DISSERTATION

PROTEIN SYNTHESIS IN SLOWED AGING: INSIGHTS INTO SHARED CHARACTERISTICS OF LONG-LIVED MOUSE MODELS

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ABSTRACT

PROTEIN SYNTHESIS IN SLOWED AGING: INSIGHTS INTO SHARED CHARACTERISTICS OF LONG-LIVED MOUSE MODELS

The following dissertation describes a series of experiments with the overall aim to understand the role that changes in protein synthesis have in slowed aging. The specific aims of the three sets of experiments were 1) to determine if chronic administration of the mTORC1 inhibitor rapamycin to mice increases proteostatic mechanisms in skeletal muscle, heart, and liver; 2) to determine if an underdeveloped anterior pituitary, caused by deletion of the *Pit-1* gene in mice, increases proteostatic mechanisms in skeletal muscle, heart, and liver of long-lived Snell dwarf mice; 3) and to determine if transient nutrient restriction during the suckling period in mice (i.e. crowded litter), increases proteostatic mechanisms in skeletal muscle, heart, and liver later in life. In Experiment #1 we found that mitochondrial proteins were preferentially synthesized in skeletal muscle and that global protein synthesis in the heart was maintained despite reduced cellular proliferation and mTORC1 activity in mice fed rapamycin compared to normal diet controls. Originally we determined that these data were indicative of an improved somatic maintenance of skeletal muscle mitochondria and the heart proteome. Since we could not account for changes to other energetic processes (e.g. metabolism), we reasoned that our data was more consistent with proteostasis, a component of somatic maintenance. In Experiment #2 we developed a novel method for assessing proteostasis and determined that Snell dwarf mice had an increase in proteostatic mechanisms across sub-cellular fractions within skeletal muscle and heart compared control mice, despite differential rates of protein synthesis in the face of decreased mTORC1. Together with our previous investigations into rapamycin fed and caloric restriction models of long-life we concluded that increased proteostatic mechanisms may be a shared characteristic of models of slowed aging. In

Experiment #3 we demonstrate that the crowded litter mouse transitions from growth to maintenance as it ages. Furthermore, in the crowded litter mouse, we demonstrate that proteostasis is not dependent upon decreased mTORC1. Our results indicate that decreased mTORC1 does not necessarily correlate to decreases in protein synthesis across all sub-cellular fractions. Discerning which proteins and the mechanism(s) of how specific proteins can be preferentially synthesized despite decreases in protein synthesis in other fractions and decreased mTORC1, may give further insight into characteristics of slowed aging. Further, we demonstrate that increases in proteostasic mechanisms are a shared characteristic of multiple unique models of slowed aging and therefore, provides a basis for future work aimed at slowing the aging process.

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CHAPTER I – INTRODUCTION/EXPERIMENTAL AIMS

The accumulation of damaged proteins over time, particularly of mitochondrial proteins, has been postulated to propagate the development of the aging phenotype (1-4). Age-dependent reductions in protein turnover are estimated to range from 20-80% (5), resulting in damaged proteins making up 20-50% of the total protein pool (1). In tissues that have limited proliferative potential, such as skeletal muscle, heart, and liver, protein homeostasis (i.e. proteostasis) is a key component of slowed, or healthy aging (4, 6-10). Therefore, interventions that promote proteostasic mechanisms could be an effective means to slow the aging process.

The overall objective of our projects was to understand how changes in protein synthesis contribute to slowed aging. Previous work in our lab had shown that life-long caloric restriction in mice, a potent and reproducible means to extend lifespan, did not change mitochondrial biogenesis, contrary to what had been suggested by others (11). Further, we showed that protein synthesis in other (non-mitochondrial) sub-cellular fractions was maintained during caloric restriction despite decreased cellular proliferation and mTORC1 activity (11, 12). The mTORC1-signaling pathway is a central regulator of protein turnover and cell cycling (13), and its inhibition is a characteristic of several models of slowed aging. Under the premise that decreased protein turnover is causative to the aging phenotype, our findings in the caloric restriction model were unexpected. A reduction in cellular proliferation suggests that energy normally devoted to cell cycling is conserved. This conserved energy may provide for the preferential synthesis of key proteins (e.g. mitochondrial proteins) during the energetic stress of caloric restriction despite decreased mTORC1, promoting slowed aging.

To expand upon our findings in the caloric restriction model, we began our studies in mice treated with rapamycin, an mTORC1 inhibitor and potential caloric restriction mimetic that extends lifespan (14). Interpreting our results as a reallocation of energetic resources towards

maintenance required the assumption that energetic contributions from other cellular processes (e.g. metabolism) remained unchanged (15, 16). Since we did not account for energetic changes in other processes within caloric restriction and rapamycin models we thought it prudent to limit further discussion to processes our data better reflected. Through further reflection and experimental insight we reasoned that our data were consistent with proteostasis (6).

Intracellular enzymatic capacity for protein repair is low so damaged proteins must be degraded then replaced through the making of new proteins (17-19). Growth requires protein synthesis to increase alongside cellular proliferation (20, 21) or recruitment of new DNA from neighboring satellite cells (22-25). Cells constantly integrate intra- and extracellular signals to coordinate the synthesis of new proteins in order to adapt to the current stressor/stimuli environment. In contrast to growth, cellular adaptation to the current environment is not necessarily dependent upon cell proliferation. Taken together, protein synthesis both equips newly made cells as well as regulates cellular proteostasis. Therefore, cellular proliferation must be accounted for along with protein synthesis in order to determine if newly made proteins are dedicated to making new cells or maintaining existing cellular structures. As a result of this new interpretation of our data, the terminology used in the subsequent manuscripts reflects this evolution in our thought process.

Using proteostasis as the new framework for interpreting our findings, we next investigated the longest-lived mouse model of slowed aging, the Snell dwarf mouse (26). Mutation of the *Pit-1* locus in Snell dwarf mice causes underdeveloped anterior pituitary glands, resulting in pronounced life-long reductions in hormonal growth factors (e.g. such as growth hormone and insulin-like growth factor-1) and mTORC1 (27, 28). Life-long reductions in growth factors and related signaling differ from caloric restriction and rapamycin models where reductions in growth signaling do not occur until later in life.

Finally, we investigated a presumed epigenetic model of long-life, the crowded litter mouse, that is characterized as having an increase in lifespan due to being nutrient restricted during the suckling period alone (29). The crowded litter model allows for comparisons of how a transient stress changes protein synthesis later in life compared to our other models where slowed aging is imparted through chronic interventions. We expand upon our previous findings in other models by also assessing crowded litter mice before they become weight stable, allowing insight into changes associated with potential restricted or catch-up growth.

In summary, the series of studies presented here began as an investigation into the role that changes in protein synthesis, particularly of mitochondrial proteins, have in slowed aging. In the end we arrived at the idea that increased proteostatic mechanisms are a shared characteristic of long-lived models. We characterize proteostasis in a pharmacological model (rapamycin) of long-life, and propose that preferential synthesis of key proteins, such as mitochondrial proteins, may be causative to slowed aging. Further, in a genetic model of long-life (Snell dwarf) we develop novel means to analyze our data that we believe is indicative of proteostasis and show that proteostatic mechanisms are increased across sub-cellular fractions. Lastly, we assess proteostasis in a presumed epigenetic model of long-life (crowded litter) and show that a transient intervention that increases lifespan also increases proteostatic mechanisms later in life, and we propose that proteostasis is not dependent on mTORC1 inhibition.

Overall Hypothesis: Proteostatic mechanisms will be increased in tissues of varying mitotic capacity across pharmacological, genetic, and presumed epigenetic models of long-life.

Specific Aim for Experiment 1: To determine if chronic administration of the mTORC1 inhibitor rapamycin to mice increases proteostatic mechanisms in skeletal muscle, heart, and liver.

Specific Aim for Experiment 2: To determine if an underdeveloped anterior pituitary, due to deletion of the *Pit-1* gene in mice, increases proteostatic mechanisms in skeletal muscle, heart, and liver of long-lived Snell dwarf mice.

Specific Aim for Experiment 3: To determine if transient nutrient restriction during the suckling period in mice, increases proteostastic mechanisms in skeletal muscle, heart, and liver later in life.

CHAPTER II – MANUSCRIPT I[1]

Assessment of mitochondrial biogenesis and mTORC1 signaling during chronic rapamycin feeding in male and female mice

Summary

Chronic inhibition of the protein synthesis regulator mTORC1 through rapamycin extends lifespan in mice, with longer extension in females than in males. Whether rapamycin treatment inhibits protein synthesis or if it does so differently between sexes has not been examined. UM-HET3 mice were fed a control (CON) or rapamycin-supplemented (Rap) diet for 12 weeks. Protein synthesis in mixed, cytosolic (cyto), and mitochondrial (mito) fractions, as well as DNA synthesis and mTORC1-signaling were determined in skeletal muscle, heart, and liver. In both sexes, mito protein synthesis was maintained in skeletal muscle from Rap despite decreases in mixed and cyto fractions, DNA synthesis, and rpS6 phosphorylation. In the heart, no change in protein synthesis occurred, despite decreased DNA synthesis. In heart and liver, Rap males were more sensitive to mTORC1 inhibition than Rap females. In conclusion, we show changes in protein synthesis and mTORC1 signaling that differ by sex and tissue.

Introduction

The protein turnover theory of aging states that aging is the result of accumulated protein damage resulting from decreased protein turnover (2). In aging, damaged proteins are estimated to make up 20-50% of the total protein pool (1) and this accumulation in damaged proteins is accompanied by a 20-80% decrease in protein turnover (5). In particular, damage to mitochondrial proteins increases with age [reviewed in (30)], and one experiment suggests that

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the synthesis of mitochondrial proteins in skeletal muscle decreases by middle age in humans (3). Thus, interventions that prevent age-related changes in protein stability and turnover could be used to slow aging.

We have recently demonstrated in mice that life-long caloric restriction (CR), a well documented means of lifespan extension in a variety of species, maintains the synthesis of mitochondrial proteins (a true measure of mitochondrial biogenesis (31)) in skeletal muscle, heart, and liver (11). In addition, DNA synthesis (indicative of cellular proliferation) decreased in heart and liver across ages (11). The simultaneous maintenance of mitochondrial biogenesis with a decrease in cellular proliferation, both energetically expensive processes, during limited energy availability (i.e. CR) suggests an increase in somatic maintenance. Thus, somatic maintenance of mitochondria could be a part of how the lifespan extension effect of CR is mediated. Although our long-term studies demonstrated maintained synthesis of mitochondrial proteins in heart and skeletal muscle during CR (11), subsequent acute (4hrs) labeling measurements of life-long CR mice show that synthesis rates were decreased in liver with CR (12). These additional findings highlight the importance of examining tissue specificity when assessing potential aging outcomes.

Protein synthesis is modulated, in part, by the highly conserved mechanistic (mammalian) target of rapamycin (mTOR) signaling pathway [reviewed in (32)]. The mTOR kinase is a protein that forms two distinct complexes, mTORC1 and mTORC2, with mTORC1 being essential for protein synthesis (33). mTORC1 is a potent cellular nutrient sensor and initiates the synthesis of new proteins only in the presence of adequate nutrients (34). mTORC1 is also involved in the regulation of cell cycling, as evident by the upregulation of mTORC1 in many cancers [reviewed in (13)]. Therefore, mTORC1 signaling is a nexus of control for both protein synthesis and cell cycling.

Inhibition of mTORC1 increases lifespan in a variety of non-mammalian species (35-37). Chronic administration of the mTORC1 inhibitor rapamycin has been shown to extend mean

and maximal lifespan in genetically heterogeneous mice (14), with a greater effect in females compared to males (14, 38), however it remains unknown what factors may be contributing to lifespan extension. Rapamycin acts through binding to its intracellular protein target, the FK506 binding protein 1A (FKBP12) (39). This drug-receptor complex destabilizes the raptor-mTOR interaction in the rapamycin sensitive mTORC1 (40), inhibiting its kinase activity leading to reduced protein synthesis (41, 42) and suppressed cellular proliferation at the G1 phase of the cell cycle (43). Though rapamycin is expected to decrease protein synthesis because of its inhibition of mTORC1, this has only been demonstrated *in vitro* (41, 42). Furthermore mTORC1 signaling varies between sexes (44), making it possible that male and female mice exhibit varying responses to mTOR inhibition through rapamycin and thus variable protein and DNA synthesis outcomes. Therefore, assessing sex-specific changes in protein and DNA synthesis may help to explain why the lifespan extension effect of chronic rapamycin feeding is more pronounced in females compared to males.

Given the rapamycins's well-characterized inhibition of mTOR signaling by rapamycin, it is imperative that chronic effects are analyzed to determine how these essential processes governed by mTOR are altered so as to gain insight into how rapamycin extends lifespan. In principle, reduced protein synthesis and cellular proliferation with rapamycin treatment could each contribute to the drug's lifespan extension effects. A decrease in global protein synthesis and cellular proliferation with chronic rapamycin feeding could lead to significant energy savings that could be allocated to somatic maintenance of key proteins, including mitochondrial proteins, thus increasing lifespan. It is yet to be determined if mitochondrial biogenesis is maintained in mice chronically fed rapamycin as observed during CR (11).

The goal of the current study was to measure tissue-specific protein and DNA synthesis simultaneously during chronic rapamycin feeding to determine what tissue(s) and/or fractions are preferentially maintained. We hypothesized that during chronic rapamycin feeding, mitochondrial biogenesis would be preferentially maintained compared to other protein fractions

and that DNA synthesis would decrease. We further hypothesized that protein and DNA synthesis responses to rapamycin feeding will differ between tissues and sexes. Understanding how chronic rapamycin treatment affects tissue specific protein synthesis, mitochondrial biogenesis, and cellular proliferation in a variety of tissues and in both sexes, could lead to potential treatments targeting somatic maintenance of mitochondrial proteins to promote slowed aging.

Materials and Methods

Animals

All procedures and conditions at the animal care facility meet or exceed the standards for animal housing as described in the Animal Welfare Act regulations, the Guide for the Care and Use of Laboratory Animals and the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching and were approved by the University of Michigan Committee on Use and Care of Animals. We used the genetically heterogeneous offspring of CB6F1 female and C3D2F1 male mice (UM-HET3), which have been well characterized in their response to chronic rapamycin feeding (14).

Routine veterinary care was provided by the Biomedical Science Research Center, Animal Research Core staff at the University of Michigan. UM-HET3 mice were fed chow mixed with rapamycin (n=12) at 14 mg/kg food (equivalent to 2.24 mg of rapamycin/kg body weight/day) or normal chow (n=12) in accordance with the original study describing lifespan extension (14). Diets were administered beginning at 4 mo of age. After 8 wks on the control (CON) or rapamycin (Rap) diet mice received $^{2}H_2O$ for 4 wks. After the labeling period and following an overnight fast, mice were anesthetized using a CO_2 overdose according to the AVMA Guidelines on Euthanasia. Complete loss of pedal reflexes was confirmed before tissues were collected. The posterior aspect of the distal hind limbs [(gastrocnemius, soleus, and

plantaris), mixed skeletal muscle], heart, liver, bone marrow from the tibia, and blood via cardiac puncture were taken and immediately frozen in liquid nitrogen for later analysis.

Labeled water

The use of ^2H_2O (heavy water) allows simultaneous assessment of multiple synthetic processes. Here, we assessed the synthesis of mitochondrial (mito), cytosolic (cyto), and mixed (mixed) protein and DNA in skeletal muscle, heart, and liver according to procedures previously described (11, 12, 45, 46). After 8 wks of the dietary intervention the animals received an intraperitoneal injection of 99% enriched ^2H_2O calculated to enrich the body water pool (assumed 60% of body weight) to 8% (11, 46). Animals were then allowed to drink *ad libitum* water enriched to 4% for the next 4 wks.

Tissue isolation - Tissue from skeletal muscle, heart and liver was fractionated according to our previously published procedures (11, 12, 47). Tissue (25-60 mg) was homogenized 1:10 in isolation buffer (100 mM KCl, 40 mM Tris HCl, 10 mM Tris Base, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, pH=7.5) with phosphatase and protease inhibitors (HALT, Thermo Scientific, Rockford, IL, USA) using a bead homogenizer (Next Advance Inc, Averill Park, NY, USA). After homogenization, the samples were centrifuged at 800 g for 10 min at 4 °C (Eppendorf, model 5415R, Hauppauge, NY, USA). The resulting pellet (mixed fraction, Mix) was washed in EtOH and H_2O . The supernatant from the 800 g spin was centrifuged at 9000 g for 10 min. 400 μl of the resulting supernatant was used as our cytosolic fraction (Cyto) and the remainder was set aside for Western blotting. The pellet from the 9000 g spin was resuspended in a second isolation buffer (100 mM KCl, 10 mM Tris HCl, 10 mM Tris Base, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.02 mM ATP, and 1.5% BSA, pH = 7.5) and centrifuged at 8000 g for 10 min at 4 °C. The supernatant was discarded, the pellet was resuspended in buffer two, and centrifuged at 6000 g

for 10 min at 4 °C. The supernatant was discarded and the remaining mitochondrial pellet was washed with H_2O. The supernatant from the 9000 g spin (Cyto) was mixed with 400 μl 14% sulfosalicyclic acid, vortexed, and centrifuged at 16,000 g for 10 min. The resulting supernatant was discarded and the pellet washed in EtOH and H_2O. Finally, 250 μl 1 M NaOH was added to all fraction pellets then placed on heat block at 50 °C and 900 rpm for 15 min.

Preparation of analytes for mass spectrometric analyses - Protein was hydrolyzed by incubation in 6 N HCl at 120 °C for 24 hrs. The hydrolysates were ion-exchanged, dried under vacuum, and then suspended in 1 mL molecular biology grade H_2O. Approximately 500 μl of suspended samples were then derivitized by adding 500 μl acetonitrile, 50 μl 1 M K_2HPO_4 (pH = 11), and 20 μl of pentafluorobenzyl bromide (Pierce Scientific, Rockford, IL, USA), and the sealed mixture was incubated at 100 °C for 1 hr. Derivatives were extracted into ethyl acetate and the organic layer was removed and dried by N_2 followed by vacuum centrifugation. Samples were reconstituted in 1 ml ethyl acetate then analyzed.

For the plasma body water concentration analysis 125 μl of plasma was placed into the inner well of o-ring screw on cap and placed inverted on a heating block overnight at 80 °C. Next, 2 μl of 10 M NaOH and 20 μl of acetone was added to all samples and 20 μl 0-20% D_2O standards and capped immediately. Samples were vortexed at low speed and sat overnight at room temperature. Samples were extracted with addition of 200 μl hexane and the organic layer transferred through anhydrous Na_2SO_4 into Gas Chromotography (GC) vials and analyzed via Electron Ionization (EI) mode using a DB-17MS column (Agilent Technologies Inc, Santa Clara, CA, USA).

GC-MS analysis of derivatized amino acids - Using negative chemical ionization (NCI), derivatized amino acids were analyzed on an Agilent 7890A GC coupled to an Agilent 5975C

MS using an Agilent DB-5MS gas chromatograph column (30 m x 0.25 mm x 0.25 micron). Samples were injected in 1 μl volumes using splitless mode (inlet temp 220°C). Helium was the carrier and methane was the reagent gas. The mass-to-charge ratios of 448, 449, and 450 were monitored for the pentafluorobenzyl-*N*,*N*-di(pentafluorobenzyl)alaninate derivative. In all cases, these mass-to-charge ratios represented the primary daughter ions that included all of the original hydrocarbon bonds from the given amino acid. ^{2}H enrichment was calculated as described previously (48). The newly synthesized fraction (f) of muscle proteins was calculated from the true precursor enrichment (p) using plasma analyzed for $^{2}H_2O$ enrichment then adjusted using MIDA (45). Protein synthesis was calculated as the change in enrichment of deuterium-labeled alanine (45) bound in muscle proteins over the entire labeling period and expressed as fraction new in 4 weeks.

