdh. There were no significant differences between any of the eight groups.

Verification of exercise training

In order to investigate adaptations to exercise, mRNA levels of PGC1α were measured. Following normalization to Gapdh, there were no differences in PGC1α mRNA levels for diet (p=0.185), wheel access (p=0.747) or selection (p=0.434), and there were also no differences in the eight group analysis. Without normalization to Gapdh, there were still no differences in diet (p=0.139), wheel access (p=0.117), or selection (0.084). Figure 6A shows data from control mice only in order to elucidate the effects of exercise on PGC1α mRNA level; no differences were observed among any of the four comparison groups. Additionally, there were no differences observed among any of the groups of selected mice (Figure 6B).

Mitochondrial copy number of the Cox1 gene was also evaluated; without normalization to a control gene (18S in the mitochondrial copy number assay), there were no differences in diet (p=0.319), wheel access (p=0.217), or selection (p=0.194). In an eight group analysis, group 6 (selected, no wheel, Western diet) had significantly lower Cox1 copy number than all other groups (Figure 7A). There also were no differences in Cox1 when normalized to 18S except in the eight group analysis. Cox1 mitochondrial copy number was not different between diet (p=0.453), wheel access (p=0.833), or selection (p=0.308), but for the eight group analysis, group 1 (control, no wheel access, regular diet) had significantly lower mtDNA copy number than groups 2 (control, wheel access, Western diet; p=0.027) and 6 (selected, no wheel access, Western diet; p=0.013; Figure 7B).


A
PGC1α/Gapdh Controls
mRNA levels (normalized to group 1)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1-Con/NoW/Reg
2-Con/Wh/W
3-Con/Wh/Reg
4-Con/NoW/W


B
PGC1α/Gapdh Selected
mRNA levels (normalized to group 5)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
5-Sel/NoW/Reg
6-Sel/NoW/W
7-Sel/Wh/Reg
8-Sel/Wh/W

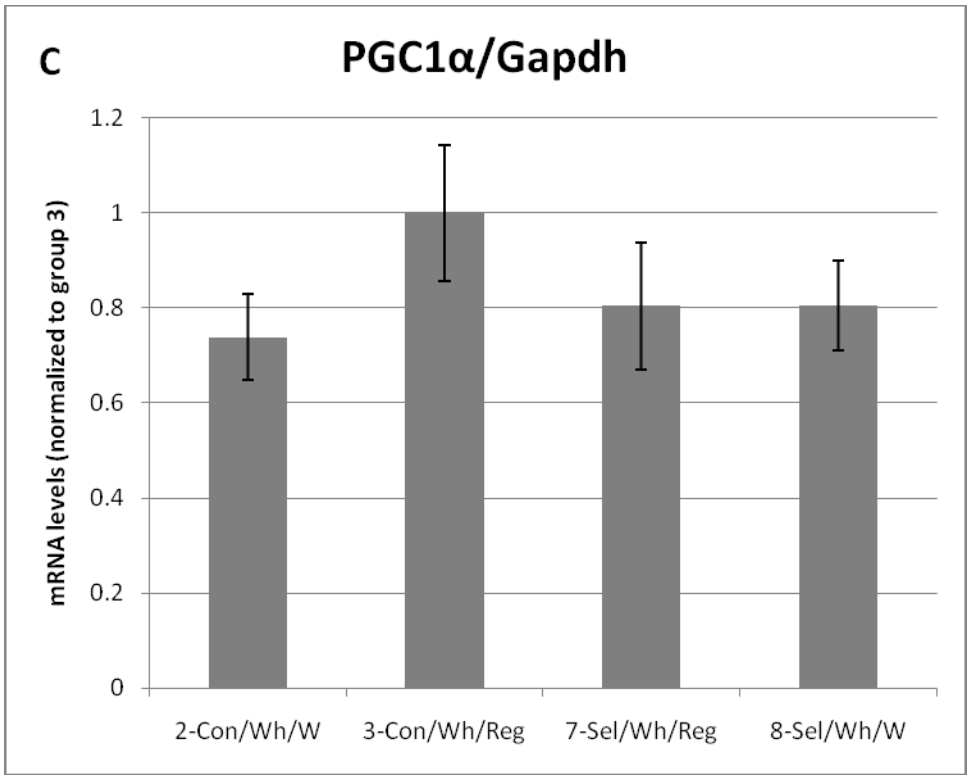
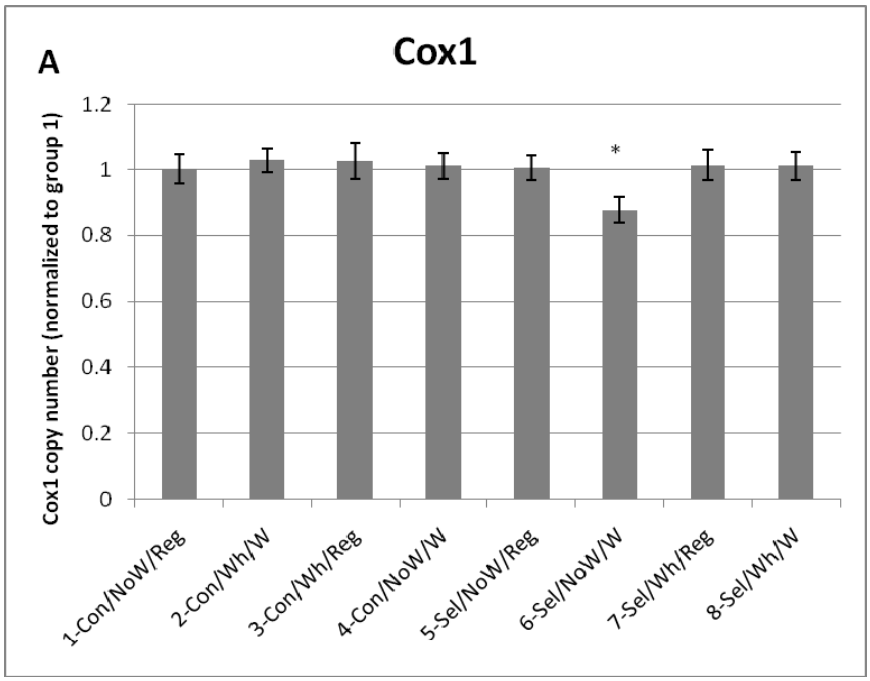


Figure 6: mRNA levels of PGC1α normalized to Gapdh. mRNA levels of PGC1α are not significantly different among any of the control groups (A). Comparisons of group 1 to 3 and group 2 to 4 resulted in both selection and diet being held constant, and these analyses also revealed no differences between the groups. There were also no differences in PGC1α mRNA levels between any of the selected groups (B). C shows that there were no differences in mRNA level between groups that differed only in wheel access (2 and 3, 7 and 8).