DNA isolation - Total DNA (~8 μg) was extracted from approximately 15 mg tissue (QiAamp DNA mini kit Qiagen, Valencia, CA, USA). The deoxyadenosine fraction was separated and analyzed for deuterium content by GC-MS as previously described (11, 45, 49). DNA from bone marrow was isolated by extracting ~300 mg from the bone marrow suspension. Bone marrow suspension was centrifuged for 10 min at 2000 g. The pellet was treated the same as the tissue DNA isolation.

DNA synthesis measurement - Determination of ^{2}H incorporation into purine deoxyribose (dR) of DNA was performed as described previously (11, 45, 49). Briefly, isolated DNA was hydrolyzed overnight at 37 °C with nuclease S1 and potato acid phosphatase. Hydrolysates were reacted with pentafluorobenzyl hydroxylamine and acetic acid and then acetylated with acetic anhydride and 1-methylimidazole. Dichloromethane extracts were dried, resuspended in ethyl acetate, and analyzed by GC/MS on a DB-17 column with NCI, using helium as carrier and methane as the reagent gas. The fractional molar isotope abundances at m/z 435 (M_0, mass isotopomer)

and 436 (M_1) of the pentafluorobenzyl triacetyl derivative of purine dR were quantified using ChemStation software. Excess fractional M_{+1} enrichment (EM_1) was calculated as:

$$EM_1 = \frac{(abundance\ m/z\ 436)_{sample}}{(abundance\ m/z\ 435.\ 436)_{sample}} - \frac{(abundance\ m/z\ 436)_{std}}{(abundance\ m/z\ 4345.\ 436)_{std}}$$

where sample and standard (std) refer to the analyzed sample and unenriched pentafluorobenzyl triacetyl purine dR derivative standard, respectively. The fractional new in 4 weeks was calculated by a comparison with bone marrow in the same animal, which represents an essentially fully turned over population of cells.

Western blotting

A portion of the Cyto fraction was used for western blot analysis. Protein concentration was determined using a bicinchoninic acid assay (Thermo Fisher, Rockford, IL, USA). Samples were diluted to the same concentration, boiled with Laemmli buffer, then 20 μg of protein was separated using 10 % SDS-PAGE at 125 V. Proteins were transferred at 4 °C (100 V for 75 min in 20 % w/v methanol, 0.02 % w/v SDS, 25 mM Tris Base, 192 mM glycine, pH=8.3) to nitrocellulose paper and incubated in 5 % Milk in Tris-buffered saline with Tween20 (TBST) for 1 hr. Antibodies were purchased from Cell Signaling Technologies (Boston, MA, USA; rpS6 phospho-Ser[240/244] #4858, rpS6 total #2217, 4E-BP1 phospho [Thr37/46] #9459, 4E-BP1 total #9452) or Santa Cruz Biotechnology (Santa Cruz, CA, USA; Actin #sc-1616). Blots were incubated overnight with primary antibodies diluted 1:1000, 1:2000 (Liver rpS6), or 1:250 (Actin). Blots were washed in TBST and incubated with anti-rabbit or anti-goat (Actin) HRP-conjugated secondary antibody diluted 1:5000 in 5 % Milk with subsequent chemiluminescence detection (West Dura; Pierce, Rockford, IL, USA). Images were captured and densitometry analyzed using a UVP Bioimaging system (Upland, CA, USA). Blots were probed for

phosphorylated proteins first, placed in stripping buffer (GM Biosciences, Rockville, MD, USA) for 5min, and then re-probed for total protein. Equal loading was verified using Ponceau-s staining as well as with actin. Due to undetectable rpS6 in skeletal muscle homogenate, a portion of the homogenate used for western blotting was acetone precipitated.

Statistics

Statistical analysis was performed using SAS (SAS Institute Inc, Cary, NC, USA) for data in Fig 1A & 2A and SPSS Statistics 20 (IBM Corporation, Armonk, NY, USA) for all subsequent data analysis. Data were assessed by two-way ANOVA. For pooled protein synthesis data in Fig 1A & 2A significant interaction effects were further analyzed via pairwise comparisons and least squares means estimate. Where applicable data were transformed to equalize variance. Significance was set at $p \leq 0.05$, and p values of < 0.10 are noted. Outliers were determined as values outside 2x standard deviation of the mean and were removed from analysis. Data is presented as means ± standard error of the mean (SEM).

Results

Protein synthesis

In skeletal muscle the main effects of fraction and treatment were significant ($p=0.048$ and $p<0.0001$, respectively) (Fig 1.1A). The interaction effect between fraction and treatment for skeletal muscle was $p=0.1$ (Fig. 1.1A), which we deemed worthy of further investigation via pair-wise comparisons. Pair-wise comparisons showed that Rap mixed and cyto fractions were significantly decreased compared to mixed and cyto from CON ($p=0.035$ and $p=0.017$, respectively), but that mito Rap was not significantly different compared to mito CON ($p=0.315$) (Fig. 1.1A). The least squares means estimate showed that the Rap vs. CON difference in mixed and cyto was significantly different ($p=0.004$ and $p=0.048$, respectively), however, the Rap vs. CON difference in mito was not significantly different ($p=0.167$). Finally, the effects of

chronic rapamycin feeding in skeletal muscle did not vary between sexes in any protein fraction (Fig. 1.1B-D). P-values are summarized in Table S1.1.

In heart, protein synthesis in Rap mice did not differ from CON in any fraction (Fig. 1.2A). There was no interaction effect between treatment and sex or a main effect of treatment between sexes in the heart (Fig. 1.2B-D). A significant sex effect was observed in the heart; females had significantly less mixed and mito protein synthesis compared to males (Fig. 1.2B & D). P-values are summarized in Table S1.2.

Protein turnover in the liver was complete after 4 weeks of deuterium labeling, making assessment of differences in CON and Rap not possible (data not shown).

DNA Synthesis

In skeletal muscle DNA synthesis was significantly decreased in Rap compared to CON independent of sex (Fig 1.3A). Of note, a trend towards a significant interaction effect for rapamycin and sex was observed ($p=0.083$) (Fig. 1.3A). DNA synthesis in heart was also significantly decreased in Rap compared to CON (Fig. 1.3B). There was significantly less DNA synthesis in the hearts of females compared to males (Fig. 1.3B). In the liver, a significant interaction effect was found for Rap and sex (Fig. 1.3C): DNA synthesis was decreased in Rap, but the effect was only observed for the males. Main effect means of the two-way ANOVA for DNA synthesis are found in Table S1.3.

mTORC1 signaling

The ratio of phosphorylated Ser235/236 rpS6 to total rpS6 (P:T) was significantly lower in skeletal muscle of Rap compared to CON independent of sex (Fig. 1.4A). Heart and liver displayed a significant interaction effect, between sex and treatment, for rpS6 (P:T) (Fig. 1.4B & C). For each of these two tissues, rpS6 phosphorylation in CON males was higher than seen in CON females, and Rap led to a decline in phosphorylation only in the male mice. Individual

assessment of phosphorylated and total rpS6 alongside their respective actin loading control is found in Fig S1.1. There was no difference in the ratio of phosphorylated Thr37/46 to total 4E-BP1 between Rap and CON in skeletal muscle, though a trend towards an effect of sex was observed (p=0.067; males higher than females) (Fig 1.5A). A significant interaction effect between sex and treatment was found for heart 4E-BP1: Rap had diminished 4E-BP1 (P:T) in heart tissue but only in the females (Fig. 1.5B). Furthermore, female mice had more 4E-BP1 than males (Fig 1.5B). For liver, no difference was found in 4E-BP1 (P:T) between Rap and CON, though females had significantly more 4E-BP1 (P:T) compared to males (Fig. 1.5C). Main effect means of the two-way ANOVA for rpS6 (P:T) and 4E-BP1 (P:T) are found in Table S1.4 and S1.5, respectively. Individual assessment of phosphorylated and total 4E-BP1 alongside their respective actin loading control is found in Fig. S1.2.

Discussion

Here we present a tissue and sex-specific assessment of protein synthesis in mixed, cyto, and mito fractions, along with DNA synthesis following 12 weeks of rapamycin feeding in mice. In skeletal muscle, chronic rapamycin feeding slows DNA synthesis and protein synthesis in mixed and cyto fractions but has no effect on mitochondrial protein synthesis (biogenesis). DNA synthesis was also inhibited in the heart but without any alteration in protein synthesis in any fraction. No sex differences were observed in response to chronic rapamycin feeding within skeletal muscle and heart. However, mTORC1-related signaling was differentially affected by chronic rapamycin feeding between sex and tissue. Collectively, we present evidence that sub-fraction and tissue-dependent maintenance of protein synthesis despite decreased cellular proliferation and mTORC1 signaling could be a distinguishing sex and tissue-dependent characteristic of chronic rapamycin-mediated lifespan extension.

Potential mechanism for rapamycin mediated lifespan extension

Chronic administration of rapamycin extends median and maximum lifespan in mice (14, 38) as well as maximal lifespan in *Drosophila melanogaster* (50) and yeast (35). In mice, chronic rapamycin feeding slows age-associated tissue degeneration (51), attenuates inflammation and stabilizes atherosclerotic plaques (52), protects against cognitive decline, and increases behaviors indicative of reduced anxiety-like symptoms (53). Therefore, chronic administration of rapamycin not only increases lifespan but also healthspan through pathways still to be elucidated in each of the affected tissues and cell types.

How chronic administration of rapamycin affects protein synthesis and cellular proliferation in various tissue types has not previously been explored in intact mice or other mammals. We have previously shown that life-long CR results in maintained mitochondrial biogenesis despite decreases in cellular proliferation and mTORC1-signaling in skeletal muscle, heart, and liver (11). A reduction in mTORC1 regulated global protein synthesis and cellular proliferation through rapamycin could result in a re-allocation of energetic resources. We hypothesized that this available energy could then be diverted towards somatic maintenance of mitochondrial proteins thus promoting longevity.

In the current study, 12 weeks of rapamycin feeding maintained mitochondrial protein synthesis, a true measure of mitochondrial biogenesis (31), in both skeletal muscle and heart of male and female mice, mirroring our previous study in life-long CR mice (11). Protein synthesis in the liver is not reported because the faster rate of protein turnover in the liver resulted in 100% new protein in our 4-week period. Therefore, we hypothesize that the maintenance of mitochondrial biogenesis is a common factor in long-lived models and should be evaluated further using additional tissues of varying mitotic capacity and models of longevity.

The maintenance of mitochondrial biogenesis with chronic rapamycin feeding should be considered in the context of the cell types contained in various tissues. Tissues that have reduced or lack of mitotic potential (i.e. heart and skeletal muscle, respectively) could be more

sensitive to accumulation in damaged proteins, potentially contributing to dysfunction and organismal senescence [reviewed in (54)]. In the current study, Rap had significantly reduced DNA synthesis in skeletal muscle and heart compared to CON; a similar change was seen in liver of male, but not female, animals. A decrease in cellular proliferation could suggest that DNA mutations are limited. Furthermore, other means may be needed to deal with an accumulation in damaged proteins over time. Autophagy, which is negatively regulated my mTOR (55) and recycles damaged organelles and proteins, may be increased with chronic rapamycin treatment within these tissues.

The similar decrease in the skeletal muscle and heart DNA synthesis is contrasted by decreased Mix and Cyto protein synthesis in skeletal muscle but no change in heart protein synthesis. These data suggests an energetic savings which in Rap mice may be directed toward somatic maintenance of the existing mitochondrial reticulum in skeletal muscle and the heart proteome. Thus the somatic maintenance of key proteins/tissues may be integral to chronic rapamycin-induced longevity.

Sexual dimorphism of lifespan extension

Rapamycin mediated lifespan extension is greater in females than males (14). Sexual dimorphic lifespan extension occurs in other long-lived models (26, 56, 57) but the reason(s) as to why is unclear. It could be that the mechanism through which longevity is increased in these models differs between sexes. Here, we show that chronic rapamycin decreased skeletal muscle protein synthesis in both male and female mice. Furthermore, in both sexes, rapamycin decreased DNA synthesis in both heart and skeletal muscle. In contrast, liver DNA synthesis was decreased only in males, suggesting that cellular proliferation in the liver of female mice is unaffected by rapamycin. Therefore, it is important to consider both sexes when assessing models of longevity as our current study has done.

Differences in mTORC1-related signaling between sexes could contribute to the greater extension in lifespan from rapamycin treatment in females. In rats, rpS6 is decreased in female EDL compared to males but rpS6 and 4E-BP1 is increased by 36mo of age (44). However, there is no sex difference in soleus 4E-BP1 (44). Therefore, we hypothesized that chronic rapamycin feeding may have sex and tissue-specific effects on mTORC1 signaling in mice. Phosphorylation of rpS6 in skeletal muscle was decreased in Rap compared to CON in both sexes. In heart and liver, however, rapamycin feeding diminished rpS6 phosphorylation only in males, a novel finding. Levels of phosphorylated rpS6 were higher in control males than in control females, consistent with data in rat EDL muscle (44). The rapamycin effect in males thus diminished rpS6 phosphorylation to the low levels already present in the control females. Chronic rapamycin feeding had quite different effects on 4E-BP1 phosphorylation. In skeletal muscle, unlike rpS6, there was no change in 4E-BP1 phosphorylation, which was also observed in the liver. Furthermore, a decrease in 4E-BP1 phosphorylation was seen only in the heart of female Rap. To our knowledge, this is the first *in vivo* report of sex and tissue-specific difference in the phosphorylation of rpS6 (heart and liver) and 4E-BP1 (heart) to chronic rapamycin feeding. Also, though rapamycin resistance has been reported for 4E-BP1 *in vitro* during long exposures (58, 59), this is the first report of *in vivo* rapamycin resistant 4E-BP1 phosphorylation varying by sex in the heart.

Furthermore, sex and tissue differences in one facet of mTORC1 signaling suggests the possibility that chronic rapamycin feeding alters other aspects of mTOR signaling (e.g. autophagy) in a sex and tissue specific manner as well. Therefore, additional investigations of the effects of chronic rapamycin on other mTOR regulated processes in relation to lifespan are warranted.

Cap-dependent translation of mRNA is dependent on the formation of the eIF4F initiation complex, which is regulated by mTORC1 by relieving 4E-BP1 inhibition of cap-dependent translation (60). The lack of a treatment effect in skeletal muscle and liver 4E-BP1 (P:T)

suggests the novel finding that mRNA within these two tissues are cap-dependently translated during chronic rapamycin feeding. It is difficult to assess what maintenance of 4E-BP1 (P:T) in the liver means in the current study due to the lack of liver protein synthesis data. However, we have recently demonstrated that short term CR results in decreased mitochondrial biogenesis within the liver (12). Thus, skeletal muscle and liver may preferentially synthesize different mRNAs via cap-dependent means during chronic suppression of mTORC1.

Perspective and conclusion

Inhibiting mTORC1 signaling via rapamycin is a robust and reproducible means of extending lifespan in a variety of species. Despite the well-known effects of rapamycin on inhibiting mTOR signaling, the effect on protein synthesis and cellular proliferation in various cellular compartments *in vivo* have not previously been described. Here, we demonstrate that in both male and female mice, chronic rapamycin feeding maintains mitochondrial biogenesis in skeletal muscle and global protein synthesis in the heart despite decreasing DNA synthesis in both tissues. Also, we demonstrate sex and tissue-specific differences exist in mTORC1 signaling as measured by phosphorylated rpS6 and 4E-BP1. Further investigation into how key proteins are preferentially translated in models of presumed mTOR-mediated longevity (e.g. dietary rapamycin & CR) may give additional insight into age-related sex and tissue dimorphisms. Also, these data are consistent with the idea of somatic maintenance as necessary for lifespan extension and that the mechanism of somatic maintenance may differ by sex and tissue. Therefore, we recommend that future studies that investigate long-lived models do so using multiple tissues and both sexes to draw conclusions.

Addendum to manuscript I

Since the publication of this manuscript, our interpretation of these data in the rapamycin model has changed. We now view our results as indicative of proteostasis, as evidenced

through the comparison of the rate of new proteins synthesized to the rate of cellular proliferation. These retrospective analyses, along with an expanded rationale, can be found in Fig 4.1 and Appendix II.

Author Contributions

J.C.D. collected data, analyzed and interpreted the data, and wrote the manuscript. F.F.P., L.M.B., and M.K.W. developed the methods and collected and analyzed the data. R.A.M. provided mouse tissues and helped to interpret the data and to write the manuscript. K.L.H, and B.F.M designed the study, directed the study, and analyzed and interpreted the data. All authors contributed critical feedback to the manuscript.

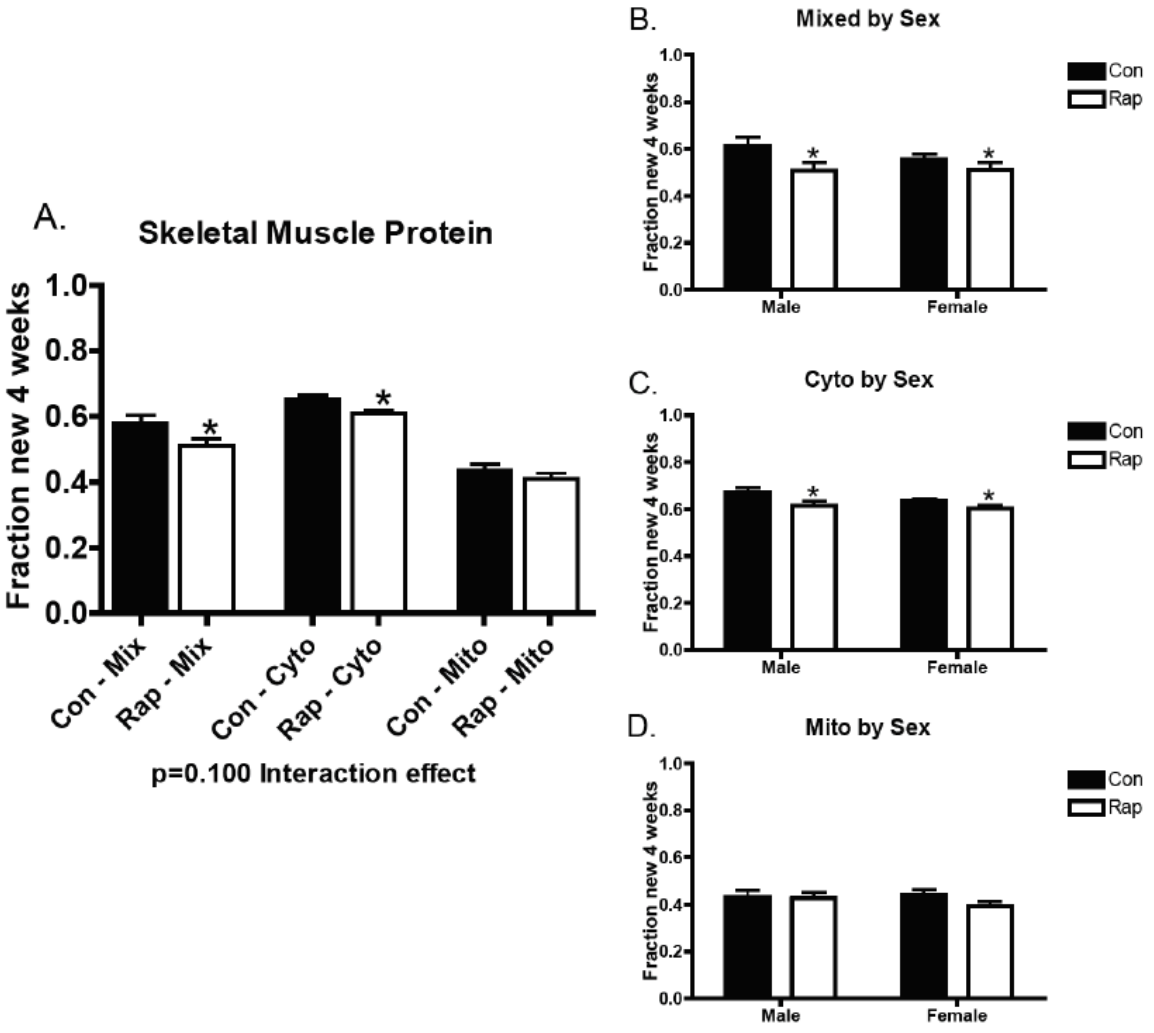

Figure 1.1. Skeletal muscle protein synthesis over 4 weeks in Rap and CON. Mixed and cyto fraction protein synthesis were decreased but mito protein synthesis was maintained in Rap mice compared to CON (A). Mixed protein synthesis was decreased in Rap compared to CON regardless of sex (B). Cyto protein synthesis was decreased in Rap compared to CON regardless of sex (C). Mito protein synthesis was maintained in Rap compared to CON regardless of sex (D). *n* = 12 per pooled group, 6 per sex/group. * = $p<0.05$ for Rap vs. CON.

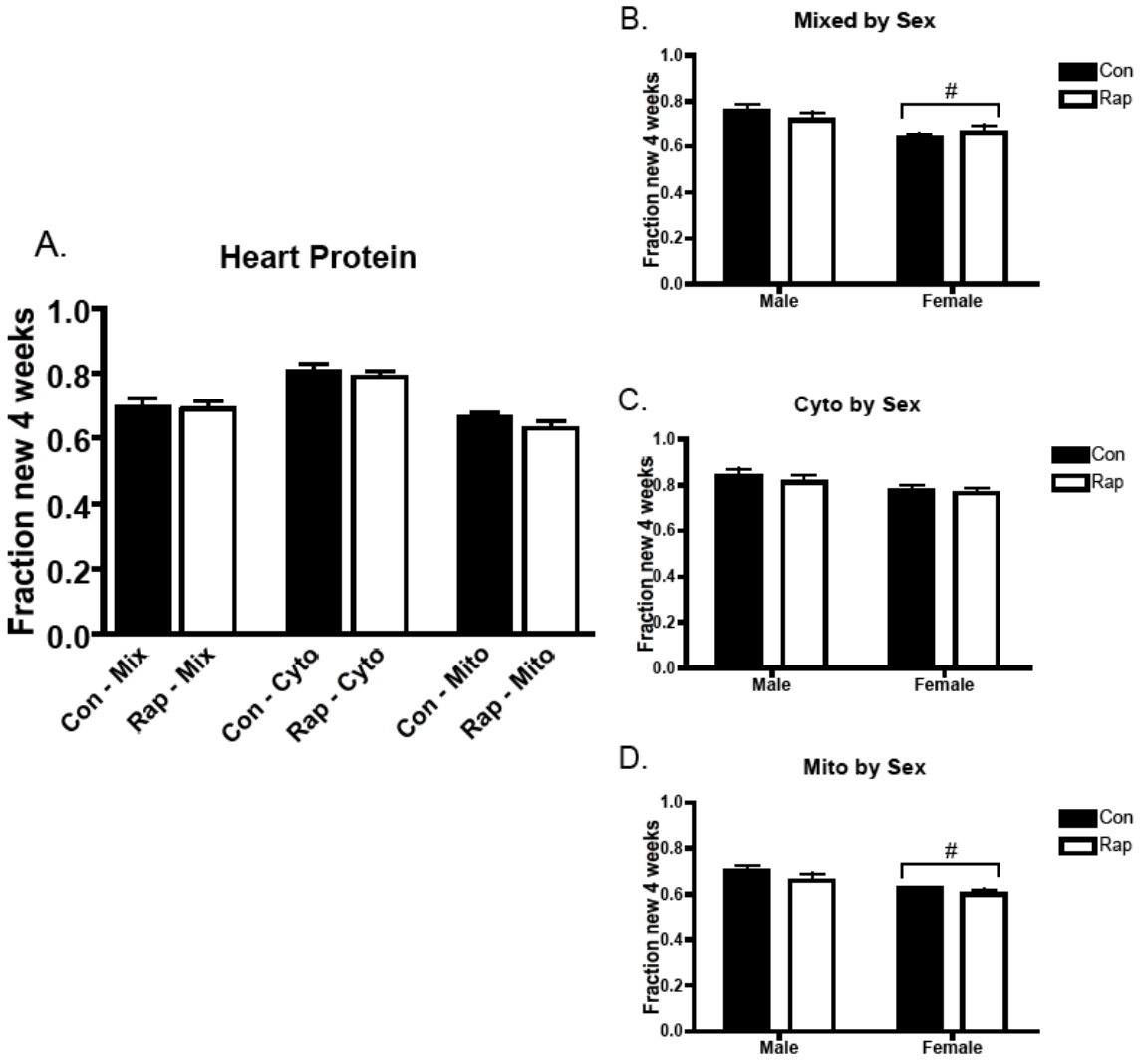

Figure 1.2. Heart protein synthesis over 4 weeks in Rap and CON. No difference was found in any fraction between Rap and CON (A). Females had less mixed protein synthesis than males (B). No sex difference in Rap and CON in cyto fraction (C). Females had less mito protein synthesis than males (D). *n* = 12 per pooled group, 6 per sex/group. # = $p<0.05$ for pooled sex difference.

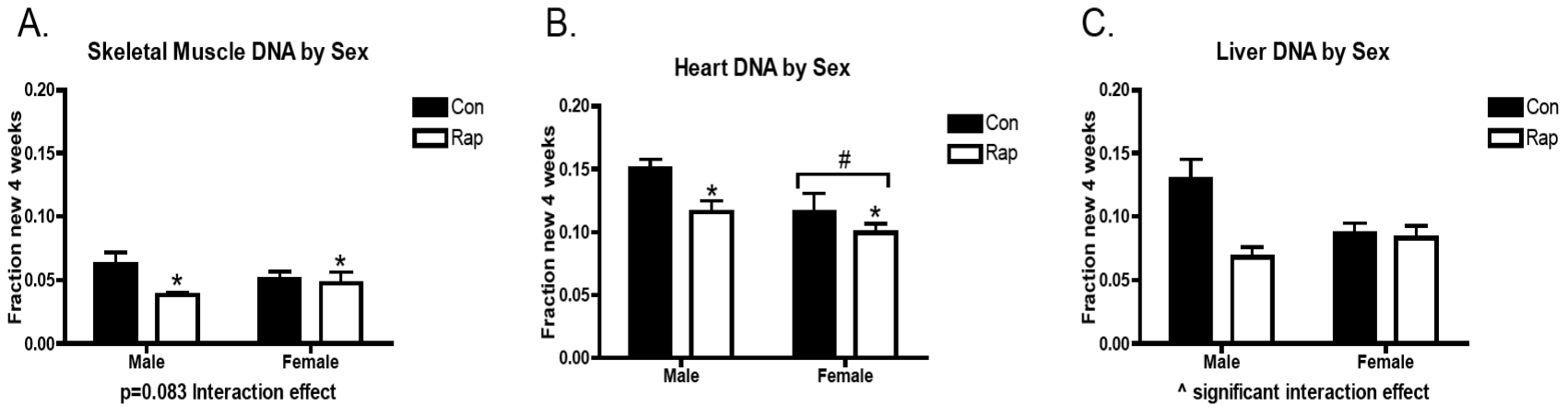

Figure 1.3. Cellular proliferation as measured by DNA synthesis over 4 weeks in Rap and CON. In skeletal muscle, Rap DNA synthesis was significantly decreased compared to CON (A). In heart, Rap DNA synthesis was significantly decreased compared to CON and females had significantly decreased DNA synthesis than males (B). In liver, a significant interaction effect between rapamycin and sex was observed (C). *n* = 11-12 per pooled group, *n* = 5-6 per sex/group. * = $p<0.05$ for Rap vs. CON; # = $p<0.05$ for pooled sex difference; ^ = $p<0.05$ for interaction effect.

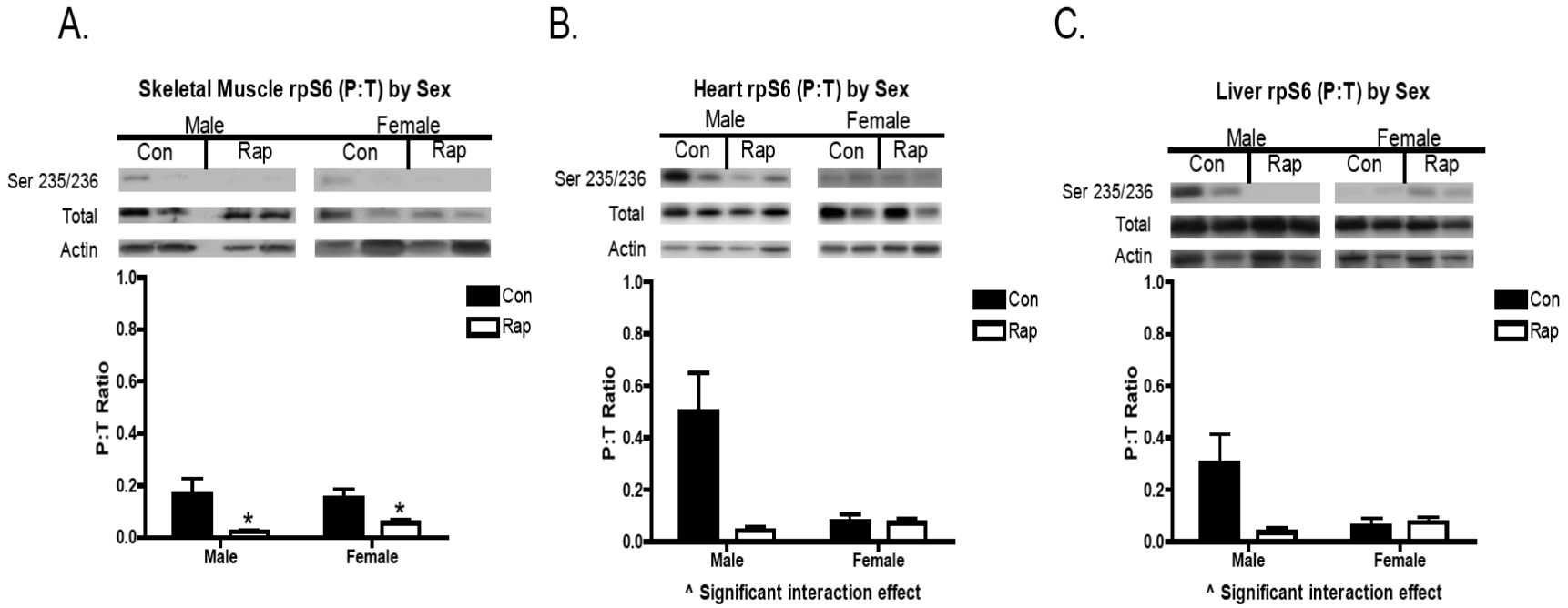

Figure 1.4. Western blotting of mTORC1 substrate rpS6. In skeletal muscle rpS6 phosphorylated to total protein (P:T) decreased in Rap compared to CON (A). A significant interaction effect for rapamycin and sex was observed in the heart and liver (B & C). *n* = 11-12 per pooled group, *n* = 5-6 per sex/group. * = $p<0.05$ for Rap vs. CON; ^ = $p<0.05$ for interaction effect.

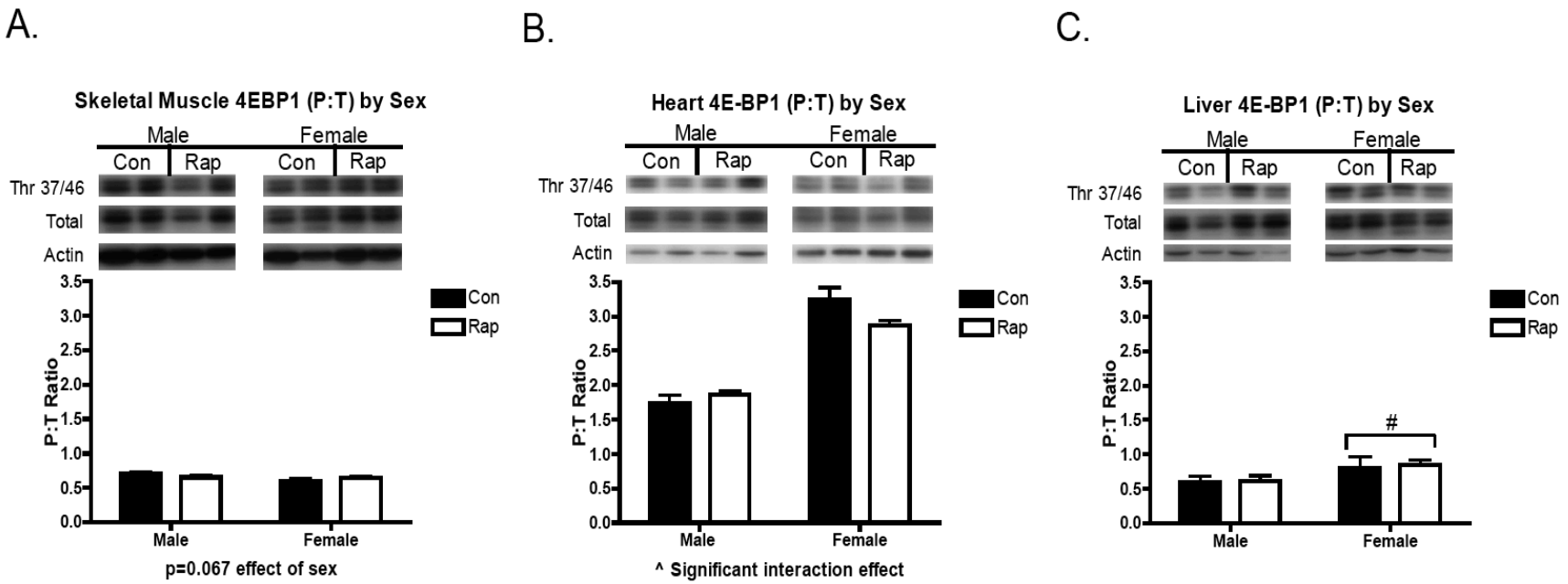

Figure 1.5. Western blotting of mTORC1 substrate 4E-BP1. Skeletal muscle 4E-BP1 phosphorylated to total protein (P:T) was not changed in Rap compared to CON (A). A significant interaction effect between rapamycin and sex was found in the heart (B). In the liver no change was observed in Rap compared to CON but females had significantly more 4E-BP1 (P:T) than males. *n* = 12 per pooled group, *n* = 6 per sex/group. Representative blots shown. * = p<0.05 for Rap vs. CON; # = p<0.05 for pooled sex difference; ^ = p<0.05 for interaction effect.

Table S1.1. Two-Way ANOVA summary for Fig 1.

Protein Synthesis
Skeletal Muscle

		p-value
Fig 1A	**Fraction**	<0.001
	Treatment	0.048
	Interaction	0.100
Fig 1B - Mix	**Treatment**	0.019
	Sex	0.748
	Interaction	0.682
Fig 1C - Cyto	**Treatment**	0.017
	Sex	0.173
	Interaction	0.465
Fig 1D - Mito	**Treatment**	0.327
	Sex	0.635
	Interaction	0.395

Table S1.2. Two-Way ANOVA summary for Fig 2.

Protein Synthesis
Heart

		p-value
Fig 2A	**Fraction**	<0.001
	Treatment	0.226
	Interaction	0.810
Fig 2B - Mix	**Treatment**	0.831
	Sex	0.008
	Interaction	0.307
Fig 2C - Cyto	**Treatment**	0.524
	Sex	0.056
	Interaction	0.816
Fig 2D - Mito	**Treatment**	0.117
	Sex	0.004
	Interaction	0.667

Table S1.3. Main effect means of DNA synthesis two-way ANOVA.

DNA Synthesis

Skeletal Muscle

Treatment	Sex	Mean	Std Dev	N
Con	Male	0.0743	0.0356	6
	Female	0.0508	0.0137	6
	Total	0.0625	0.0285	12
Rap	Male	0.0381	0.0053	6
	Female	0.0473	0.0212	6
	Total	0.0427	0.0147	12

Heart

Treatment	Sex	Mean	Std Dev	N
Con	Male	0.1506	0.0174	6
	Female	0.1153	0.0371	6
	Total	0.1330	0.0332	12
Rap	Male	0.1159	0.0215	6
	Female	0.0995	0.0176	6
	Total	0.1077	0.0206	12

Liver

Treatment	Sex	Mean	Std Dev	N
Con	Male	0.1296	0.0174	6
	Female	0.0871	0.0371	6
	Total	0.1084	0.0332	12
Rap	Male	0.0678	0.0200	6
	Female	0.0833	0.0232	6
	Total	0.0756	0.0222	12

Table S1.4. Main effect means of rpS6 two-way ANOVA. All data shown has been log transformed to equalize variance.

rpS6

Skeletal Muscle

Treatment	Sex	Mean	Std Dev	N
Con	Male	-0.8767	0.3173	5
	Female	-0.8979	0.3040	6
	Total	-0.8882	0.2943	12
Rap	Male	-1.6734	0.2052	6
	Female	-1.3286	0.3319	5
	Total	-1.5167	0.3123	12

Heart

Treatment	Sex	Mean	Std Dev	N
Con	Male	-0.3969	0.3469	6
	Female	-1.2356	0.3440	6
	Total	-0.8544	0.5469	12
Rap	Male	-1.4872	0.3790	6
	Female	-1.2249	0.3322	6
	Total	-1.356	0.3663	12

Liver

Treatment	Sex	Mean	Std Dev	N
Con	Male	-0.7806	0.6118	6
	Female	-1.3509	0.3689	6
	Total	-1.0398	0.5747	12
Rap	Male	-1.6744	0.4721	6
	Female	-1.2125	0.378	6
	Total	-1.4644	0.4762	12

Table S1.5. Main effect means of 4E-BP1 two-way ANOVA. Skeletal muscle data was cube transformed to equalize variance.