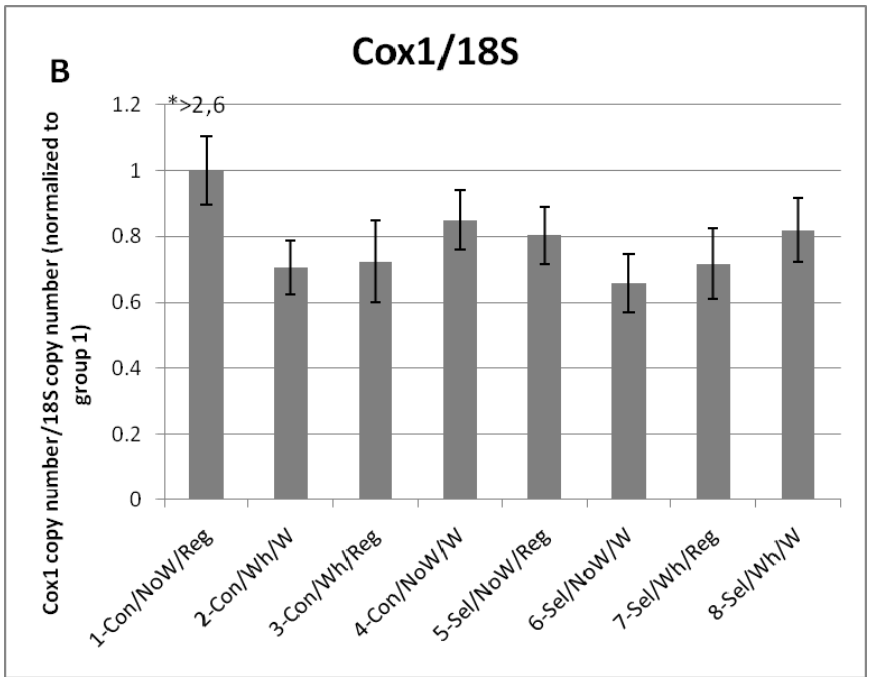


Figure 7: mtDNA copy number of Cox1, normalized and not normalized to 18S. Cox1 copy number is lower in group 6 (selected, no wheel, Western diet) compared to all other groups when not normalized to 18S (A). With normalization to 18S, group 1 (control, no wheel, regular diet) has higher copy number compared to groups 2 (control, wheel access, Western diet) and 6 (selected, no wheel, Western diet).

Chapter 4: Discussion

The present study is the first to examine the combined effects of diet and exercise on telomere length and telomere binding proteins in mouse skeletal muscle. Additionally, the impact of many generations of selection for high voluntary wheel running on telomere biology was investigated. This study did not confirm our hypothesis that eight weeks of voluntary exercise impacts telomere length in mouse skeletal muscle, though diet did have an unexpected effect on telomere length. The PGC1α mRNA level and Cox1 mtDNA copy number data suggest that wheel access had little effect on mitochondrial adaptations to exercise, which may explain the lack of change in telomere length due to exercise training. This is the first study to demonstrate longer telomeres in young mice fed Western diet compared to those fed a standard diet.

A key finding from the present study is that in mice fed a Western diet for eight weeks in early life, skeletal muscle telomere length was significantly longer compared to those fed a regular diet. This finding is in contrast to our initial hypothesis that exercise (wheel access), regular diet, and selection for high voluntary wheel running would result in longer telomeres compared to mice who were sedentary (no wheel access, though typical cage roaming activity was allowed and not measured), ate a Western diet, and were control-bred. Additionally, we showed that group 6 (selected, no wheel, Western diet) mice had longer telomeres than any of the four regular diet groups (groups 1, 3, 5, 7; see Figure 1D), which further supports the effects of diet on telomere length. There was also a tendency for selected mice to have longer telomeres than controls (see Figure 1C), indicating that the selection over 52 generations may affect telomere biology in some way that results in telomere lengthening.

Poor diet is thought of as a risk factor for cellular aging, but Western diet appears to positively affect telomere length in the present study. In a human study of leukocyte telomere length in patients with coronary artery disease, also considered a risk factor for cellular aging, telomere length increased in some individuals, remained constant in others, and in still others, decreased after a five year follow-up (54). The individuals with the shortest telomeres at baseline tended to have the longest telomeres after 5 years, and vice versa. In another study with these same mice (generation 52), Western diet increased wheel running in selected mice, and selected mice on Western diet had greater caloric intake compared to controls on Western diet (105). Minamino and colleagues recently showed that excessive calorie intake leading to obesity resulted in accumulation of oxidative stress in the adipose tissue of mice with type 2 diabetes-like disease and also led to increased activity of senescence-associated beta-galactosidase, higher expression of p53 and increased production of proinflammatory cytokines (107). Thus, we propose that in our relatively young mice, a reversal of telomere length would result had the mice been allowed to age (i.e., the mice on a Western diet would, after a length of time, actually demonstrate the shortest telomeres). The Western diet may have resulted in elevated oxidative stress, inflammation, etc. and over a lifetime this would result in telomere shortening. However, in young animals, the effects of diet may be manifested not as short telomeres but instead, dysfunction may be revealed as telomeres that are actually longer than those of their counterparts fed a regular diet.

We observed no effect of exercise training on telomere length. Mice may have been too young (approximately 12 weeks of age at sacrifice) and/or the exercise stimulus too short (8 weeks) to result in changes in telomere length as a result of the exercise

intervention. In previous animal studies, neither an exercise stimulus of 3 weeks nor 6 months resulted in telomere shortening in myocardium (162) or aorta (161), though telomerase activity was higher in the exercised mice and mRNA levels of telomere-regulatory proteins were altered as a result of the exercise training (162).

Several studies have shown differences in telomere length with exercise (34, 39, 88, 97), but no difference was shown in skeletal muscle in the current study. It is possible that the exercise intervention was too short or that the exercise was not of an adequate intensity to elicit a training effect. Werner and colleagues showed no differences in telomere length with three weeks or six months of wheel running in mice in myocardium (162) and in aortas (161), which supports this potential explanation of the lack of differences in telomeres with exercise. One other possibility is that the control mice had low physical activity levels and the selected mice exercised at very high levels, resulting in a similar "inverted U" shape to that seen by Ludlow and colleagues (97), where both low and high physical activity levels were associated with shorter telomeres compared to moderately active individuals. The large gap in distance run between selected and control mice (see Table 1) supports this hypothesis. In a study of voluntary wheel running patterns of several strains of mice, the strain that ran the least ran approximately 1.5 kilometers per night and the strain that ran the most ran about 7.5 kilometers per night, on average (92). The control mice of the present study ran little more on average than the low group in the Lerman and colleagues study (92) while the selected group ran considerably more than the highest running strain, suggesting that the difference in running distances between the controls and selected mice might result in the "inverted U" relationship described by Ludlow and colleagues (97). The current study might simply

lack the middle, moderately active group and thus all telomere length results appear similar. Unfortunately, time course analyses that might have addressed this issue were not possible using the samples for the present study.