4E-BP1

Skeletal Muscle

Treatment	Sex	Mean	Std Dev	N
Con	Male	0.3503	0.0818	6
	Female	0.2274	0.0973	6
	Total	0.2889	0.1071	12
Rap	Male	0.2845	0.1096	6
	Female	0.2717	0.0351	6
	Total	0.2781	0.0779	12

Heart

Treatment	Sex	Mean	Std Dev	N
Con	Male	0.5975	0.2144	6
	Female	0.8009	0.3820	6
	Total	0.6992	0.3139	12
Rap	Male	0.6093	0.1895	6
	Female	0.8440	0.1734	6
	Total	0.7266	0.2121	12

Liver

Treatment	Sex	Mean	Std Dev	N
Con	Male	1.7409	0.2653	6
	Female	3.247	0.4332	6
	Total	2.494	0.8579	12
Rap	Male	1.8584	0.1240	6
	Female	2.8676	0.1787	6
	Total	2.363	0.5470	12

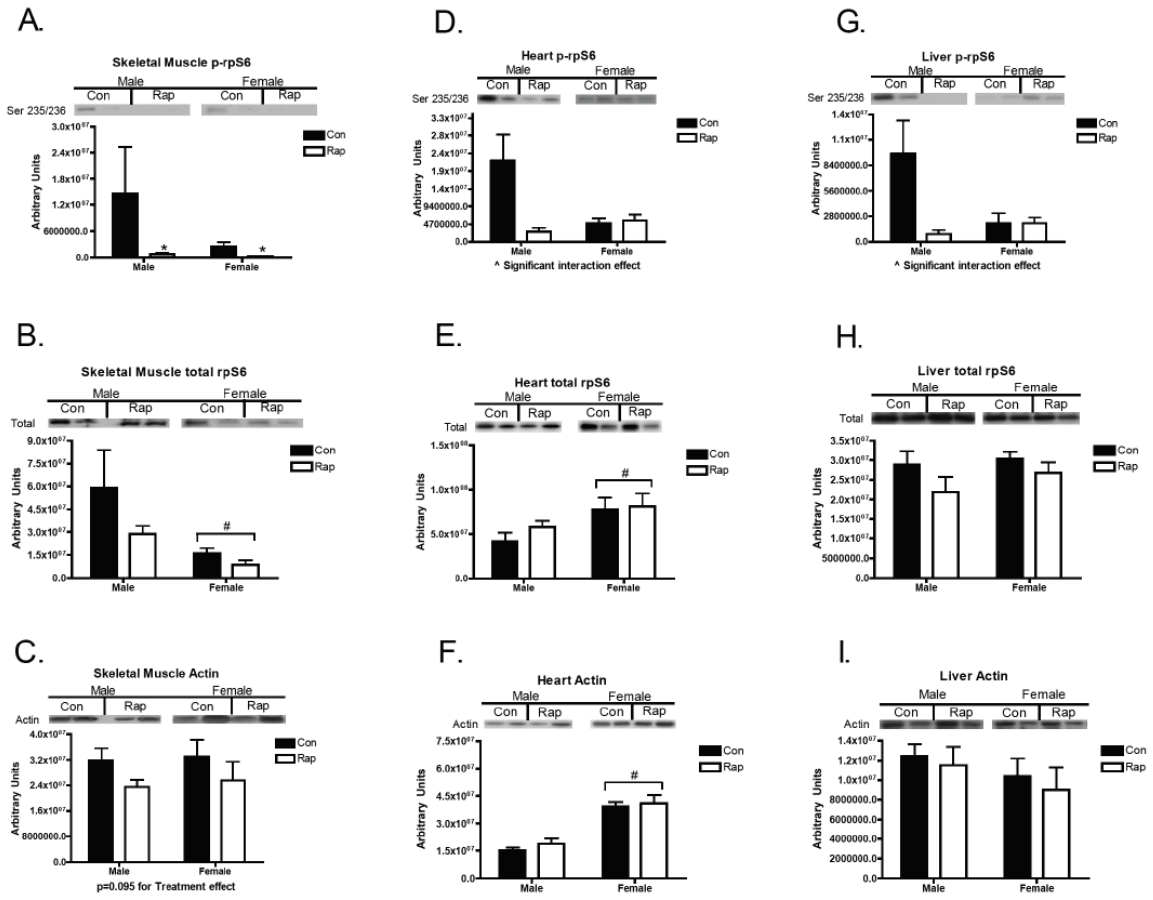

Figure S1.1. Western blotting of phospho (Ser235/236) rpS6 and total rpS6. Skeletal muscle rpS6 and respective actin loading control A-C. Skeletal muscle p-rpS6 data was rank transformed and total rpS6 was log transformed to equalize variance. Heart rpS6 and respective actin loading control D-F. Heart p-rpS6 data was log transformed to equalize variance. Liver rpS6 and respective actin loading control G-I. Liver p-rpS6 data was log transformed to equalize variance. *n* = 11-12 per pooled group, *n* = 5-6 per sex/group. Representative blots shown. * = p<0.05 for Rap vs. CON. # = p<0.05 for pooled sex difference; ^ = p<0.05 for interaction effect.

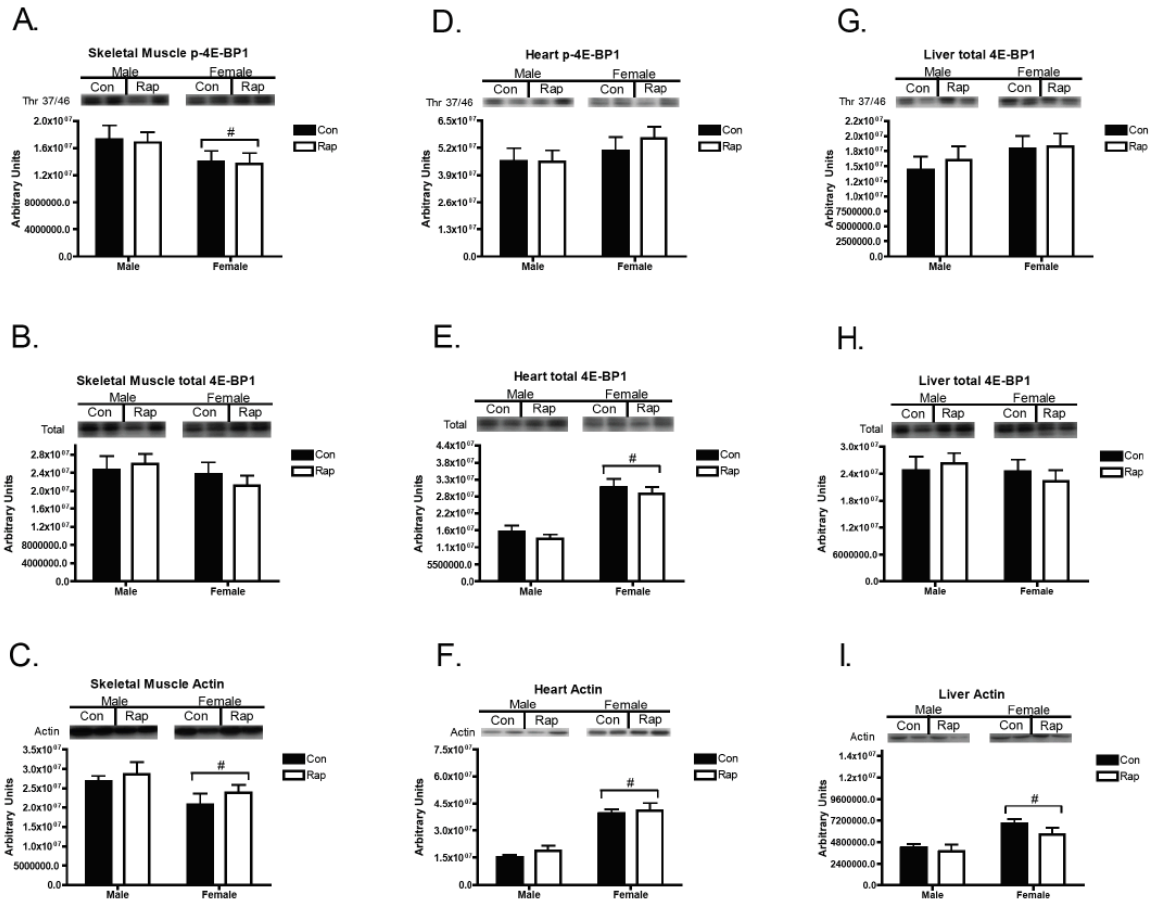

Figure S1.2. Western blotting of phospho (Thr36/47) and total 4E-BP1. Skeletal muscle 4E-BP1 and respective actin loading control A-C. Heart 4E-BP1 and respective actin loading control D-F. Liver 4E-BP1 and respective actin loading control G-I. n = 12 per pooled group, *n* = 6 per sex/group. * = p<0.05 for Rap vs. CON. # = p<0.05 for pooled sex difference; ^ = p<0.05 for interaction effect.

CHAPTER III – MANUSCRIPT II

Novel assessment of proteostasis in skeletal muscle and heart of Snell dwarf mice

Summary

Proteostasis is a key factor thought to contribute to slowed aging. We have shown evidence of increased proteostatic mechanisms across several models of long-life, suggesting that proteostasis may be a shared characteristic of slowed aging. The Snell dwarf mouse (Snell) has an underdeveloped anterior pituitary due to a mutation in the *Pit-1* locus. The Snell model has pronounced lifelong reductions in multiple hormonal growth factors and mTORC1 as well as the largest lifespan extension of long-lived rodent models. We hypothesized that proteostatic mechanisms would be increased in Snell compared to control (Con). We assessed protein synthesis in mixed (Mix), cytosolic (Cyto), and mitochondrial (Mito) fractions simultaneously with DNA synthesis via D_2O labeling as well as mTORC1 signaling in skeletal muscle, heart, and liver over two weeks in both sexes. Protein synthesis in Snell mice was decreased in all fractions assessed in skeletal muscle compared to Con. In the heart, protein synthesis in Snell was decreased in Cyto but increased in Mix and Mito fractions compared to Con. No differences in protein synthesis were found between Snell and Con in the liver. DNA synthesis in Snell was lower in skeletal muscle and heart but not liver compared to Con. The protein to DNA ratio, indicative of proteostasis, was increased 3-fold in Snell skeletal muscle and heart. Snell rpS6 was decreased in heart and liver compared to Con. No effect of sex was seen in this study. Together with our previous investigations in long-lived models, these data suggest proteostasis is a shared characteristic of slowed aging.

Introduction

Protein turnover decreases with age, resulting in a progressive accumulation of damaged proteins and propagation of the aging phenotype (1, 2). Maintaining protein homeostasis (i.e. proteostasis) through coordination of mRNA translation, protein synthesis, folding, and compartmental targeting is a key component of slowed, or healthy aging (4, 10). Therefore, interventions that promote proteostatic mechanisms could be effective in the promotion of slowed aging.

Enzymatic capacity for protein repair is low, meaning damaged proteins must be removed (e.g. autophagy, etc) and replaced by the synthesis of new proteins (17-19). Therefore the synthesis of new proteins is essential to cellular proteostasis (10). Though protein synthesis is a critical component of proteostasis, assessment of protein synthesis alone may not give sufficient insight into proteostasis. For example, in proliferative tissues, cell division requires an increase in protein synthesis to ensure equality between daughter cells (20). Similarly, in post-mitotic tissues such as skeletal muscle, hypertrophy requires an increase in protein synthesis along with the recruitment of DNA from resident stem cells so as to maintain the myonuclear domain (61). Adaptation to the intra- and extracellular environment in various cell types requires increases in protein synthesis to maintain cellular homeostasis, which are not necessarily dependent upon cell proliferation. Therefore assessing proteostasis in both proliferative and post-mitotic tissues should include measurements of both protein synthesis and DNA synthesis (i.e. cellular proliferation).

The mTOR-signaling pathway integrates nutrient and hormonal signaling to regulate protein turnover (e.g. protein synthesis, cell cycle, and autophagy) via two multi-protein complexes, mTORC1 and mTORC2 (13). Life-long caloric restriction (CR) and chronic administration of rapamycin (Rap) both inhibit mTORC1 and increase lifespan in a sexually dimorphic manner (14, 62). Using deuterium oxide (D_2O) in both long-lived CR and Rap models, we found evidence of increased proteostatic mechanisms alongside decreased

mTORC1 signaling (11, 12, 63). Therefore, decreased mTORC1 signaling may be necessary for proteostasis in models of long-life.

The Snell dwarf mouse model has a mutation of the *Pit-1* locus that results in an underdeveloped anterior pituitary, causing reductions in anterior pituitary hormone production (e.g. growth hormone) and reduced mTORC1 signaling (27, 28). Compared to their respective controls, the Snell dwarf mouse has the greatest increase in lifespan among rodent models of slowed aging (56). Though female Snell dwarfs significantly outlive their male counterparts, both sexes of Snell mice have a much longer lifespan extension compared to Rap and CR models (14, 62). The pronounced reduction in mTORC1 signaling and hormonal growth factors throughout the lifespan in Snell, in contrast to CR and Rap interventions that begin later in life, make the Snell model ideal for expanding the concept of increased proteostatic mechanisms as a key mediator of slowed aging. Therefore, we hypothesized that proteostatic mechanisms, as assessed by DNA and protein synthesis, would be increased in Snell compared to controls (Con) and would correspond with decreased mTORC1-related signaling. We also assessed these parameters by sex to determine if proteostasis is reflective of the sexual dimorphic lifespan extension in Snell.

Materials and Methods

Animals

All procedures and conditions at the animal care facility meet or exceed the standards for animal housing as described in the Animal Welfare Act regulations and the Guide for the Care and Use of Laboratory Animals. All procedures were approved by the University Committee on Use and Care of Animals at the University of Michigan. Routine veterinary care was provided by the Biomedical Science Research Building staff. Snell dwarf and control mice were produced by crossing C3H/HeJ-Pit^{dwJ}/+ females with DW/J-Pit1$^{dw/=}$ males producing (DW/J x C3H/HeJ)F_1 mouse offspring (64). The homozygous dw/dw dwarf offspring were compared to

littermate controls using both dw/+ and +/+mice as indistinguishable controls. After weaning (21-23 days), mice were housed in single-sex groups of 3-4 mice per cage (64). Animals were kept on a 12-hour light and dark cycle and fed ad libitum.

At 7 months of age (age-matched to our previous investigations in long-lived models (11, 12, 63, 89), Snell dwarf and control mice (n=5 per sex, n=10 per group) received deuterium oxide (D_2O) for two weeks. After the labeling period and following an overnight fast, mice were euthanized using a CO_2 overdose according to the AVMA Guidelines on Euthanasia. Complete loss of pedal reflexes was confirmed before tissues were collected. The posterior aspect of the distal hind limbs (gastroc complex - gastrocnemius, soleus, and plantaris [i.e. mixed skeletal muscle]), heart, liver, bone marrow from the tibia, and plasma via blood from cardiac puncture were taken and immediately frozen in liquid nitrogen for later analysis.

Deuterium Oxide

We assessed protein synthesis in mitochondrial enriched (Mito), cytosolic (Cyto - other cytosolic organelles with the exception of mitochondria and nucleus), and mixed (Mix; nuclei, plasma membranes, and contractile proteins [heart and skeletal muscles]) subcellular fractions from skeletal muscle heart and liver (11, 12, 45, 46, 63, 89). Also, we assessed DNA synthesis according to procedures previously described (11, 12, 45, 46, 63, 89). Animals received an intraperitoneal injection of 99% enriched D_2O to enrich the body water pool (assumed 60% of body weight) to 5% (11, 46, 89). Animals then received 8% D_2O in their drinking water with *ad libitum* access for 2 weeks.

Protein Synthesis Measurement

Protein isolation - Tissues were fractionated according to our previously published procedures (11, 12, 47, 63, 89). Tissues (15-50 mg) were homogenized 1:10 in isolation buffer (100 mM KCl, 40 mM Tris HCl, 10 mM Tris Base, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, pH=7.5) with

phosphatase and protease inhibitors (HALT, Thermo Scientific, Rockford, IL, USA) using a bead homogenizer (Next Advance Inc, Averill Park, NY, USA). After homogenization, sub-cellular fractions were isolated via differential centrifugation as previously described (11, 12, 63, 89). Once fraction pellets were isolated and purified, 250 μl 1 M NaOH was added and pellets were incubated for 15 min at 50 °C and 900 rpm.

Preparation of analytes for mass spectrometric analyses - Protein was hydrolyzed by incubation for 24 hrs at 120 °C in 6 N HCl. The hydrolysates were ion-exchanged, dried under vacuum, and resuspended in 1 mL molecular biology grade H_2O. 500 μl of suspended samples were derivatized (500 μl acetonitrile, 50 μl 1 M K_2HPO_4 (pH = 11), and 20 μl of pentafluorobenzyl bromide (Pierce Scientific, Rockford, IL, USA)), sealed, and incubated at 100 °C for 1 hr. Derivatives were extracted into ethyl acetate. The organic layer was removed and dried by N_2 followed by vacuum centrifugation. Samples were reconstituted in 1 ml ethyl acetate then analyzed.

To determine body water enrichment, 125 μl of plasma was placed into the inner well of o-ring screw cap and inverted on heating block overnight. 2 μl of 10 M NaOH and 20 μl of acetone was added to all samples and to 20 μl 0-20% D_2O standards then capped immediately. Samples were vortexed at low speed and left at room temperature overnight. Extraction was performed by the addition of 200 μl hexane. The organic layer was transferred through anhydrous Na_2SO_4 into GC vials and analyzed via EI mode using a DB-17MS column.

GC-MS analysis of derivatized amino acids – The pentafluorobenzyl-*N*,*N*-di(pentafluorobenzyl) derivative of analnine was analyzed on an Agilent 7890A GC coupled to an Agilent 5975C MS as previously described (11, 12, 49, 63, 89). The newly synthesized fraction (f) of proteins was calculated from the true precursor enrichment (p) using plasma analyzed for D_2O enrichment

and adjusted using mass isotopomer distribution analysis (MIDA) (45). Protein synthesis was calculated as the ratio of deuterium-labeled to unlabeled alanine (45) bound in proteins over the entire labeling period and expressed as fraction new in 2 weeks.

DNA synthesis measurement

DNA isolation – Approximately 100 ng/ul (heart, gastroc complex) and 500ng/ul (liver) of total DNA was extracted from ~20 mg tissue (QiAamp DNA mini kit Qiagen, Valencia, CA, USA). DNA from bone marrow was isolated by extracting ~300 mg from the tibial bone marrow suspension and centrifuged for 10 min at 2000 g.

Preparation of analytes and GC-MS analysis of derivatized purine deoxyribose - Isolated DNA was hydrolyzed overnight at 37 °C with nuclease S1 and potato acid phosphatase. Hydrolysates were reacted with pentafluorobenzyl hydroxylamine and acetic acid and then acetylated with acetic anhydride and 1-methylimidazole. Dichloromethane extracts were dried, resuspended in ethyl acetate, and analyzed by GC/MS as previously described (11, 45, 49, 63, 89). The fraction of new DNA in 2 weeks was calculated by comparison with bone marrow (representing an essentially fully turned over cell population and thus the precursor enrichment) in the same animal (11, 63). From the synthesis rates of protein and DNA, we calculated the protein synthesis to DNA synthesis ratio to be indicative of proteostasis. This ratio illustrates how much new protein is made in relation to the rate of cellular proliferation (new DNA) during the labeling period.

Western blotting

Western blots were completed on a portion of the Cyto fraction. Protein concentration was determined using a bicinchoninic acid assay (Thermo Fisher, Rockford, IL, USA). Samples were diluted to equal concentrations, boiled with Laemmli buffer, and then 30-60 μg of protein

was separated using 10 % SDS-PAGE at 100 V. Proteins were transferred at 4 °C (100 V for 75 min in 20 % w/v methanol, 0.02 % w/v SDS, 25 mM Tris Base, 192 mM glycine, pH=8.3) to nitrocellulose and incubated in 5 % nonfat dry milk in Tris-buffered saline with Tween20 (TBST) for 1 hr. Antibodies were purchased from Cell Signaling Technologies (Boston, MA, USA; rpS6 phospho-Ser[235/236] #4858, rpS6 total #2217, 4E-BP1 phospho [Thr37/46] #9459, 4E-BP1 total #9452) or Santa Cruz Biotechnology (Santa Cruz, CA, USA; (β-tubulin #sc-5274). Blots were incubated overnight with primary antibodies diluted 1:500 (skeletal muscle rpS6), 1:1000, 1:2000 (Liver rpS6), or 1:500 (β-tubulin). Blots were washed in 1x TBST and incubated with anti-rabbit or anti-mouse (β-tubulin) HRP-conjugated secondary antibody diluted 1:5000 in 5% milk with subsequent chemiluminescence detection (West Dura; Pierce, Rockford, IL, USA). Images were captured and densitometry analyzed by UVP Bioimaging system (Upland, CA, USA). Blots were probed for phosphorylated proteins first, placed in stripping buffer (GM Biosciences, Rockville, MD, USA) and then re-probed for total protein. Equal loading was verified using ponceau-s staining and β-tubulin. Due to undetectable rpS6 in skeletal muscle and some heart samples, a portion of the homogenate was acetone precipitated to concentrate proteins then analyzed via western blotting.

Statistics

Statistical analysis was performed using Prism V4.0c (GraphPad Software, Inc. La Jolla, CA, USA). Protein synthesis data grouped by subcellular fraction and all sex comparisons were assessed by two-way ANOVA. Combined sexes DNA synthesis data was assessed via two-sided Student's t-test. Significance was set at $p < 0.05$, and p-values of ≤ 0.10 are noted. Data are presented as means ± standard error of the mean (SEM).

Results

Protein and DNA Synthesis

In skeletal muscle, Snell dwarf mice had significantly lower rates of protein synthesis in all fractions compared to Con (Fig 2.1A). In the Mix fraction there was a significant interaction between condition (Snell versus Con) and sex (Fig 2.1B). There were no sex differences in protein synthesis in Cyto or Mito fractions (Fig 2.1C, D). Independent of condition, males had significantly greater rates of protein synthesis in Cyto compared to females (Fig 2.11C).

In heart, there was a significant interaction between condition (Snell versus Con) and tissue fraction, with greater rates of proteins synthesis in Mix and Mito fractions and lower rates of Cyto protein synthesis in Snell dwarfs compared to Con (Fig 2.2A). However, when separated by sex, there was not a significant condition effect in Mix or Cyto (Fig 2.2B, C). In both female and male Snells, Mito protein synthesis was increased compared to their respective Con (Fig 2.2D). Independent of condition, males had significantly greater rates of protein synthesis in the Mix and Mito fraction compared to females (Fig 2.2B, D).

In liver, Snell had a trend ($p=0.067$) for lower protein synthesis in all sub-cellular fractions assessed compared to Con (Fig 2.3A). When separated by sex a trend ($p=0.060$) for Snell dwarfs having lower protein synthesis was found only in Mix (Fig 2.3B). Independent of condition, male protein synthesis was significantly increased compared to females in Cyto and trended towards an increase in Mix ($p = 0.06$) and Mito ($p = 0.076$) (Fig 2.3B, C, D).

DNA synthesis rates were significantly less in Snell compared to Con in both heart and skeletal muscle, but were not different from Con in liver (Fig 2.4A). Decreased DNA synthesis in Snell compared to Con was also present when the data were separated by sex (Fig 2.4B, C, D).

In both skeletal muscle and heart, there was a significant increase in the protein to DNA synthesis ratio in Snell compared to Con (Fig 2.5A, C), which was not different between sexes

(Fig 2.5B, D). In the liver, there was no difference in the protein to DNA synthesis ratio between Snell and Con or when separated by sex (Fig 2.5E, F).

mTORC1 signaling

Phosphorylation of the mTORC1 substrate, ribosomal protein S6 (rpS6), was significantly decreased in heart and liver, as well as in both sexes of Snell compared to Con (Fig 2.6B, C). Even with acetone precipation we were unable to assess a visible band for rpS6 in skeletal muscle. Phosphorylation of another mTORC1 substrate, eukaryotic initiation factor 4E binding protein 1 (4E-BP1), was not statistically different in any tissue or between sexes (Fig 2.6 D, E, F), though a slight interaction trend between condition (Snell) and sex was observed in skeletal muscle ($p=0.098$) (Fig 2.6 D, E).

Discussion

Here we present a tissue and sex-specific assessment of proteostasis via DNA and protein synthesis in long-lived Snell dwarf mice. We tested the hypothesis that Snell mice would exhibit increased proteostatic mechanisms across multiple tissues; consistent with our findings in other slowed aging models (11, 12, 63, 89). Also, we investigated if proteostatic mechanisms would be more pronounced in female compared to male Snell mice given their female-biased sexual dimorphic lifespan. We demonstrate that proteostatic mechanisms, as assessed by protein to DNA synthesis ratio, is indeed increased by 3-fold in skeletal muscle and heart, but independent of sex, in Snell compared to Con. Additionally, the mTORC1-substrate, rpS6, was decreased in heart and liver. Collectively, our data further suggest proteostasis is a shared characteristic of slowed aging but cannot explain the sex difference in Snell lifespan (11, 12, 63, 89).

Proteostasis in Snell dwarf mice.

Proteostasis involves the coordinated control between concentration, location, and conformation of proteins within cells (6) and has recently become a key outcome in understanding the aging process (6-9). Protein synthesis, along with protein folding, trafficking, and degradation, is an important process capable of regulating proteostasis (6). Since the intrinsic capacity of cells to enzymatically repair proteins is low (17, 18), damaged proteins must instead be removed (e.g. proteosome, autophagy, etc.) and replaced through the synthesis of new proteins (19). Therefore protein synthesis has an essential role in maintaining cellular stability and repair, and thus proteostasis (10).

Proliferative cells must double in mass through increased protein synthesis in order to ensure equality between daughter cells (21). In post-mitotic cells (e.g. skeletal muscle), increases in protein synthesis are accompanied by recruitment of new DNA from resident stem cells in order to maintain the myonuclear domain (23-25). Proteostasis requires constant integration of multiple intra- and extracellular signals in order to coordinate the synthesis of new proteins to adapt to the current environment, which is not necessarily dependent on cellular proliferation. Therefore, protein synthesis should be assessed in parallel with cellular proliferation in order to determine if new proteins are dedicated to cell doubling or maintenance of existing cellular structures.

In the current study, we used D_2O to simultaneously assess both protein and DNA synthesis across multiple tissues and between sexes (11, 12, 45, 46, 49, 63) in the long-lived Snell dwarf mouse. We show the novel finding that protein synthesis is decreased in all subcellular fractions, including mitochondria, in skeletal muscle. A decrease in mitochondrial protein synthesis (i.e. mitochondrial biogenesis (31)) is counter to the idea of slowed aging (3). However, when decreased mitochondrial, as well as Mix and Cyto, protein synthesis rate is considered in relation to the decreased rate of DNA synthesis in skeletal muscle, the ratio of new proteins to new DNA synthesized is approximately 3-fold greater in Snell compared to Con.

To us, this suggests that new proteins are synthesized in existing cells, thus maintaining existing cellular structures, including mitochondria, (i.e. proteostasis) in Snell dwarf skeletal muscle. In the heart, protein synthesis in Mix and Mito fraction is increased, whereas Cyto protein synthesis is decreased in Snell compared to Con. Again, the new protein to new DNA synthesis ratio shows a 3-fold increase in proteostatic mechanisms across sub-cellular fractions in Snell heart despite the differential protein synthesis response between fractions compared to Con. Our findings of increased proteostatic mechanisms in Snell mice are in accordance with long-lived CR and Rap mouse models of the same age (11, 12, 63), thus suggesting that proteostasis is a shared characteristic of long-lived models.

An increase in proteostatic mehcanisms within Snell heart and skeletal muscle is contrasted by the lack of statistical significant differences in the liver between Snell and Con. Liver has a much higher protein turnover rate than skeletal muscle and heart (11, 12, 63, 89), thus making it challenging to capture synthesis rates in liver protein while still allowing for sufficient incorporation of deuterium into DNA across tissues. In the current study, one liver sample in Con was fully turned over, while others were >98% new. Combined with the significant decrease in rpS6 phosphorylation in Snell liver, we therefore suspect that a true difference in liver protein synthesis between Snell and Con is present that we were unable to capture because of our extended labeling period. Thus, a shorter labeling period may give further insights into proteostasis within Snell liver.

mTORC1-related signaling

The mTORC1 signaling pathway is thought to be a key regulator of proteostasis due to its control over protein turnover and cell cycling (13). Furthermore, decreased mTORC1 signaling is a common characteristic of long-lived models (11, 14, 28, 63, 65). We found that rpS6 phosphorylation was decreased in heart and liver from Snell mice compared to Con but were unable to detect rpS6 in skeletal muscle. Also, we did not find statistically significant

differences in 4E-BP1 phosphorylation within any tissue. The translation of mRNA, which is subject to mTORC1 control, is prone to errors in amino acid incorporation at a rate of 1 in every 10^3 to 10^4 codons mistranslated (66), and may increase with age creating more abhorrent polypeptides (4). Rapamycin induced decreases in rpS6 phosphorylation *in vitro* has been shown to regulate proteostasis through slowing the rate of elongation, thus increasing the fidelity of translated polypeptides (67). However, as we have demonstrated here and elsewhere (11, 63), decreased rpS6 phosphorylation *in vivo* does not always correlate to decreased protein synthesis across sub-cellular fractions or tissues. Taken together, our findings in Snell suggest that increased proteostatic mechanisms is not necessarily dependent on decreased mTORC1 (89), but rather on the investment of new proteins towards repair rather than growth. How proteins are differentially synthesized despite decreased mTORC1 is of significant interest and may be important in slowed aging (63).

Proteostasis and sex in Snell dwarf mice

With the exception of protein synthesis in the Mix fraction of skeletal muscle, there were no sex differences in protein or DNA synthesis, or mTORC1 signaling in Snell. Given the sexual dimorphic lifespan of Snell dwarf mice this was an unexpected finding. Though an interaction between sex and protein synthesis was noted in the skeletal muscle mix fraction, this sex difference did not persist in the ratio of new proteins to new DNA synthesis. However, the findings presented here do not rule out the possibility that sex-differences in synthetic outcomes of other tissues may contribute to sexual dimorphic lifespan or that the longer lifespan in Snell females may be due to other factors not directly related to proteostasis.

Summary and Conclusions

Manipulating gene targets (e.g. *Pit1* in Snell dwarf mice) can have multiple downstream effects. Since the pleiotropic outcomes of gene manipulations are sometimes unanticipated,

synthetic rates provide excellent insight into complex models such as the Snell dwarf. By comparing the amount of new protein to the rate of cell proliferation, we illustrate that proteostatic mechanims are uniformly increased across sub-cellular fractions in Snell skeletal muscle and heart even though rates of protein synthesis were different between fraction and tissue. How proteins are differentially synthesized despite decreased mTORC1 may be important in slowed aging but further suggests that proteostasis may not be dependent on decreased mTORC1. Collectively, we provide novel evidence using kinetic measurements that suggest proteostasis is a shared characteristic of slowed aging.

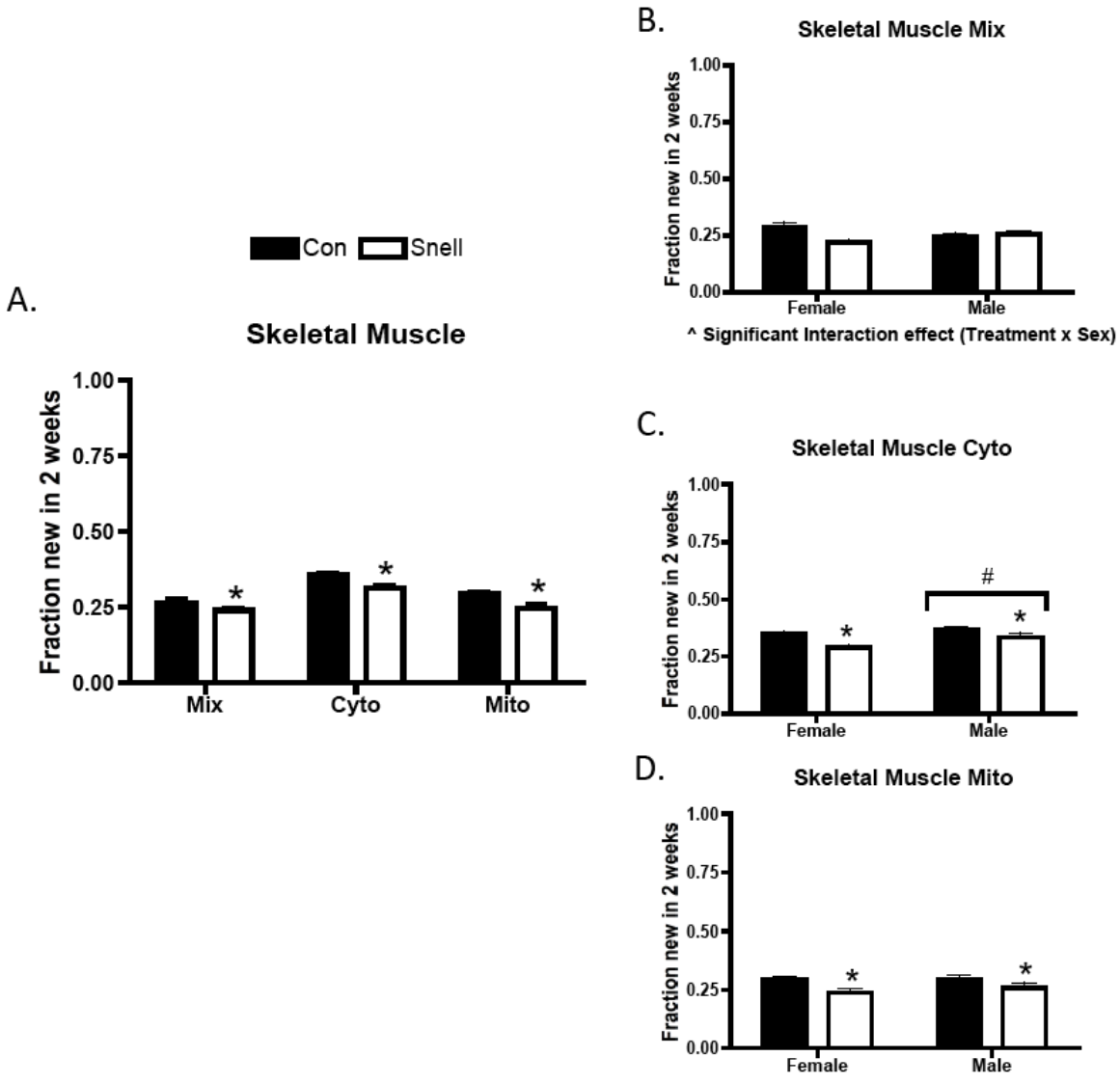

Figure 2.1. Protein synthesis in skeletal muscle and between sexes over 2 weeks in Snell and Con. Protein synthesis was significantly decreased in Snell compared to Con in Mix, Cyto, and Mito fraction (A). In Mix there was a significant interaction between condition (Snell) and sex (B). Protein synthesis was decreased in both female and male Snell compared to their respective Con in Cyto and Mito (C, D). Independent of condition, Cyto protein synthesis was increased in males compared to females (C). n = 5 per sex, n = 10 per group. * = $p<0.05$ for Snell vs CON; # = $p<0.05$ difference between sexes independent of condition; ^ = $p<0.05$ Interaction between condition and sex.

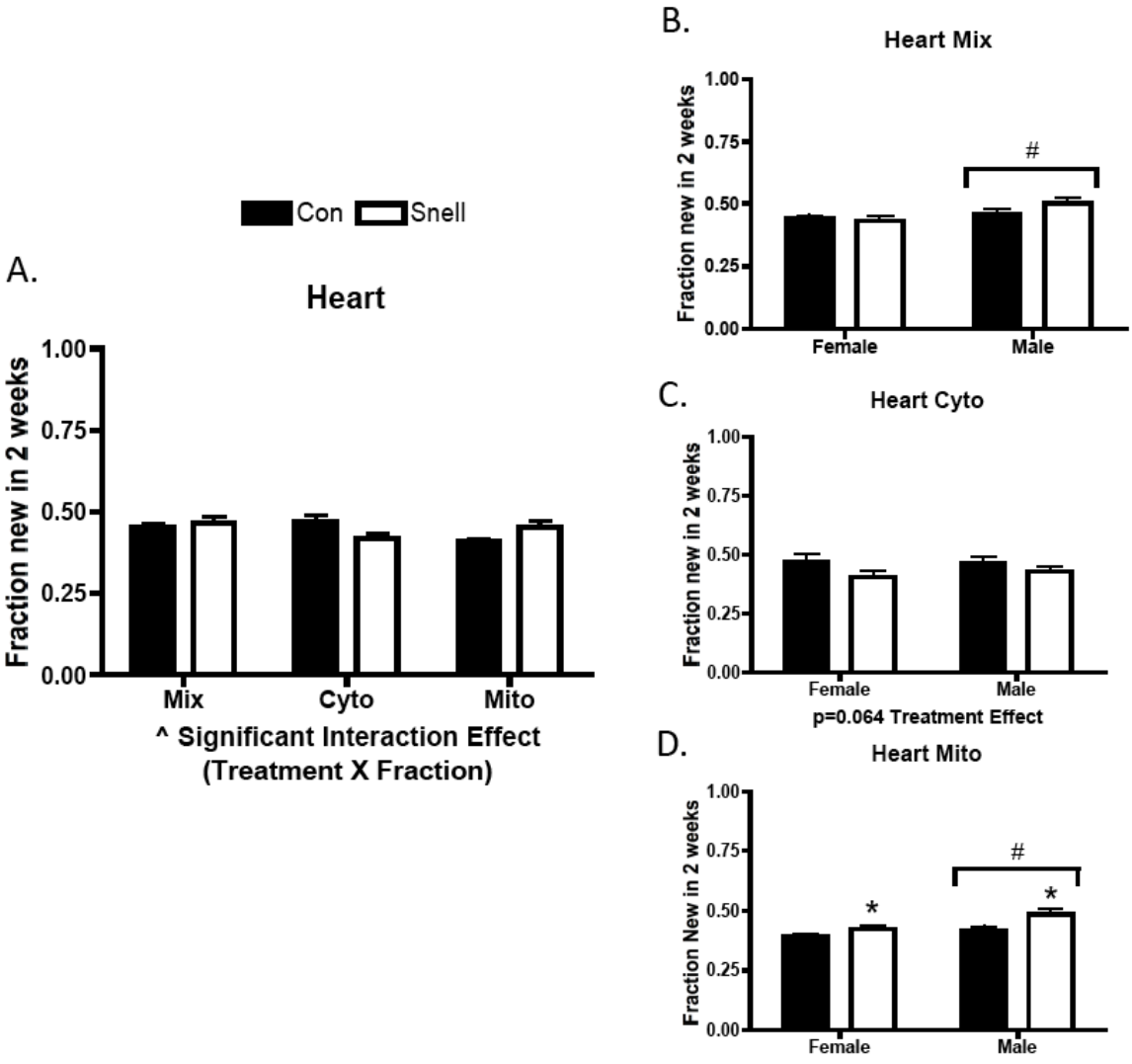

Figure 2.2. Protein synthesis in heart and between sexes over 2 weeks in Snell and Con. There was a significant interaction in protein synthesis between condition (Snell) and fraction (A). Mix protein synthesis was significantly increased in pooled males compared to pooled females (B). A trend (p=0.064) of decreased protein synthesis was observed for both sexes in Cyto (C). Mito protein synthesis was increased in both sexes (C). Males independent of condition had increased protein synthesis compared to females (C). n = 5 per sex, n = 10 per group. * = $p<0.05$ for Snell vs Con; # = $p<0.05$ difference between sexes independent of condition; ^ = $p<0.05$ Interaction between condition and sex.