Telomere length by itself has limited utility in understanding telomere biology, as evidenced by the Farzaneh-Far et al. (54) study that showed that in older coronary artery disease patients, leukocyte telomere length increased in some individuals, remained constant in others, and in still others, decreased at follow-up five years later. Werner and colleagues found altered telomerase activity, expression of Trf2, p53, TERT, and Ku70 in the myocardium of mice provided a running wheel for 21 days despite no differences in telomere length (162). The mice began their exercise training at eight weeks of age, so were similar in age to those in the current study at sacrifice, suggesting that exercise may not alter telomere length in very young animals (161, 162). In baboons, seven weeks of high-fat diet resulted in decreased replicative potential of endothelial cells with no change in telomere length (147). Another study in humans showed that six months of aerobic exercise training in obese, middle-aged women did not result in telomere length changes, though antioxidant enzyme levels did rise in the exercising subjects (148). A study of Terc (the gene that encodes telomerase RNA) knockout mice demonstrated that telomere dysfunction rather than mean telomere length is associated with aging and disease phenotypes (72). We suggest that telomere length alone may not be indicative of cellular health, but that other factors, including telomerase activity, as well as gene and protein expression of telomere-stabilizing proteins must be measured in addition to length in order to understand the full picture of telomere activity and cellular health in response to a stimulus.

For the present study, we chose not to measure telomerase enzyme activity in these skeletal muscle samples. First, telomerase should not be active in post-mitotic skeletal muscle, so it is likely that any detectable levels of telomerase are from satellite cells or other replicating cells mixed into the muscle homogenates. Telomerase has been reported in post-mitotic tissues including skeletal muscle in several studies (Radak et al 2001; Ludlow et al 2008; Werner et al 2008), but we suspect that it is satellite cells that are altering telomerase activity in these studies and thus suggests that telomerase is a marker of satellite cell telomerase activity, which was not an aim of the present study. The Werner papers (161, 162), though interesting, showed differences in telomerase activity and not telomere length in myocardium and aorta, not skeletal muscle. Additionally, the triceps surae muscle homogenates used in this study contained mixed fiber types as well as numerous cell types, limiting our ability to distinguish telomerase activity of muscle cells from other cell types.

In the current study, the patterns of differences in Trf1 mRNA levels were similar when levels were normalized to Gapdh and when they were left un-normalized (Figures 2A, 4A). For both analyses, group 1 (control, no wheel, regular diet) had higher mRNA levels than all other groups, except in the normalized analysis, where group 1 was not different from group 3. For the Trf2 analyses, normalization to Gapdh resulted in group 8 (selected, wheel access, Western diet) having lower mRNA levels than groups 3 (control, wheel, regular diet) and 4 (control, no wheel, regular diet). When left un-normalized, Trf2 mRNA levels for group 8 were significantly lower than groups 5, 6, or 7, which are the three other groups from selected lines. Group 1 (control, no wheel, regular diet) had the highest levels of Trf1 mRNA while the opposite group, 8 (selected,

wheel, Western diet) had the lowest levels of Trf2. The reason for this finding is unclear. However, Werner et al. demonstrated that both 21 days and six months of wheel running in mice resulted in an up-regulation of Trf2 but not Trf1 in myocardium (162) and 21 days of running up-regulated Trf2 but not Trf1 in the thoracic aorta and circulating mononuclear cells (161). The two Werner et al. papers suggest that Trf1 is less sensitive than Trf2 to wheel running, and our data similarly indicate that Trf1 and Trf2 may be differentially regulated in response to environmental stimuli. The shelterin components Pot1a and Pot1b were not different in any analyses when normalized to Gapdh or when not normalized. To our knowledge, changes in these genes have not been evaluated in response to exercise, so it is unclear whether Pot1a and Pot1b mRNA levels are not altered with exercise or if the 8 week training period was inadequate to elicit changes.

p53 is a pro-apoptotic transcription factor that has been shown to be down-regulated in exercising mice compared to sedentary mice in myocardium (162) and in the thoracic aorta (161). In the present study, there were no differences in p53 mRNA levels in any analyses, with normalization. This result is surprising; we would expect to see differences in p53 with diet to confirm our hypothesis that telomere dysfunction is the reason for higher T/S ratio in mice fed Western diet. However, it may be that oxidative stress or levels of proinflammatory cytokines are altered in the Western diet groups, but these were not measured in the present study. Vaanholt and colleagues showed that while wheel running did not affect antioxidant levels (superoxide dismutase and glutathione peroxidase) in the liver, higher daily energy expenditure was associated with higher antioxidant levels (155). Since selected mice expended more energy than control mice due to breeding for high wheel running (105), we expected selected mice to have higher

antioxidant levels than controls in order to compensate for higher oxidative stress due to environmental stimuli, of which p53 is a marker (5). However, Bronikowski and colleagues (18) measured mRNA levels of the antioxidant enzyme catalase in the liver of generation 16 of these animals bred for high wheel running and found that exercising controls had higher antioxidant activity than sedentary controls or any of the selected groups, though the exercising mice ran for 20 months. Our p53 results did not mimic the results of any similar studies, and though the reason for this is unclear, it may again be that the exercise/diet stimulus was too brief or that the mice were too young for the full, expected effects to occur.

The mRNA levels of PGC1α and mtDNA levels of Cox1 were not different across the exercise versus sedentary groups, which was entirely unexpected. The very high running distances of the selected lines argue against a hypothesis that the training stimulus was inadequate, though these animals have been selected over 52 generations for high voluntary running behavior. One possibility is that the selected lines did not adapt because they are at a ceiling for mitochondrial function. Mice from this breeding experiment that have a mini-muscle trait and are represented in the current study in the selected lines, where hindlimb muscle mass is reduced by 50 percent, have more oxidative fibers than mice without mini-muscles even without training (164). And, it may be that the control lines did not adapt because they ran rather low distances given their selection against voluntary wheel running. Coupled with the modest sensitivity of the semi-quantitative PCR method used in the present study, subtle differences related to training may not have been seen. Regardless, the lack of a clear impact of exercise training on markers of oxidative metabolism may have impacted the results of the other

measured variables, either indicating an inadequate stimulus or unique genetic contributions that counteracted the adaptations expected from the stimulus. These results are difficult to interpret but indicate that neither wheel access nor diet resulted in greatly altered mitochondrial content or function.

This study is the first to describe the effects of the combination of diet and exercise training on telomere length and telomere-stabilizing proteins. The evolution of telomeres and the related shelterin complex over 52 generations of breeding for high voluntary wheel running was also investigated. We saw limited changes in mRNA expression of proteins that make up the shelterin complex. We did not measure protein expression directly, so it is possible that protein differences were present but were not reflected by the mRNA levels. Another limitation is the lack of baseline tissues for measurement of telomere length and mRNA expression. We do not know how these measures changed from baseline since we could not measure telomere length and gene expression at age 4 weeks, before the intervention. Other limitations include the use of only male mice; it is not clear that we would see the same results in females, particularly if running times were different between the sexes. Finally, powdering of the entire triceps surae muscle group does not allow determination of muscle-specific or fiber-type-specific changes in dependent variables.

Our primary hypotheses were not supported in this study, and potentially, the young age of these mice may have impacted the lack of expected results. We have suggested that if allowed to age, the mice fed Western diet would have the shortest telomeres due to the role of high oxidative stress. A future study could include a similar design with the addition of a timeline for animal sacrifice. Every two to three weeks, a

group of mice would be removed from their intervention for sacrifice so that the effects of time and age, rather than just wheel, diet, and selection, could be evaluated. Overall, we conclude that stressful environmental stimuli such as Western diet result in telomere dysfunction, which may present as altered telomere length, even lengthening. These data in total indicate the importance of diet and genetic history in cellular health.

In conclusion, diet does affect telomere length and shelterin mRNA levels though not in the manner we expected. Western diet resulted in longer telomeres in young mice, suggesting telomere dysfunction, or abnormal regulation, is induced by this poor diet. Telomere length was unaffected, however, by selection for high voluntary wheel running or wheel access. Shelterin proteins were not consistently regulated by diet, wheel access, or selection, suggesting that the components may be differentially regulated by environmental factors. This evidence suggests an important role of diet in telomere biology, though the time course of these changes needs to be elucidated.

Chapter 5: Review of Literature

The purpose of this review is to synthesize the literature in the area of telomeres and their associated enzyme, telomerase, and review changes in response to oxidative stress as present in aging, disease, physical activity, dietary changes, chronic stress, etc. The topics covered in this review include a general overview of telomeres and telomerase, telomeres and aging, telomeres and disease, telomeres and exercise/physical activity, telomeres and diet, and telomeres and other environmental factors. Finally, an overview of selective breeding experiments similar to the one used in the current study is provided.

Telomeres

In 1965, Leonard Hayflick published a now famous study describing the inability of cells to indefinitely divide in culture (70). This phenomenon was coined the Hayflick Limit, and has since been attributed to the loss of telomeres, repeat sequences of DNA found on the ends of linear DNA that shorten with each DNA replication (and cell division). In humans, there are thousands of $(TTAGGG)_n$ repeat sequences on the end of each chromosome that make up the telomere (11, 171). Telomeres are the solution to the "End Replication Problem", the fact that DNA polymerase is unable to create a complementary strand to synthesize the end of the 5' lagging strand thus resulting in incomplete DNA replication at the ends of the chromosome (118, 159). Telomeres thus act as a buffer in order to protect the cell's important genetic information contained in the chromosome from being lost during cell division, with telomeres progressively shortening with each cell division (40, 67, 95).

Telomere shortening is seen in aging in numerous post-mitotic tissues including muscle and liver despite that they do not undergo DNA replication; this may be explained by telomere shortening in other cell types that are mixed in with the homogenate (i.e. satellite cells, white blood cells). However, progressive telomere shortening does not occur in some tissue types. These include stem and immune cells due to the presence of telomerase (81, 146) an enzyme that synthesizes telomeric repeats onto the 3' end of telomeres (110) . In cells without telomerase, telomere shortening eventually results in the inability to further proliferate and permanent cell cycle arrest; this state is termed cellular senescence, and it coincides with the Hayflick limit in which cells may no longer divide (36, 72). In cells that require the ability to continually replicate (e.g., stem cells), telomerase allows for maintenance of telomere length and repeated rounds of cell division with induction of senescence. Cancer cells, for example, generally express telomerase and are able to divide indefinitely.

The ends of mammalian chromosomes are associated with a six protein complex called shelterin (43). Shelterin is a complex of proteins that provide a marker to distinguish chromosome ends from DNA breaks requiring repair, and the amount of shelterin is proportional to the length of the telomere (43). The 3' end of the chromosome is a single stranded overhang that forms a structure called the t-loop (66, 111, 138). The current thinking is that the 3' overhang invades double-stranded telomeric DNA and complementary base pairs bind, forming a lariat-like structure (43). Shelterin specifically binds to telomeric DNA through the actions of telomeric repeat binding factor one and two (TRF1 and TRF2); TRF1 has an affinity for double-stranded TTAGGG repeats (37, 172) and TRF2 is able to form a loop-like structure when an appropriate substrate is

provided (149). Similar to Trf1 and Trf2, Pot1 binds TTAGGG sequences, but in single-strand form, and two other proteins, Tin1 and Tpp hold the Trf1, Trf2, and Pot1 together (42). Shelterin is required for recruitment of telomerase (150), and is responsible for enabling the cell to differentiate between its natural chromosome end and DNA breaks as well as repressing DNA repair at chromosome ends (120).

Telomerase

Telomerase was first discovered on Christmas Day, 1984 by then-graduate student Carol Greider. Just a few years later, she and her mentor Elizabeth Blackburn published their findings that telomerase isolated from Tetrahymena protected telomeres in five other species (65). Telomerase is highly expressed in germ cells but not in somatic cells (63) and high levels of activity are reported in 90 percent of malignant tumor cells. In 2009, Greider, Blackburn, and Jack Szostak won the Nobel Prize in Physiology or Medicine for the discovery of telomerase and how it protects telomeres.

Telomerase contains two subunits: hTER (human telomerase RNA) and the protein catalytic subunit hTERT (human telomerase reverse transcriptase). It also contains a telomere-associated protein, TP1, and all of these components are necessary for telomerase activity (160). TP1 and hTER are expressed in all cells, but hTERT is only expressed in cells with telomerase activity. Thus, hTERT is a limiting factor in telomerase activity in post-mitotic tissues (17, 160). Telomerase functions to replenish the ends of DNA chromosomes (64, 170) but also may function to protect telomeres in a non-lengthening manner (173). Introduction of telomerase extends lifespan in normal human cells (17), and mice without telomerase RNA show signs of premature aging (16, 32) Mice with the mTR gene knockout, which do not produce telomerase RNA, have

extreme telomere shortening and chromosome end fusions resulting in genomic instability as well as other health concerns including impaired hematopoiesis, atrophy of the intestinal epithelium, low spermatogenesis, and decreased regenerative capacity in response to wounds (Rudolph et al. 1999). However, higher than normal telomerase levels in fibroblasts in culture were actually related to the induction of cellular senescence (61), suggesting that there may be a specific range of telomerase expression that results in telomere lengthening.