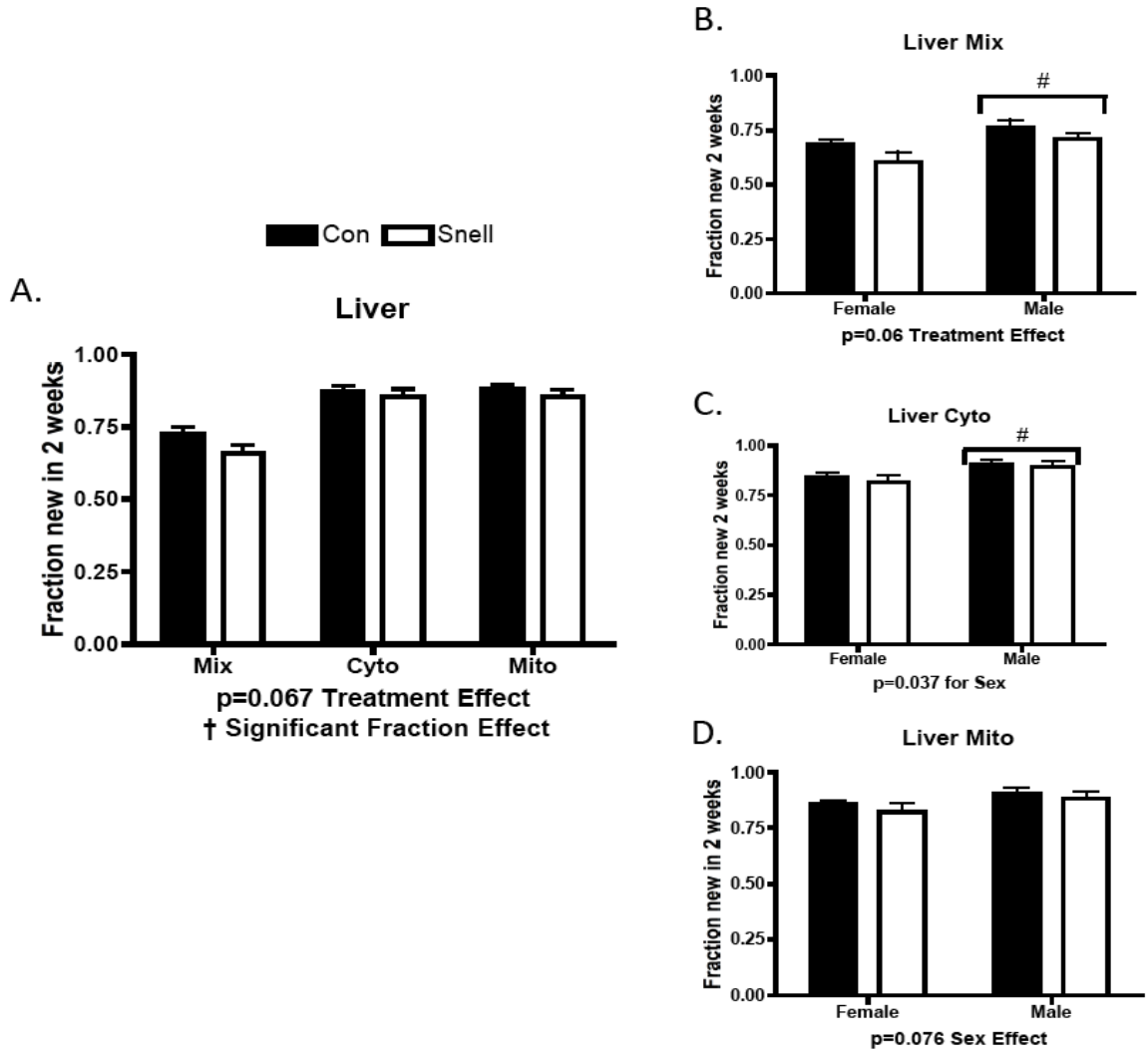

Figure 2.3. Protein synthesis in liver and between sexes over 2 weeks in Snell and Con. A trend (p=0.067) of decreased protein synthesis was observed across fractions in Snell compared to Con and protein synthesis was significantly different between fractions (A). A trend (p=0.060) for decreasing protein synthesis in both sexes within mix was observed; also mix protein synthesis was significantly higher in males compared to females, independent of condition (B). Males had higher Cyto protein synthesis compared to females, independent of condition (C). In Mito there was a trend (p=0.076) for increased protein synthesis in males compared to females independent of condition (D). n = 5 per sex, n = 10 per group, except for Con Cyto n = 9 corresponding to n = 4 in male Con Cyto. * = $p<0.05$ for Snell vs Con; † = p,0.05 difference between fractions; # = $p<0.05$ difference between sexes independent of condition.

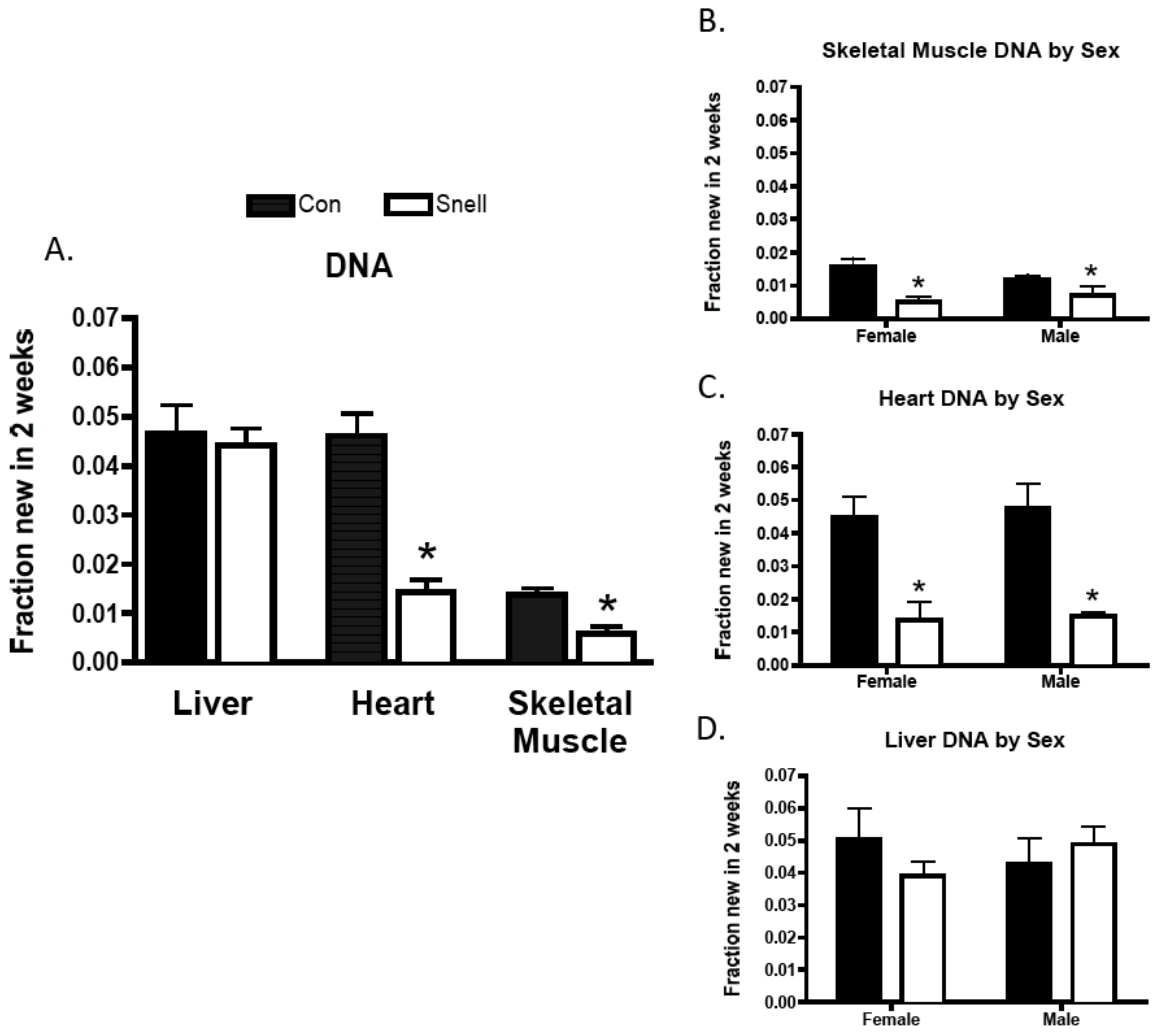

Figure 2.4. DNA synthesis in skeletal muscle, heart, and liver and between sexes over 2 weeks in Snell and Con. DNA synthesis was significantly decreased in skeletal muscle and heart but no change was observed in liver DNA synthesis between Snell compared to Con (A). When separated by sex, the decrease in DNA synthesis in Snell compared to Con was conserved in females and males in skeletal muscle and heart (B, C). No sex differences in DNA synthesis were observed in the liver (D). n = 5 per sex, n = 10 per group. * = $p<0.05$ for Snell vs Con.

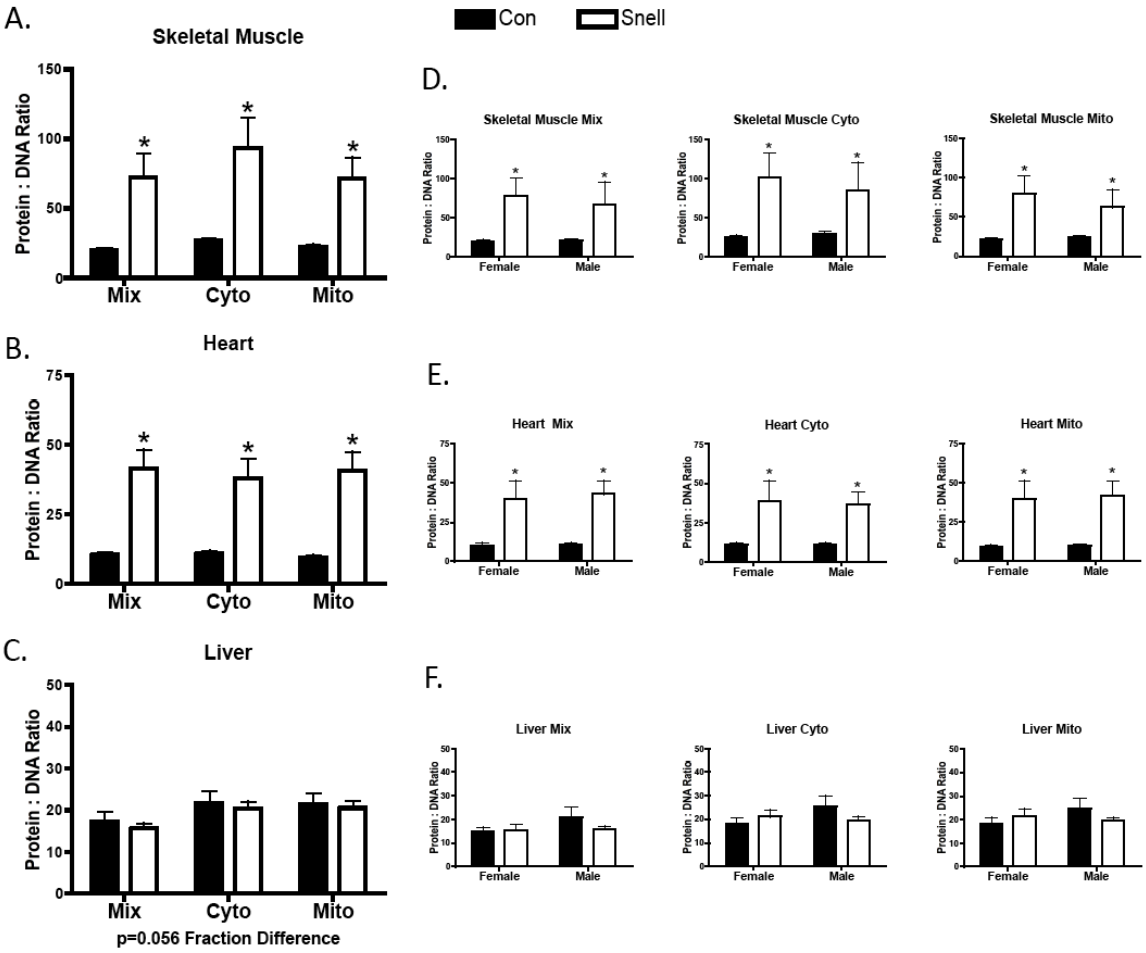

Figure 2.5. Protein to DNA synthesis ratios in skeletal muscle, heart, and liver and between sexes in Snell and Con. In skeletal muscle and heart there was a significant increase in the protein to DNA ratio in mix, cyto, and mito fractions (A, B), which was conserved in both sexes (C, D). No change in the protein to DNA ratio between Snell and Con was observed in liver or when separated by sex (E, F). n = 5 per sex, n = 10 per group. * = $p<0.05$ for Snell vs Con.

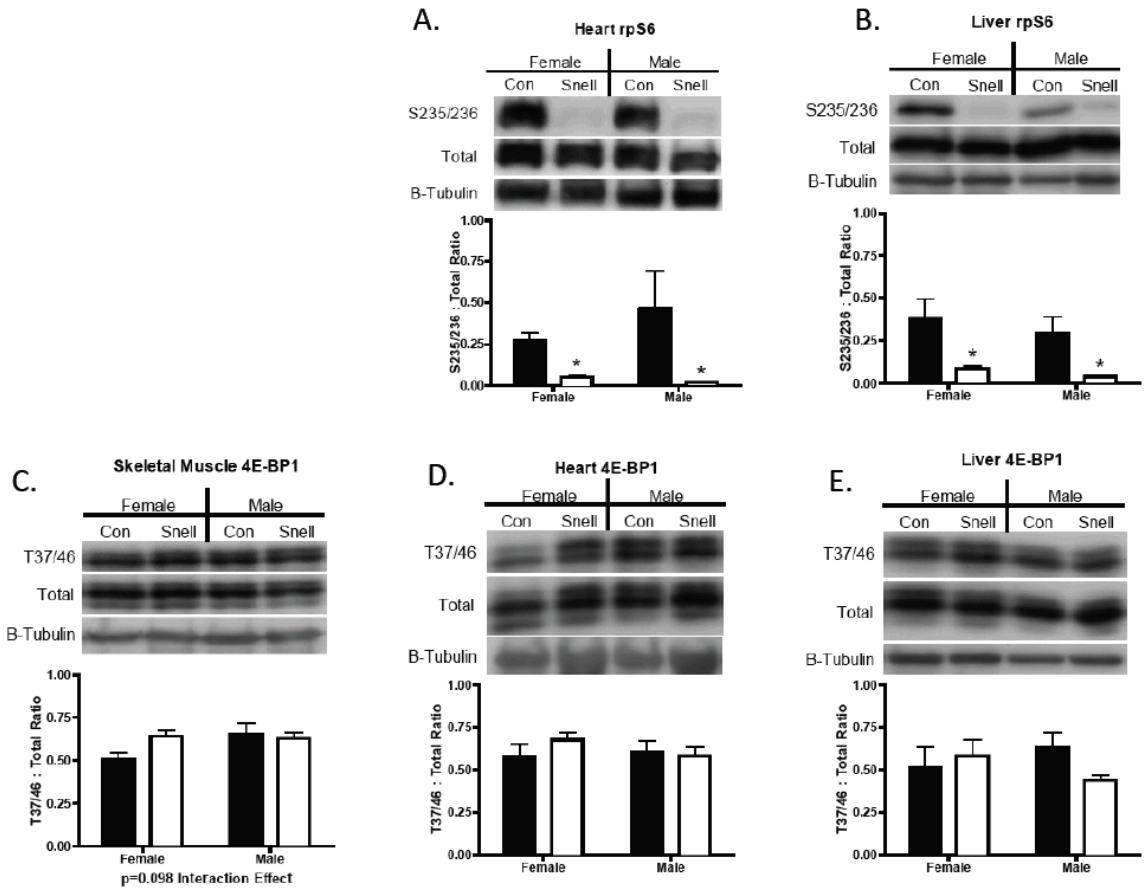

Figure 2.6. Western blotting of mTORC1 substrates rpS6 and 4E-BP1 in skeletal muscle, heart, and liver of Snell and CON. rpS6 phosphorylation was significantly decreased in heart and liver, which was conserved in both sexes (A, B). We were unable to detect rpS6 in skeletal muscle. An interaction trend in skeletal muscle (p=0.098) 4E-BP1 between condition (Snell) and sex was observed (C). No changes in 4E-BP1 were found in heart or liver between Snell and Con liver (D, E). n = 5 per sex, n = 10 per group. * = $p<0.05$ for Snell vs Con.

CHAPTER IV – MANUSCRIPT III

A novel protein and DNA synthetic response in the long-lived crowded litter mouse

Summary

Increasing litter size in mice, termed crowded litter (CL), imposes a transient energetic stress during suckling but increases lifespan through unknown mechanism(s). We have previously shown protein and DNA synthesis changes (indicative of proteostasis) alongside mTOR inhibition, in long-lived caloric restriction and rapamycin models. Therefore, we hypothesized that proteostasis may be a shared characteristic of other long-lived models, including CL mice. We assessed protein and DNA synthesis, and mTORC1 signaling in skeletal muscle, heart, and liver of 4 and 7 month (mo) CL mice. At 4 mo, DNA synthesis was greater in gastrocnemius complex of CL compared to control (CON) with no differences in protein synthesis in any tissue between groups. By 7 mo, CL had less DNA synthesis in heart and greater protein synthesis, including mitochondrial, in heart, liver, and skeletal muscle compared to CON. Furthermore, in 7 mo CL, phosphorylation of rpS6 and 4E-BP1 was increased compared to CON. These data suggest that between 4 and 7 mo in CL, new proteins are directed towards proteostasis rather than new cells, independent of mTORC1 inhibition. Together with our previous investigations, these data in CL mice support the notion that proteostasis is a shared characteristic of long-lived models.

Introduction

A progressive age-dependent reduction in protein turnover results in an accumulation of damaged proteins and propagates the development of the aging phenotype (1, 2). In tissues that have a limited proliferative capacity (e.g. heart and skeletal muscle), the ability to maintain

functional proteins (i.e. proteostasis) has become a key outcome in aging research as a means to maintain homeostatic function with age (6, 7). Thus, interventions that maintain protein stability could lead to slowed aging.

In proliferative tissues, growth is accomplished by cell replication, which requires the duplication of DNA and of cellular machinery through increased protein synthesis [reviewed in (20)]. In non-proliferative tissues (e.g. skeletal muscle) growth is accomplished by increased protein synthesis, which may be followed by recruitment of satellite cell nuclei to maintain a constant cytoplasmic to nuclear ratio (22). Since there is low enzymatic capacity for protein repair within cells, damaged proteins must be removed and subsequently replaced through synthesis (17, 18). Therefore, in addition to protein folding, trafficking, and degradation, the making of new protein is a primary means by which to regulate proteostasis (6). In order to gain insight into proteostasis the simultaneous assessment of DNA synthesis (i.e. cellular proliferation) along with protein synthesis gives context to how new proteins are used; either for new cells/growth or maintaining existing cellular structures.

The mechanistic (formerly mammalian) target of rapamycin (mTOR) signaling pathway promotes growth by increasing protein synthesis and cellular cycling via two multi-protein complexes, mTORC1 and 2 [reviewed in (13)]. Caloric restriction (CR) (68, 69) and chronic rapamycin administration (14, 51, 70) suppress mTORC1 activity and increase lifespan and healthspan in various species. Our lab has shown that in lifelong CR (11, 12) and chronically rapamycin treated mice (63), mTORC1 activity is reduced, DNA synthesis is decreased (i.e. decreased growth), but mitochondrial biogenesis and global protein synthesis are maintained. Therefore in CR mice and mice exposed to rapamycin, the maintenance of protein synthetic rates, despite lower levels of DNA synthesis and mTORC1 signaling, suggests that protein synthesis is directed toward the maintenance of existing cellular structures rather than growth (11, 12, 63). It is unknown if other long-lived models exhibit an increase in proteostatic mechanisms.

The nutritional environment of the developing fetus can have a profound impact on gene expression throughout the lifespan of the offspring, affecting healthspan and longevity (71-73). Furthermore, it has been demonstrated that changing litter size or post-natal maternal protein intake can change offspring lifespan, suggesting that some degree of genomic plasticity also remains during the postnatal suckling period (29, 73-76). For example, increasing litter size by 50% for the first 3 weeks of life, a model termed crowded litter (CL), extends mean and maximal lifespan (29). The increase in litter size presumably imposes a transient caloric restriction period until the pups are weaned and given free access to food (29). How a transient energetic stress at such a young age extends lifespan is unknown. Therefore, we hypothesized that in CL mice there would be an increase in proteostatic mechanisms that is consistent with other long-lived models in that protein synthesis in the liver, heart, and skeletal muscle would be maintained despite decreased DNA synthesis and mTORC1 signaling.

Materials and Methods

Animals

All procedures and conditions at the animal care facility meet or exceed the standards for animal housing as described in the Animal Welfare Act regulations, the Guide for the Care and Use of Laboratory Animals, and were approved by the University Committee on Use and Care of Animals at the University of Michigan. We used the genetically heterogeneous offspring of CB6F1 female and C3D2F1 male mice (UM-HET3), which have been well characterized for the development of the CL model (29, 75).