Telomeres and Cellular Aging

Extended telomere shortening results in eventual cellular senescence, or the inability of the cell to further divide, as the cells are irreversibly locked in the G1 phase of the cell cycle. However, though cellular senescence may be responsible for aspects of aging (27), it is also thought to be a protective mechanism to prevent tumor development and unlimited cell division capacity (117). According to Ohtani et al (117), two types of hypotheses have been proposed to explain mechanisms of cellular senescence. First, senescence is due to oxidative damage or stress, and second, senescence is a process that is programmed into the genes to occur at certain times. In either situation, when the telomere becomes critically short, DNA damage checkpoints, including a cell cycle arrest checkpoint signaled by p53, are activated (158). Depending on the type of cell, activation of this p53/p21 pathway results in cell cycle arrest (38) or apoptosis (89). Fibroblasts with non-functional p53 have extended lifespans, but this results in even more telomere shortening and a second checkpoint, called the crisis or mortality stage, is induced (168). The result is extreme telomere dysfunction, chromosomal instability, and

cellular death, though these checkpoints prevent uncontrolled cellular growth and thus also act as mechanisms of tumor suppression.

DNA damage that occurs as a result of oxidative stress and/or inability of cells to repair DNA likely plays an important role in the etiology of many diseases including cancer, diabetes, and arteriosclerosis (169). Accumulation of DNA damage may occur in aging via reactive oxygen species (ROS), defects in DNA damage repair mechanisms, and/or exposure to certain environmental factors including irradiation and toxins (158). It has been suggested that maintenance of genomic integrity in response to stressful events is vital to protect from early aging and/or disease (139). Increasing levels of DNA damage occur during aging (144), and the number of senescent cells rises as well (47). In wild type mice, there is a decrease in the expression of DNA repair genes with aging (31). Activation of the ATM/Chk2 and ATR/Chk1 checkpoints leads to activation of the p53 pathway (see Figure 1—(158)), resulting in apoptosis or senescence and prevention of uncontrolled or unregulated cellular growth. Several pathways have been studied as potential regulators of the response to DNA damage. The ATM pathway results in altered expression of p53 and downstream components, and is described in the next paragraph in greater detail. The ATR pathway is responsible for maintenance of replicative performance. In a mouse model with null ATR, the mouse is unable to maintain replicative polymerases at replication forks (21, 153). The ATR knockout mouse developed an early aging phenotype (140) and had decreased stem and progenitor cell pools and decreased capacity for tissue renewal (86, 140). This pathway is also necessary for embryonic development and is important in tumor suppression (20).

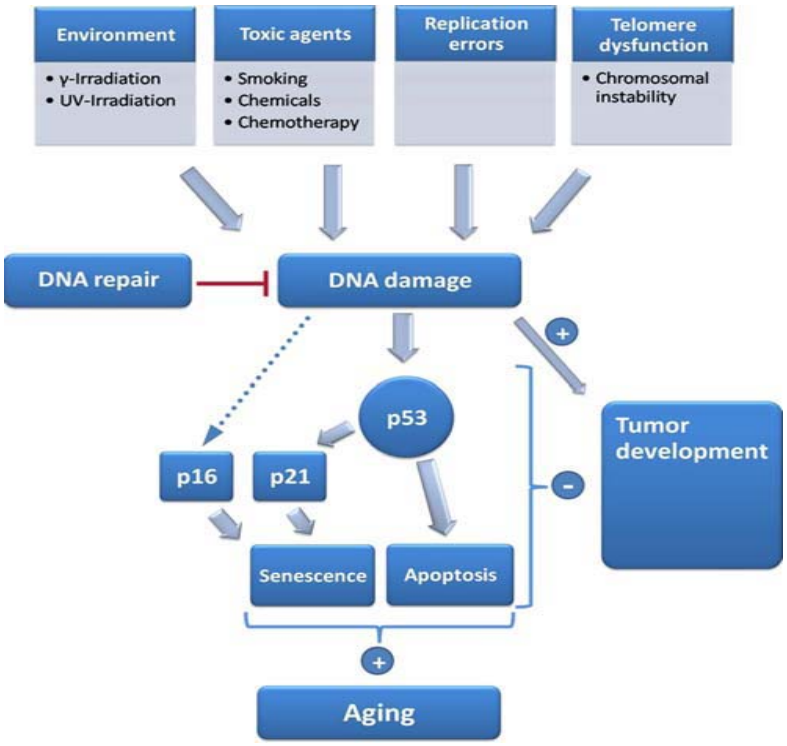


Figure 8: Role of p53 in DNA Damage. From (158). Environmental stimuli, toxic chemicals, errors in DNA replication, and dysfunctional telomeres all contribute to DNA damage, which signals the pro-apoptotic transcription factor p53, which can result in either apoptosis or senescence via the stimulation of p21.

The tumor suppression protein p53 has the ability to induce cell cycle arrest or apoptosis in response to environmental stimuli including DNA damage, hypoxia, oxidative stress, or activation of oncogenes (5), and fifty percent of human tumors have mutations in the p53 gene (84). The absence of p53 results in cellular immortalization and predisposition to neoplastic transformation (62). Additionally, evidence suggests that p53 represses hTERT by altering the expression of p21, E2F and Rb (41). In telomerase deficient mice, p53 expression is elevated, resulting in a triggering of senescence or apoptosis (56). The same study indicated that reduced p53 expression in mice resulted in restored hair grown, skin renewal, wound healing, and increased epidermal stem cell motivation, all of which were consequences of telomerase deficiency (2009). Another study in mice focused on effects of p53 deletion on the intestine. The mice also had dysfunctional telomeres and a decreased overall lifespan, as well as impaired deletion of chromosome unstable intestinal stem cells (9). The results included higher levels of

apoptosis, altered differentiation of epithelial cells, and premature intestinal failure (9). Clearly, the p53 pathway is important in the maintenance of cellular lifespan, though the mechanisms by which it works are not yet fully understood. Downstream of p53, p21 regulates senescence. Late generation Terc knockout mice in which p21 has been eliminated experience rescue of hematopoeisis and ability to generate intestinal villi, both of which are consequences of aging in the knockout, though there is no rescue of telomere length or DNA damage levels (38).

Telomeres and Human (Organismal) Aging

Increasing age is related to higher rates of chronic disease such as cardiovascular disease, type II diabetes, cancer, and sarcopenia. Decreased ability to perform activities of daily living also often reduces quality of life. Telomeres have been implicated in aging due to their shortening with subsequent cell divisions in somatic cells, and older people have shorter telomeres than their younger counterparts (45).

In humans, evidence suggests that telomere shortening acts as a mechanism for counting cell divisions, which does not seem to be true in mice (167), which have longer telomeres (71). This suggests that mechanisms for cellular senescence may differ in humans and mouse models. Additionally, Armanios and colleagues (4) demonstrated that short telomeres are passed down to offspring and that mice with short telomeres but a normal genetic profile for telomerase develop defects in hematopoietic and immune systems, suggesting that short telomeres can cause aging-related defects without telomerase defects. The evidence for genetically-programmed senescence is less established than the hypothesis of oxidative damage build-up, but is growing and makes this idea, particularly in humans, a reasonable one.

Telomere dysfunction and cellular senescence have also been tied to age-associated diseases. In elderly Caucasian men, leukocyte telomere shortening rate was found to correlate to mortality rates due to cardiovascular disease (53). Patients with chronic heart failure (CHF) have shorter telomeres, higher levels of cellular senescence, and more cell death in myocardial tissue as compared to normal hearts (35), and telomere length is as much as 25 percent shorter in patients with CHF (116). Additionally, telomere shortening is related to disease advancement in liver cirrhosis (163), ulcerative colitis (114), and hematopoietic stem cell-associated disorders (22). Finally, diseases of premature aging often affect either telomerase function or the DNA repair system (115). Dyskeratosis congenital (109), aplastic anemia (100), Werner syndrome, Bloom syndrome and ataxia telangiectasia patients all share a short telomere phenotype (115).

Telomeres and Disease

Independent of aging, short telomeres have been associated with cardiovascular disease (165), atherosclerosis (142), and obesity (82). Additionally, bone marrow failure syndromes have been related to mutations in hTERT, hTERC (human telomerase RNA, also called hTER) and proteins involved in telomere maintenance (48). In one of these syndromes, the rare Dyskeratosis Congenital (DC), patients manifest symptoms of premature aging including increased risk of cancer (3), and have short telomeres (2, 49) as well as mutations in the telomere-associated dyskerin protein, hTERT, and hTERC (101).

Telomeres and telomerase are also particularly interesting in the field of cancer research. A high level of telomerase enzyme activity is characteristic of most tumors (15) and tumor cells have short telomeres (69). Chromosome instability is thought to be

related to tumor growth (25) and in human tumor cells, the telomerase RNA template is a mutant, resulting in telomere uncapping (94). Short leukocyte telomere length is a risk factor for solid tumors (excluding breast cancer) (73, 96), and DC patients had 11 times higher risk for certain cancers (3). Mutations in the TERT gene were found in nine percent of acute myeloid leukemia patients (24), and genome wide association studies (GWAS) identified a relationship between lung, bladder, prostate, and cervix cancers and a TERT polymorphism, though the increased risk was relatively small (127).

More generally, telomere length and activity of the shelterin complex that protect the telomere have been linked to cellular health. Telomeres and telomerase are protective against events promoting instability of the chromosome (13). Overexpression of a TERT mutant protein results in the inability of telomerase to work properly, and leads to premature senescence and apoptosis in primary human fibroblasts or keratinocytes (80). Telomeres are also involved in DNA damage response pathways; one of two processes, nonhomologous end-joining (NHEJ) or homologous recombination, is used by the cell to repair breaks in chromosomal DNA (12).

Telomeres and Exercise/Physical Activity

Physical activity is known to reduce various markers of stress (124) as well as reduce mortality in humans (14) and extend lifespan in animals (90). Only recently, however, has physical activity been studied in relation to telomere length and telomere biology. In several studies, physical activity has been shown to modulate telomere length both in human and animal models. Collins and colleagues (39) demonstrated that muscle homogenate telomere length is shorter in athletes with fatigued athlete myopathic syndrome (FAMS), a condition of exercise-associated chronic fatigue, compared to age

and training-matched controls. Another group demonstrated that higher levels of leisure time physical activity are associated with longer telomere length, even after controlling for age, sex, body mass index, smoking, socioeconomic status, and physical activity at work, and the finding was validated in a group of twin pairs discordant for physical activity (34).

Ludlow and colleagues showed that telomeres are shorter in humans who do not exercise regularly as compared to the telomeres of people who exercise with moderate exercise energy expenditure (97). Their results also showed shorter telomeres for highly active subjects as compared to those who exercise at moderate intensities, and the authors concluded that a moderate level of physical activity may be protective against telomere shortening (97). Since then, Werner et al. (162) published findings indicating that six months of voluntary wheel running induces an increase in telomerase activity and the up-regulation of telomere related proteins in the mouse myocardium, with no change in telomere length. This was the first study of its kind in the mouse. Even more recently, VO_2max, a measure of aerobic capacity, was shown to be associated with leukocyte telomere length in older adults, and thus aerobic exercise may prevent age-related decline in leukocyte telomere length (88). A study of highly trained endurance runners demonstrated that the runners who spent the greatest number of years and total hours training had the shortest mean terminal restriction fragment (a measure of telomere length), and the authors concluded that chronic endurance exercise may act as a stressor to skeletal muscle, resulting in the change in telomere length (126). Kadi & Ponsot (74) hypothesize that high levels of physical activity without the presence of unusual oxidative stress levels may either result in unchanged or lengthened skeletal muscle telomeres.

However, not all studies have shown regulation of telomere length by exercise or physical activity. A study in power-lifters showed no difference in mean telomere length between the lifters and control subjects (75). Another study showed that six months of aerobic exercise training did not result in telomere length changes, though antioxidant enzyme levels did rise in the exercising subjects (148). Across quartiles of physical activity, there was no difference in telomere length after controlling for age, body mass index, smoking, self-perceived socioeconomic ranking, and sex in a population of Chinese men and women over 65 years old (166). It is unclear why these studies did not show similar differences in telomere length as in other studies, though it is possible that the exercise stimulus was not significant enough to elicit a change. Further research is required to determine the amount, type, frequency, etc. of exercise necessary to elicit these changes.

Few studies have addressed changes in telomere-binding and telomere-related proteins in response to exercise. Three weeks of wheel running resulted in increased telomerase expression in the thoracic aorta of C57 mice as well as increased numbers of circulating mononuclear cells, increased Trf2 and Ku70 expression, and a decrease in apoptosis as demonstrated by decreased in Chk2, p16, and p53 expression (161). In the same study, a group of endurance athletes were studied to validate the results in humans. In leukocytes, expression of telomerase and telomere stabilizing proteins (e.g., Trf2, Ku70) were higher than in non-athletes, and there was a decreased expression of cell cycle inhibitors, indicating similar changes in humans and mice in response to exercise. In another study by the same group, three weeks of wheel running increased telomerase enzyme expression and protein expression of TRF2 and TERT in mouse myocardium, as

well as decreased the mRNA expression of apoptotic proteins Chk2, p53, and p16 (162). In mice that ran for 6 months, similar changes (higher protein expression of TRF2 and TERT and decreased mRNA expression of Chk2, p53, and p16) occurred and all exercise-induced changes were abrogated in Terc and eNOS knockout mice (162).