Routine veterinary care was provided by the Biomedical Science Research Building staff. UM-HET3 litters were culled to 8 and an additional 4 mice from separate litters were added, resulting in a 50% increase in litter size (29). Control (CON) UM-HET3 mice were maintained in a litter size of 8 pups. After the 3-week suckling period, female mice were weaned onto a chow diet and CL and CON were placed in their own respective cages (4

mice/group/cage) with *ad libitum* access to food and water. At 4 months (mo) (not weight stable, still growing) and 7 mo of age (weight stabilizing, and same age as our previous investigations in long-lived models) (11, 29, 63), CL (n=4, per age group) and CON (n=4, per age group) mice received deuterium labeled water (D_2O) for two weeks. After the labeling period and following an overnight fast (7 mo only), mice were euthanized using a CO_2 overdose according to the AVMA Guidelines on Euthanasia. Complete loss of pedal reflexes was confirmed before tissues were collected. The posterior aspect of the distal hind limbs (gastroc complex - gastrocnemius, soleus, and plantaris [i.e. mixed skeletal muscle]), tibialias anterior (TA, 7 mo animals only, predominately Type II fibers), heart, liver, bone marrow from the tibia, and plasma via blood from cardiac puncture were taken and immediately frozen in liquid nitrogen for later analysis.

The 4 mo mice were not fasted the night before sacrifice. Therefore, in order to assess mTORC1 signaling at a younger age, an additional cohort of approximately 3 mo CL and CON (n=4, respectively) were sacrificed following an overnight fast and gastroc complex and liver were harvested as already reported.

Labeled water

We assessed protein synthesis in mitochondrial enriched (Mito), cytosolic (Cyto), and mixed (Mix; nuclei, plasma membranes, and contractile proteins [heart and skeletal muscles]) subcellular fractions, and whole tissue DNA synthesis in skeletal muscle, heart, and liver according to procedures previously described (11, 12, 45, 46, 63). Animals received an intraperitoneal injection of 99% enriched D_2O to enrich the body water pool (assumed 60% of body weight) to 8% (11, 46). Animals were then received 8% D_2O in their drinking water with *ad libitum* access for the next 2 weeks.

Tissue isolation - Tissue were fractionated according to our previously published procedures (11, 12, 47, 63). Tissues (25-60 mg) were homogenized 1:10 in isolation buffer (100 mM KCl, 40 mM Tris HCl, 10 mM Tris Base, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, pH=7.5) with phosphatase and protease inhibitors (HALT, Thermo Scientific, Rockford, IL, USA) using a bead homogenizer (Next Advance Inc, Averill Park, NY, USA). After homogenization, sub-cellular fractions were isolated via differential centrifugation as previously described (11, 12, 63). Once fraction pellets were isolated and purified, 250 μl 1 M NaOH was added and pellets were incubated for 15 min at 50 °C and 900 rpm.

Preparation of analytes for mass spectrometric analyses - Protein was hydrolyzed by incubation for 24 hrs at 120 °C in 6 N HCl. The hydrolysates were ion-exchanged, dried under vacuum, and resuspended in 1 mL molecular biology grade H_2O. 500 μl of suspended samples were derivatized (500 μl acetonitrile, 50 μl 1 M K_2HPO_4 (pH = 11), and 20 μl of pentafluorobenzyl bromide (Pierce Scientific, Rockford, IL, USA)), sealed, and incubated at 100 °C for 1 hr. Derivatives were extracted into ethyl acetate. The organic layer was removed and dried by N_2 followed by vacuum centrifugation. Samples were reconstituted in 1 ml ethyl acetate then analyzed.

To determine body water enrichment, 125 μl of plasma was placed into the inner well of o-ring screw on cap and placed inverted on heating block overnight. 2 μl of 10 M NaOH and 20 μl of acetone was added to all samples and to 20 μl 0-20% D_2O standards and capped immediately. Samples were vortexed at low speed and left at room temperature overnight. Extraction was performed by the addition of 200 μl hexane. The organic layer was transferred through anhydrous Na_2SO_4 into GC vials and analyzed via EI mode using a DB-17MS column.

GC-MS analysis of derivatized amino acids – The pentafluorobenzyl-*N*,*N*-di(pentafluorobenzyl) derivative of analnine was analyzed on an Agilent 7890A GC coupled to an Agilent 5975C MS as previously described (11, 12, 49, 63). The newly synthesized fraction (f) of proteins was calculated from the true precursor enrichment (p) using plasma analyzed for D_2O enrichment and adjusted using mass isotopomer distribution analysis (MIDA) (45). Protein synthesis was calculated as the ratio of deuterium-labeled to unlabeled alanine (45) bound in proteins over the entire labeling period and expressed as fraction new in 2 weeks.

DNA isolation – Approximately 75 ng/ul (heart, gastroc complex, and TA) and 400ng/ul (liver) of total DNA was extracted from ~15 mg tissue (QiAamp DNA mini kit Qiagen, Valencia, CA, USA). DNA from bone marrow was isolated by extracting ~300 mg from the tibial bone marrow suspension and centrifuged for 10 min at 2000 g.

DNA synthesis measurement - Determination of ^2H incorporation into purine deoxyribose (dR) of DNA from whole tissue and bone marrow was performed as described previously (11, 45, 49, 63). Briefly, isolated DNA was hydrolyzed overnight at 37 °C with nuclease S1 and potato acid phosphatase. Hydrolysates were reacted with pentafluorobenzyl hydroxylamine and acetic acid and then acetylated with acetic anhydride and 1-methylimidazole. Dichloromethane extracts were dried, resuspended in ethyl acetate, and analyzed by GC/MS as previously described (11, 49, 63). The fraction new in 2 weeks was calculated by comparison with bone marrow (representing an essentially fully turned over cell population and thus the precursor enrichment) in the same animal (11, 12, 63).

Western blotting

A portion of the Cyto fraction from 7 mo and the additional 3 mo, fasted cohort was used for western blot analysis. Protein concentration was determined using a bicinchoninic acid

assay (Thermo Fisher, Rockford, IL, USA). Samples were diluted to the same concentration, boiled with Laemmli buffer, then 30-45 μg of protein was separated using 10 % SDS-PAGE at 100 V. Proteins were transferred at 4 °C (100 V for 75 min in 20 % w/v methanol, 0.02 % w/v SDS, 25 mM Tris Base, 192 mM glycine, pH=8.3) to nitrocellulose paper and incubated in 5 % nonfat dry milk in Tris-buffered saline with Tween20 (TBST) for 1 hr. Antibodies were purchased from Cell Signaling Technologies (Boston, MA, USA; rpS6 phospho-Ser[235/236] #4858, rpS6 total #2217, 4E-BP1 phospho [Thr37/46] #9459, 4E-BP1 total #9452, phospho-p90 RSK (S380) #12032, RSK total #9355, phospho-mTOR [S2448] #2971) or Santa Cruz Biotechnology (Santa Cruz, CA, USA; (β-tubulin #sc-5274). Blots were incubated overnight with primary antibodies diluted 1:500 (skeletal muscle rpS6, all mTOR), 1:1000, 1:2000 (Liver rpS6), or 1:500 (β-tubulin and RSK). Blots were washed in 1x TBST and incubated with anti-rabbit or anti-mouse (β-tubulin) HRP-conjugated secondary antibody diluted 1:5000 in 5% milk with subsequent chemiluminescence detection (West Dura; Pierce, Rockford, IL, USA). Images were captured and densitometry analyzed by UVP Bioimaging system (Upland, CA, USA). Blots were probed for phosphorylated proteins first, placed in stripping buffer (GM Biosciences, Rockville, MD, USA) and then re-probed for total protein, except for total mTOR, which we could not detect and therefore S2448 mTOR was normalized to β-tubulin. Equal loading was verified using ponceau-s staining and β-tubulin. Due to undetectable rpS6 in skeletal muscle homogenate, a portion of the homogenate was acetone precipitated then analyzed via western blotting.

Statistics

Statistical analysis was performed using Prism V4.0c (GraphPad Software, Inc. La Jolla, CA, USA). Protein synthesis data were assessed by two-way ANOVA. DNA synthesis and western blotting were assessed via two-sided Student's t-test. Variances were normalized via

log-transformation where necessary. Significance was set at $p < 0.05$, and p values of ≤ 0.10 are noted. Data are presented as means ± standard error of the mean (SEM).

Results

Protein and DNA synthesis

In the gastroc complex, heart, and liver of 4 mo CL mice, there were no differences in protein synthesis within the Mix, Cyto, or Mito fractions compared to 4 mo CON (Fig 3.1A, C, E). By 7 mo of age there was a dramatic change, in that gastroc complex, heart, and liver (Fig 3.1B, D, F) as well as TA (Fig S3.1A) all had significantly greater rates of protein synthesis in Mix, Cyto, and Mito fractions compared to 7 mo CON (Fig 3.1B, D, F). Pooled fractions (Con and CL) were significantly different from each other in all tissues and ages (Fig 3.1A-F).

In the 4 mo CL, DNA synthesis was significantly greater in the gastroc complex compared to CON (Fig 3.2A). DNA synthesis in heart trended greater in the heart of 4 mo CL compared to 4 mo CON, but did not reach statistical significance ($p=0.070$; Fig 3.2A). Liver DNA synthesis in 4 mo CL was not different from 4 mo CON (Fig 3.2A). At 7 mo, CL mice had significantly lower rates of DNA synthesis in the heart but not in gastroc and liver (Fig 3.2) or TA (Fig S3.1B).

mTOR signaling

At 3 mo (additional fasted cohort), there were no differences between rpS6 and 4E-BP1 phosphorylation between groups in either gastroc complex or liver (Fig 3.3). At 7 mo, phosphorylation of ribosomal protein S6 (rpS6) was significantly increased in heart and liver (Fig 3.4B, C). Phosphorylation of eukaryotic initiation factor 4E binding protein 1 (4E-BP1) was significantly increased in gastroc complex (Fig 3.4D) and TA (Fig S3.2B). Though they did not reach statistical significance, two-sided significance values for rpS6 in TA (Fig S3.2A), and for 4E-BP1 in heart (Fig 3.4E) were $p < 0.06$. Phosphorylation of mTOR S2448, indicative of

mTORC1 activity (77), was not different between 7 mo CL and CON in the gastroc complex, heart, or liver (Fig 3.5A, B, C), or in TA (Fig S3.2C). Phosphorylation of p90 ribosomal S6 kinase (RSK), which phosphorylates rpS6 at T389, and 4E-BP1 at T37/46 (78, 79), was not different between CON and CL in heart and liver (Fig 3.6A, B). We were unable to detect RSK phosphorylation in either gastroc complex or TA.

Discussion

We tested the hypothesis that CL mice would exhibit increased proteostatic mechanisms, in that protein synthesis would be maintained despite decreased DNA synthesis and mTORC1 signaling, consistent with other long-lived models. At 4 mo of age, protein synthesis was not different between CL and CON, but DNA synthesis was significantly greater in the gastroc complex and tended to be greater in the heart of CL compared to CON. At 7 mo of age, however, protein synthesis was significantly greater in all tissues and fractions while DNA synthesis was significantly less in the heart of CL compared to CON. Furthermore, at 3 mo of age, the phosphorylation of mTORC1 substrates was not different between groups in any tissue, while at 7 mo of age rpS6 (in heart and liver) and 4E-BP1 (in gastroc complex and TA) was greater in CL compared to CON. Collectively, we demonstrate for the first time that the transient energetic stress of a 50% increase in litter size leads to alterations in protein and DNA synthesis, indicative of increased proteostatic mechanisms, in mice at 7 mo of age. Tissue dependent increases in mTORC1 activity in the long-lived CL model are in contrast to the reduced mTORC1 activity seen in long lived caloric restricted, rapamycin-treated, and $S6K1^{-/-}$ mice (11, 14, 63, 65), and the unaltered mTORC1 activity in long-lived methionine-restricted mice (29). Therefore, proteostasis in CL may be accomplished by different mechanism(s) compared to lifelong CR and chronic rapamycin feeding.

Postnatal feeding and growth

Gene expression during *in utero* development can be profoundly affected by maternal nutrition, with lifelong effects on the healthspan and longevity of offspring (71-73). Changes in lifespan and healthspan through litter reduction in rats (3-4 pups/litter) [reviewed in (74)] as well as litter enlargement (i.e. CL) experiments in mice (29, 75) suggest that nutritional status during early development can have long-lasting effects. Whereas reducing litter size increases pups' caloric intake leading to a vast array of pathologies associated with poor healthspan [reviewed in (74)], CL is the antithesis. CL extends mean and maximal lifespan (29) and upregulates the transcription of at least some phase I xenobiotic enzymes (75), which are protective against environmental toxins. However, it is not known how an increase in litter size affects the synthetic processes that contribute to growth and increased lifespan and healthspan.

In studies of proliferative cells, new DNA may be indicative of tissue growth through cell replication [reviewed in (20)]. In our studies using models of slowed aging (11, 12, 63), human exercise (49), and our current data in CL mice, we have repeatedly demonstrated synthesis of new DNA in post-mitotic cardiac and skeletal muscle tissues. In addition, we have found that the rates of DNA synthesis in these tissues can change with interventions that slow aging or with exercise. The use of D_2O to measure DNA synthesis is reflective of S-phase synthesis and not DNA repair (46). Therefore, we propose two possibilities for measurable changes in DNA synthesis in post-mitotic tissues: 1) there is some mitotic ability in cardiac myocytes and skeletal muscle myofibers that are not fully appreciated, or 2) there are other cellular sources of new DNA in these tissues that change based on our interventions. For example, it appears that cardiomyocytes have an ability to replicate their own DNA (80, 81), or incorporate DNA from other neighboring cell types (e.g. satellite cells) (82, 83). In addition, it appears that the regulation of cardiac DNA synthesis may vary by stage of development (84). Importantly though, a potential increase in heart DNA synthesis in 4 mo CL mice is in agreement with progenitor cell-independent cardiomyocyte enlargement and/or number (80, 81), which is

indicative of growth. Similarly, skeletal muscle is primarily made up of post-mitotic, multinucleated myofibers, but also contains resident proliferative satellite cells, pericytes, interstitial cells, and myoendothelial cells (85-87). Previous work on exercising humans in our lab suggest that it is unlikely that other cell types or mitochondrial DNA synthesis contribute significantly to our measurement of total DNA synthesis in skeletal muscle using D_2O (49). When myofiber size increases, there is an increase in satellite cell recruitment to stabilize the myonuclear domain (49, 61), which isotopic labeling has been shown to reflect (88). Therefore, skeletal muscle growth is characterized by increased protein synthesis, increasing myocyte size, and resident satellite cell duplication with subsequent donation of DNA to maintain the myonuclear domain. To summarize, we believe that changes in DNA synthesis in post-mitotic tissues provides novel insights into growth regulation of long-lived models and may be indicative of cardiomyocyte and skeletal muscle myofiber proliferation, however, the contribution of other sources of new DNA remains a possibility.

Proteostasis within long-lived models

Proteostasis has become a key outcome in aging research as a means to maintain homeostatic function with age (6, 7). Low enzymatic capacity for protein repair within cells means that damaged proteins must be removed and then replaced through synthesis of new proteins (17, 18). Therefore, the making of new protein is a key mediator of proteostasis (6). In our present study we found changes in DNA synthesis in post-mitotic tissues that are consistent with our previous investigations (11, 12, 49, 63), and that indicate there is a period of growth, or catch up growth, to 4 mo but evidence of increased proteostatic mechanisms by 7 mo in CL mice. In light of our previous investigations in long-lived models, we posit that a reduction in cellular proliferation allows for newly synthesized proteins to be dedicated to proteostasis (6, 11, 12, 63) of skeletal muscle mitochondria and the heart proteome (11, 63), contributing to increased lifespan and healthspan. Collectively, this led us to hypothesize that decreased

cellular proliferation and preferential maintenance of protein synthesis may be a shared characteristic of long-lived models. However, the long-lived CL mouse at 7 mo is characterized by increased protein synthesis in all tissues and subcellular fractions within skeletal muscle, heart, and liver, differing from long-lived CR and chronic rapamycin fed mouse models of the same age (11, 63). We also highlight elevated rates of mitochondrial protein synthesis, indicative of mitochondrial biogenesis (31), which are consistent with our previous work in other long lived models and hypothesized to be key to slowed aging (11, 38, 63). Increases in protein synthesis are necessary for growth to ensure duplication of cellular machinery upon cell division [reviewed in (20)]. Increased protein synthesis while DNA synthesis is decreased (heart) or unchanged (gastroc complex, TA, and liver) in 7 mo CL suggests increased proteostatic mechanisms since the increased synthesis of new proteins is not supplying an elevated rate of cell division. It is important to note that the CL model is generated from mice of the same heterogeneous background (UM-HET3) as in our previous investigation of chronic rapamycin feeding (63), and thus strain differences cannot account for their differing protein and DNA synthetic responses. In summary, the CL model supports the concept of increased proteostatic mechanisms (6, 11, 12, 63) as described in our previous studies using long-lived CR and chronic rapamycin models. Further, we speculate that in CL mice, a tissue-dependent tradeoff from growth to proteostasis occurs between 4 mo and 7 mo of age that is characterized by increased protein synthetic rates in all subcellular fractions despite decreased or unchanged rates of DNA synthesis (11, 12, 63).

CL growth signaling

mTOR is a central regulator of protein turnover, cell cycle, and mRNA translation [reviewed in (13)]. Inhibiting or downregulating mTORC1 signaling is suggested to be integral to the lifespan extension imparted by CR and chronic rapamycin treatment (11, 12, 14, 28). Expanding upon our protein synthesis findings in the long-lived CL mouse, we assessed two

mTORC1 substrates, rpS6 and 4E-BP1, which are involved in ribosome biogenesis and translation initiation, respectively. In a young, fasted cohort (approximately 3 mo of age) of CL and CON mice, phosphorylation of the mTORC1 substrates rpS6 and 4E-BP1 was not different between 3 mo CL and CON in any tissue, corroborating the protein synthesis measurement in 4 mo CL. In contrast to our previous findings in age-matched lifelong CR and chronic rapamycin treatment, in 7 mo CL mice rpS6 phosphorylation was greater in heart and liver while 4E-BP1 phosphorylation was greater in the gastroc complex and TA compared to 7 mo CON (11, 12, 63). Together these data suggest the novel finding that mTORC1 activity increases from 4 to 7 mo of age in the long-lived CL mouse.

Both mTORC1 and the MAPK substrate RSK can phosphorylate rpS6 at S235/236 and 4E-BP1 at T37/46 (78, 79). Therefore, we assessed markers of both mTORC1 (mTOR S2448) and RSK (S380) activity. mTOR phosphorylation at S2448 was not statistically different between 7 mo CL and CON in any tissue. RSK phosphorylation at S380 was not different in heart or liver and was undetectable in the gastroc complex and TA. Given the wide variance in some tissues (Fig 5A, C and Fig 6), it is possible that a type II error is limiting our ability to observe true differences between RSK and mTORC1 phosphorylation in 7 mo CL and CON. However, regardless of the upstream regulator (mTORC1 or RSK), the increase in rpS6 and 4E-BP1 phosphorylation is consistent with the increased rates of protein synthesis observed in CL at 7 mo. That proteostasis and extended lifespan occur in the CL mouse despite tissue-dependent increases in mTORC1-related signaling leads us to believe that: 1) a decrease in mTORC1 signaling may not be necessary for lifespan extension (29), 2) there are alternative pathways that lead to increases in proteostatic mechanisms.

Summary and conclusion

Here we demonstrate in long-lived CL mice, that a transient energetic stress during the suckling period results in long-term alterations in protein and DNA synthesis that differ between

4 and 7 mo. We propose that between 4 and 7 mo there is a switch from growth to proteostasis. We also present novel evidence that in CL mice the increase in proteostatic mechanisms at 7 mo is accomplished independently of reduced mTORC1 activity in some tissues. These data highlight the importance of assessing pathways thought to be integral to slowed aging, such as mTOR, at multiple time points across the lifespan. Collectively, we expand on the body of evidence establishing CL as a novel model of longevity that may provide valuable insights into growth and proteostasis and could aid in the development of novel strategies to promote slowed aging.