Despite the numerous general health benefits of exercise, it has been suggested that acute, strenuous exercise may actually induce oxidative stress due to increased levels of ROS and reactive nitrogen species (RNS) (91, 119). Oxidative alteration of lipids, proteins, and nucleic acids occurs as a result of excessive ROS accumulation, and both acute exercise and chronic exercise training result in alterations in antioxidant levels and DNA repair gene expression (141). Niess and colleagues (113) showed DNA damage in half marathon runners 24 hours after the race. In another study of treadmill running to exhaustion, some subjects had DNA damage but returned to baseline after 72 hours post exercise (68). A study of eight subjects showed no change in amount of DNA damage immediately post or three hours after two and a half hours of treadmill running at 75 percent of VO2max. Results of studies of DNA damage in response to competitive exercise were mixed as well (129), though it is clear that at least in some instances, exercise does result in ROS production leading to DNA damage; however, in none of the cases was the damage persistent (129). It may be that very low or high levels of exercise result in elevated oxidative stress; this explanation seems plausible to explain the "U-shaped" curve of the Ludlow et al. study (97) where moderately active subjects had the longest telomeres, as well as the Collins and colleagues paper (39) where athletes with FAMS (and presumably high ROS levels) had short telomeres.

Telomeres and Diet

Telomeres have been studied even less in regards to diet than physical activity. Increased body mass is related to shorter white blood cell telomere length in humans (33, 108), while calorie restriction is known to increase lifespan across species (128). Caloric restriction has also been shown to attenuate age-associated telomere shortening in the lens epithelium of aged rats (122). Poor eating behavior is related to increased biochemical stressors (inflammation and oxidative stress), which in turn lead to increased abdominal adiposity (99, 136). The increased fat storage leads to insulin insensitivity, which imparts even greater increases of inflammation and free radical production. A vicious cycle ensues, and can lead to premature aging and associated diseases, since oxidative stress and inflammation are associated with shorter telomere length (46).

Several studies have shown that prenatal nutrition impacts levels of oxidative stress throughout the lifespan and may also affect longevity. In rats, maternal diet was associated with offspring's aortic telomere length as well as oxidative stress levels and antioxidant activity (152). Pregnant rats that received a low protein diet had offspring with improved insulin sensitivity and higher antioxidant levels compared to the offspring that underwent in utero growth restriction (102). The low protein diet offspring also lived longer than their counterparts whose mothers had restricted diets during pregnancy (87).

In baboons, seven weeks of high-fat diet resulted in decreased replicative potential of endothelial cells without change in telomere length (147). Another study demonstrated that processed meat, known to contain high levels of sodium, was the only dietary predictor of telomere length measured by T/S ratio (112). In both pre and post-menopausal women, high levels of dietary restriction, which is associated with higher

levels of cortisol, weight gain, and perceived stress, was correlated to short telomeres compared to women with lower DR, independent of BMI, age, and smoking (79). Mirabello and colleagues (108) reported that prostate cancer patients with healthy lifestyle habits including lower body mass index (BMI), higher exercise levels, healthy diets, and lack of tobacco use were associated with longer leukocyte telomere lengths. In women, leukocyte telomere length was positively associated with dietary fiber intake and was inversely related to linoleic acid (a polyunsaturated fat) intake as was waist circumference (28). Overall, it appears that poor diet resulting in higher levels of oxidative stress may be a risk factor for accelerated telomere shortening and cellular aging (50).

Telomeres in Skeletal Muscle

Though skeletal muscle is a post-mitotic tissue, it is not a stable tissue due to the fact that satellite cells are recruited as needed for the muscle to adapt to various activities (74). Muscular strength and endurance decline with age, and in marathon runners and weight lifters, the decline begins approximately at age 40 yr (55). The size of type II muscle fibers is diminished in older individuals and limb muscles are between 25 and 35 percent smaller in older men and women compared to younger sex-matched controls (93). Along with these changes, muscle satellite cell content has been reported to decrease after age 70 yr in some studies in humans (76, 130, 157) but not in others (123, 137).

Decreases in skeletal muscle telomere length have been reported in endurance athletes suffering from a condition of chronic fatigue (39), in mdx mice (which exhibit symptoms of muscular dystrophy due to lack of the protein dystrophin) compared to wild

type mice (98), and in aged adults compared to younger controls (45). It may be that the degree of oxidative stress rather than biological age or intensity and amount of exercise training predicts skeletal muscle telomere length (74). For example, short telomeres have been shown in people with diseases associated with high levels of oxidative stress including diabetes mellitus (143) and heart failure (46).

Other Predictors of Telomere Activity

Other predictors of telomere length include socioeconomic status (SES), cigarette smoking, stress levels, and sex. A study in female twins showed that low SES, smoking, obesity, and low physical activity were all related to shorter white blood cell telomere length (33). Interestingly, Woo and colleagues (166) showed that older Chinese men living in Hong Kong who had higher self-rated SES actually had shorter telomeres than their lower SES counterparts. Paternal age at the offspring's birth was also related to telomere length; the older the father, the longer the telomere, though the mechanism is unknown (83). Chronic stress has been related to risk factors for both cardiovascular disease and decreased immunity (104, 145) and is also related to telomere length and telomere biology. Epel and colleagues (51) recruited pre-menopausal women who were mothers to a healthy or a chronically-ill child and measured chronic stress levels (self-evaluated), with the assumption that the mothers of the chronically ill children would have higher perceived stress levels. In fact, degree of perceived stress and chronicity of stress were both associated to higher oxidative stress levels, shorter telomere length, and lower telomerase activity, all stressors that may promote onset of age-related illness (51). Chronic stress promotes aging and possibly age-associated diseases via oxidative stress,

alterations in eating behavior resulting in insulin resistance and inflammation, and an imbalance in anabolic and catabolic hormones (50).

Selective Breeding Experiments

In 1998, Dr. Ted Garland's laboratory at the University of California, Riverside published a paper describing a breeding line of mice selected for high voluntary wheel running behavior (151). Today, over 50 generations of mice have been bred as a part of that study, and selected mice run, on average, 2.7 times greater distance per day than control bred mice (57, 135). These lines of mice have provided the opportunity for dozens of studies of physical activity, in particular the relationship between wheel running performance and many dependent variables. Running speed and VO2max during voluntary exercise was significantly higher in selected mice compared to controls, though maximal running speeds were not different (131, 134). Leptin levels were significantly lower in the selected mice compared to controls (59), and maximal oxygen consumption was evaluated in the selected and control mice at various levels of PO_2 (133). Ventricle size was a positive predictor of VO_2max at three different PO_2 levels, and blood hemoglobin concentration predicted VO_2max (positively) in S lines but negatively in control lines (133).

Vaanholt and colleagues (154) put the mice on a high fat diet, and the mice, with the exception of selected females, reduced food intake and increased body fat mass and plasma leptin levels. Selected females consumed more calories without affecting body mass or plasma leptin levels. They also had higher daily energy expenditure (DEE), elevated spontaneous cage activity (seven-fold higher than controls) and higher resting metabolic rate (RMR) on the HFD. There was no difference in anti-oxidant activity

though daily energy expenditure was positively associated with antioxidant capacity (154). In a study of energy costs of running in these mice, selection resulted in reduced energy costs of running, and running distances increased 50%-90% in selected lines (132). Selection also resulted in a 23% increase in daily energy expenditure (DEE) in males and a 6% increase in females after controlling for body mass, and the cost of increased running was different between males and females because selected females evolved by running faster without much change in total running time, while selected males spent 40% more time running than control males. Numerous other studied have been conducted on these mice as well.

However, the mice in these studies were never subjected to more than 6 days of wheel running; they were only *selected* for high voluntary wheel running rather than actually trained (with the exception of one study in generation 16 mice, which were trained). In 2009, Meek et al. for the first time described the effects of diet and exercise training on these mice (105). Male mice were fed either a Western (42 percent kJ from fat) or regular (14 percent kJ from fat) diet and were allowed access to a running wheel or kept in a standard cage for a total of eight weeks. The Western diet did not change the amount that the control bred mice ran, but the selected mice on the Western diet ran up to 75 percent more than those on the regular diet, and the difference was mostly seen in an increase in time spent running. In another study where half of the studied mice were given wheel access, life expectancy was evaluated. Median life expectancy was greater for selected females than for control females, and sedentary males had the highest early adult mortality (19). Active control males had the lowest mortality across the lifespan (until the end of life) and active selected males had the lowest median life expectancy.

The current study is only one of only a handful to evaluate the effects of physical activity and selection (diet was included in the current study as well) for wheel running on dependent variables. Further characterization of these animals and their phenotypes is necessary to evaluate the combined effects of these variables on evolution patterns and in this case, cellular responses as measured by telomere lengthand mRNA levels of telomere binding proteins.

Summary

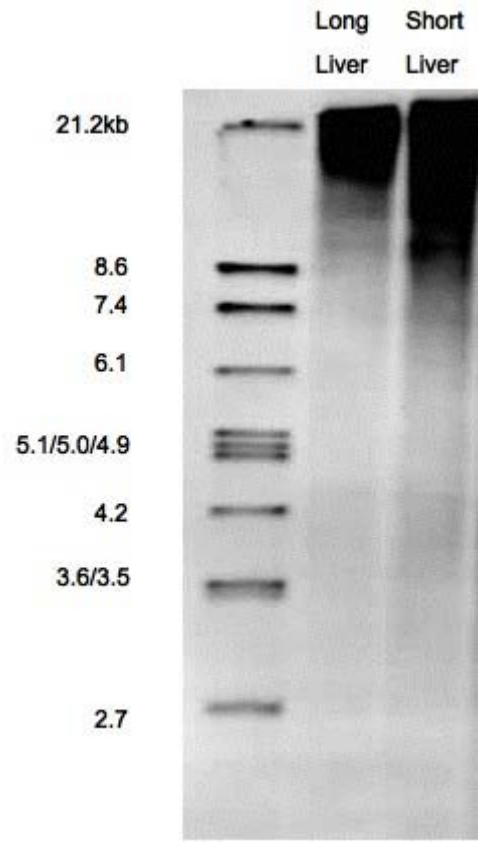
Lifestyle factors including physical activity levels, diet, smoking, and chronic stress as well as disease and age affect telomere length and the associated proteins. Healthy lifestyle may slow cellular aging and have positive effects on treatment of disease, disease risk (decrease), and/or prevent or delay side effects of increasing age.

However, telomere length alone may not be reliable as a marker of cellular health or an indicator of the cell's response to environmental stimuli. Werner and colleagues (162) found altered telomerase activity, expression of Trf2, p53, TERT, and Ku70 in mice provided a running wheel for 21 days despite no differences in telomere length. In baboons, seven weeks of high-fat diet resulted in decreased replicative potential of endothelial cells with no change in telomere length (147). And as previously mentioned, a human study of leukocyte telomere length in coronary artery disease patients showed that telomere length increased in some individuals, remained constant in others, and in still others, decreased at follow-up five years later (54). Another study showed that six months of aerobic exercise training did not result in telomere length changes, though antioxidant enzyme levels did rise in the exercising subjects (148). We suggest that other variables, including telomerase activity, gene and protein expression of telomere-

stabilizing proteins, etc must be measured in addition to length in order to understand the full picture of telomere activity in response to environmental stimuli.

There is no clear consensus of the effects of diet and/or exercise on telomere biology and cellular health and aging. Healthy lifestyle including regular exercise and good dietary habits may be helpful in preventing, delaying, or reducing cellular aging. The present thesis attempts to address the question of the combined effects of diet, physical activity, and selection for high voluntary wheel running on telomere dynamics. We measured telomere length in addition to mRNA levels of shelterin proteins and the prop-apoptotic transcription factor p53. In addition, we sought to determine whether the interventions resulted in clear mitochondrial adaptations to exercise. The results of this study will expand the literature on the effects of diet and exercise, alone or in combination, on these telomere-related outcomes.

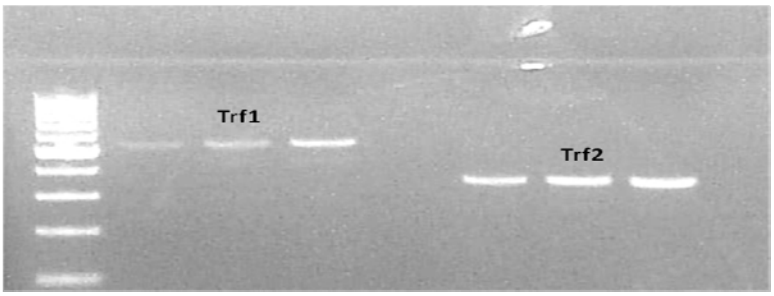
Appendix A:



Southern blot for validation of real-time PCR method for measurement of T/S ratio. See Table below for kilobase measurements (Southern blot) and T/S ratios (PCR technique) for each sample.

	Kilobases (measured by Southern blot)	**T/S Ratio (measured by real-time PCR assay)**
Liver Short	20.2	1.86
Liver Long	8.8	0.26

Appendix B:



Trf1 and Trf2 linearity checks. Samples on the left side of the gel are Trf1 and samples on the right are Trf2. For both genes, a two-fold difference in amount of cDNA used in the assay was used in each consecutive sample (0.5, 1.0, and 2.0 uL of cDNA were used). For Trf1, the fold differences are 2 units between each consecutive sample, and for Trf2, the fold differences are 1.2 units.

Appendix C:

Representative gel pictures of Trf1, Trf2, and Gapdh for the eight intervention groups. Group numbers correspond to those reported in the Results section.

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