Addendum to manuscript III

Retrospective analyses of proteostasis, as evidenced through the comparison of the rate of new proteins to the rate of cellular proliferation, can be found in Fig 4.1 and Appendix II.

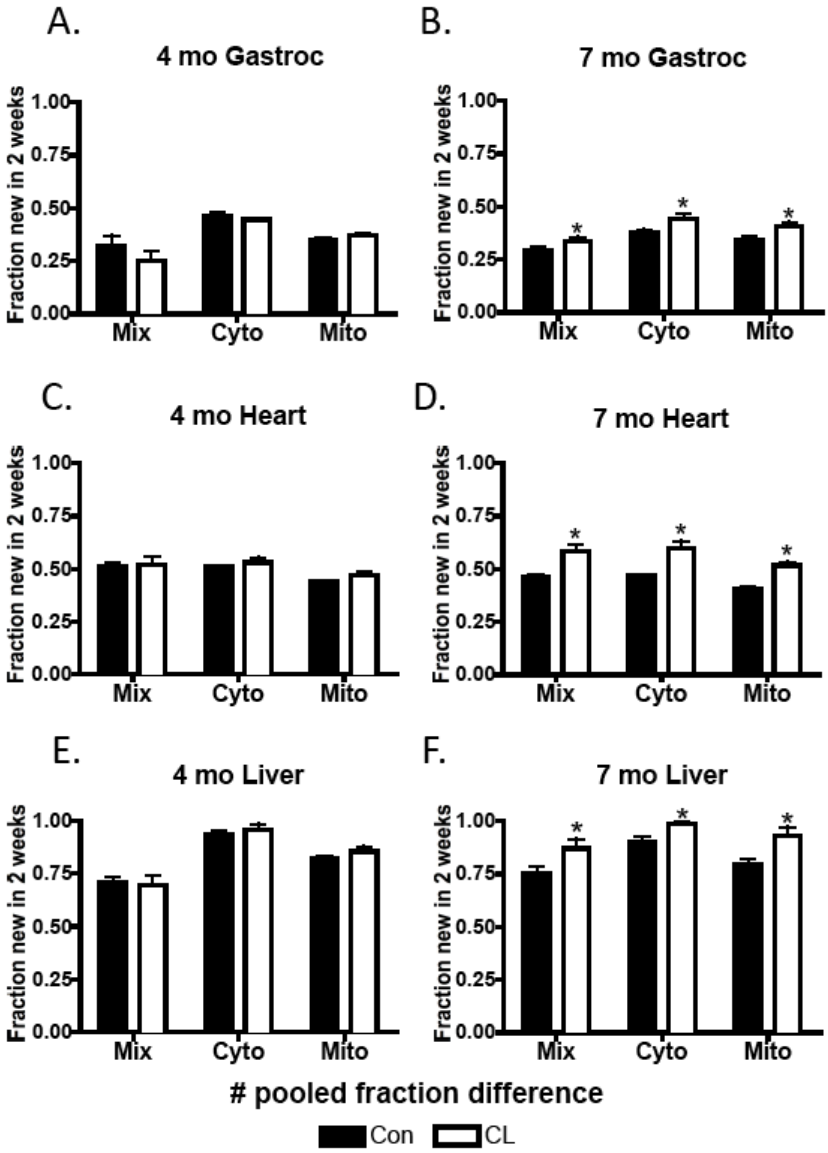

Figure 3.1. Protein synthesis in gastroc complex, heart, and liver over 2 weeks in 4 mo and 7 mo CL and CON. There were no differences in protein synthesis between groups in any tissue between 4 mo CL and 4 mo CON (A, C, E). Protein synthesis was significantly higher in all tissues and fractions in 7 mo CL compared to 7 mo CON (B, D, F). n=4 per group and fraction except liver. Liver 7 mo CL Cyto and Mito n=2 because in 2 animals these fractions were fully turned over in the liver (i.e. 100% new) after 2 weeks. * = $p<0.05$ for CL vs. CON; # = $p<0.05$ difference between pooled (Con and CL) fractions in all tissues at both 4 and 7 mo.

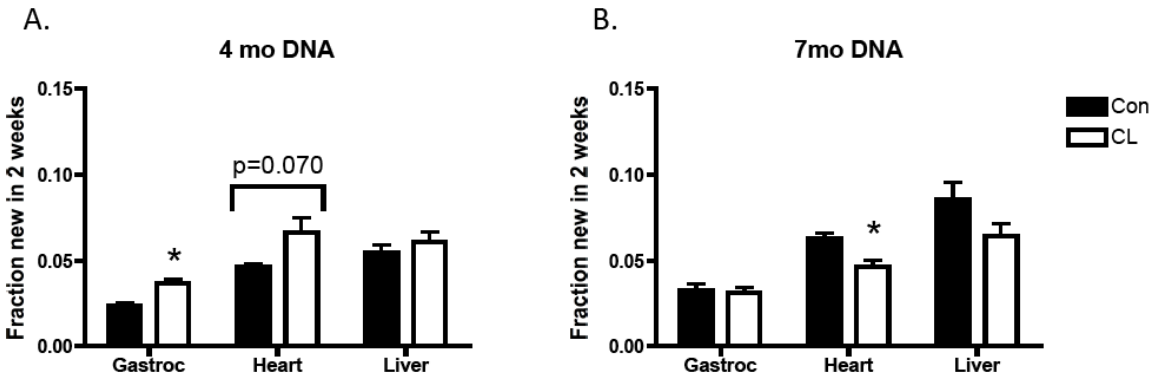

Figure 3.2. DNA synthesis in gastroc complex, heart, and liver over 2 weeks in 4 mo and 7 mo CL and CON. Gastroc complex DNA synthesis was significantly greater in 4 mo CL compared to 4 mo CON (A). Heart DNA synthesis in 4 mo CL tended to be greater (p=0.070) than 4 mo CON (A). There were no differences in liver DNA synthesis between 4 mo animals (A). In the 7 mo animals, DNA synthesis was significantly less in CL heart compared to CON (B). No significant differences were observed between 7 mo CL and 7 mo CON in gastroc complex or liver (B). DNA synthesis rates in heart tissues from 4 mo mice were log transformed to normalize variance. n=4 in both ages and groups except in 7 mo CON gastroc complex and 7 mo CL gastroc complex, heart, and liver n=3 due to one data point being excluded as an outlier. * = $p<0.05$ for CL vs CON.

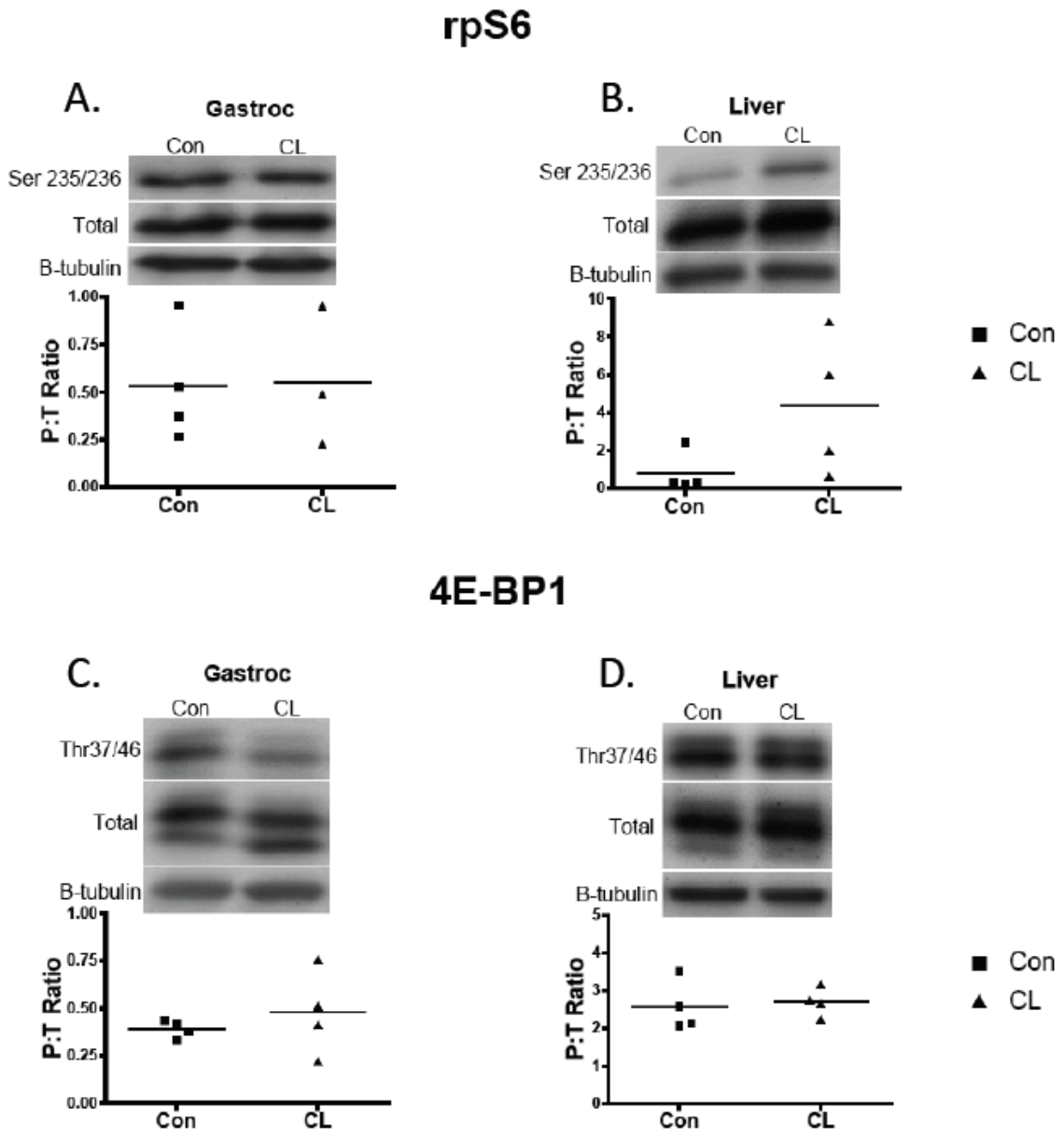

Figure 3.3. Western blotting of mTORC1 substrates rpS6 and 4E-BP1 in fasted 3 mo CL mice. No difference between 3 mo CL and CON was found in either rpS6 or 4E-BP1 phosphorylation in gastroc complex (A, C) or liver (B, D). Data are expressed as a ratio of phosphorylated:total protein. β-tubulin is shown to verify equal loading of protein. Gastroc rpS6 in CL has an n=3 because one band was not visible to assess and thus was excluded to avoid misrepresenting the data. n=4 per all other groups.

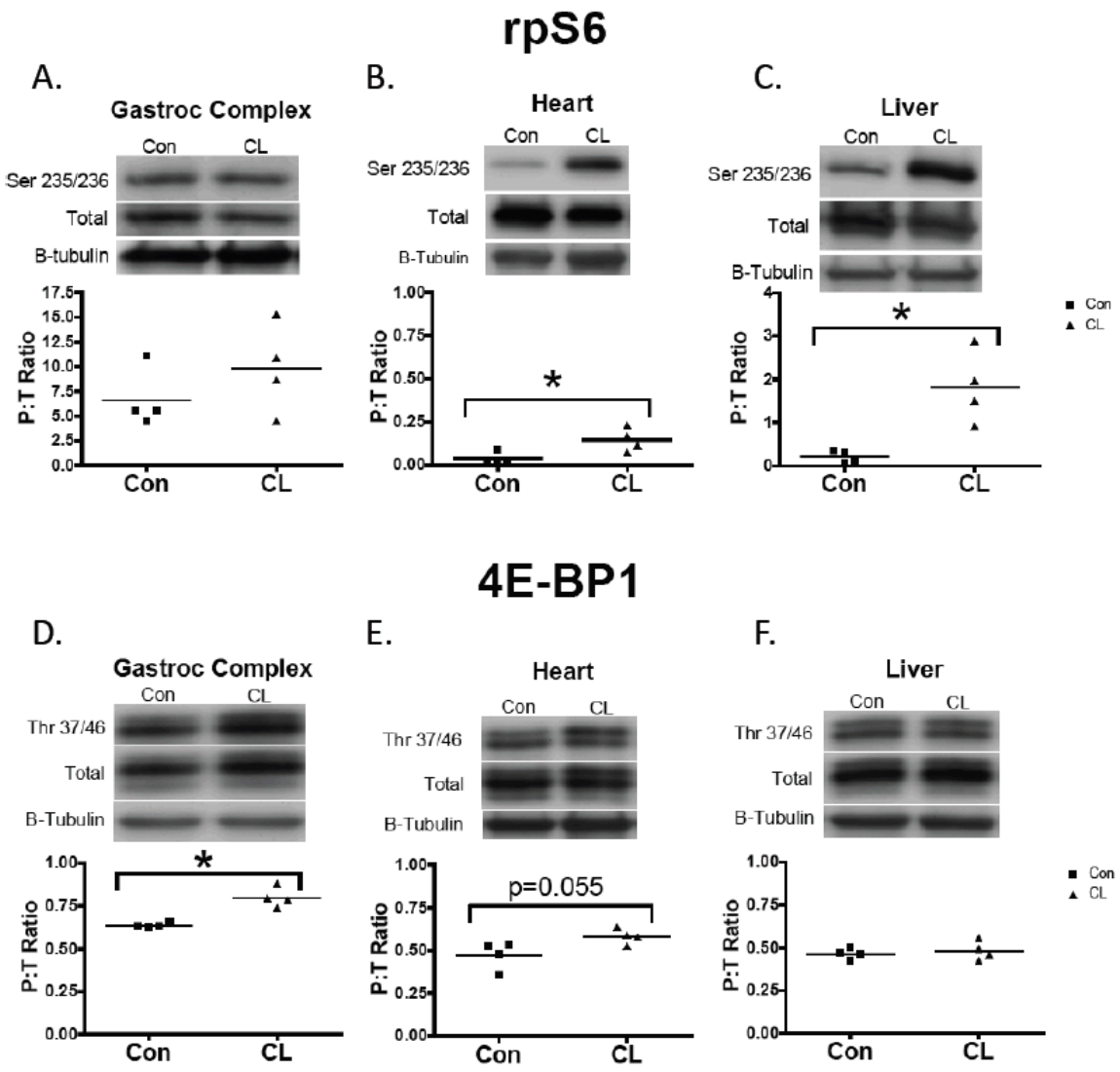

Figure 3.4. Western blotting of mTORC1 substrates rpS6 and 4E-BP1 in 7 mo CL mice. rpS6 phosphorylation was significantly greater in heart and liver in 7 mo CL compared to CON (B and C). 4E-BP1 phosphorylation was significantly greater in gastroc complex and tended to be greater in heart (0.055) in 7 mo CL compared to 7 mo CON (D and E). Data are expressed as a ratio of phosphorylated:total protein. β-tubulin is shown to verify equal loading of protein. Liver rpS6 was log transformed to normalize variance. n=4 per group. * = $p<0.05$ for CL vs CON.

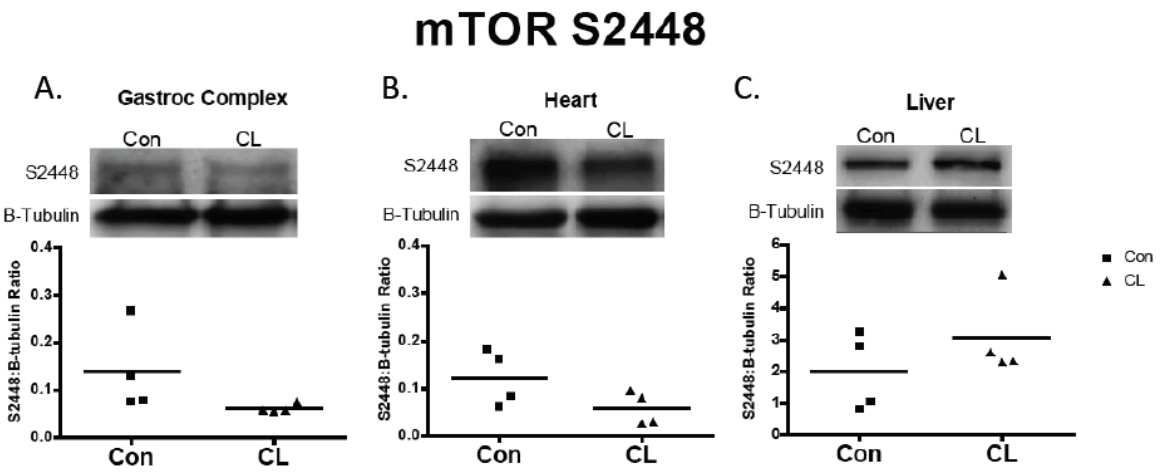

Figure 3.5. Western blotting of mTOR in 7 mo CL mice. mTOR phosphorylation at S2448 was not statistically significant in any tissue between groups at 7 mo of age (A-C). Data are expressed as a ratio of phosphorylated mTOR to β-tubulin because total mTOR protein was not visible. n=4 per group. * = $p<0.05$ for CL vs CON.

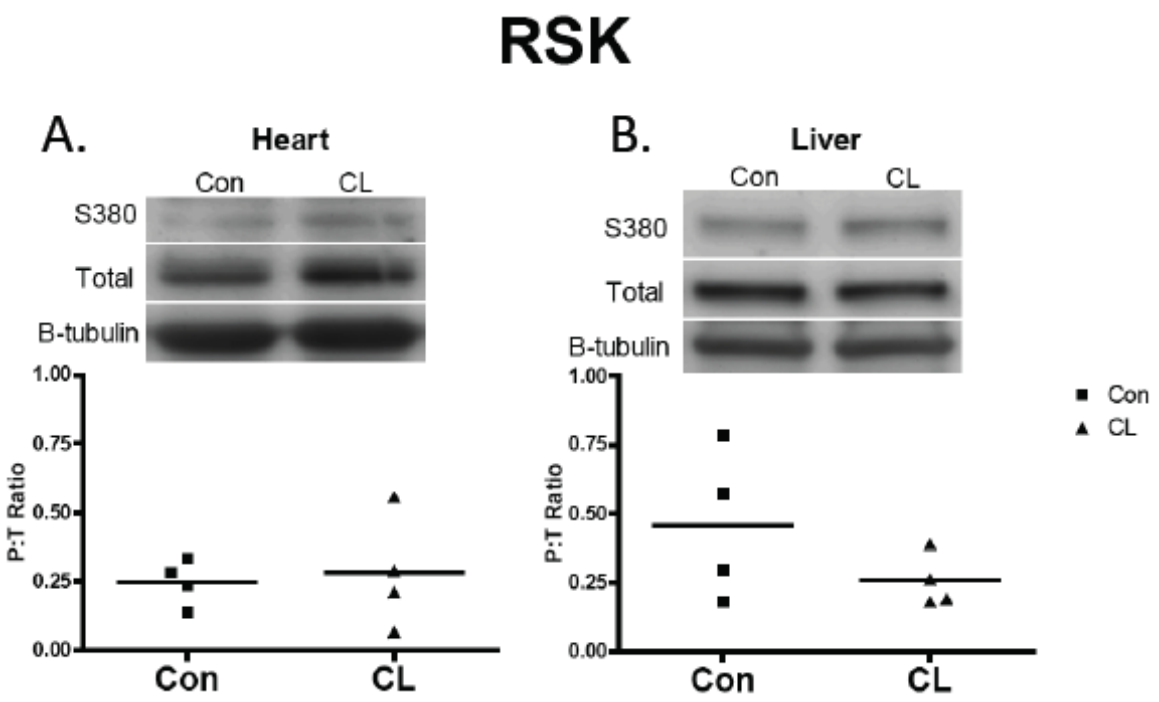

Figure 3.6. Western blotting of RSK in 7 mo CL mice. RSK phosphorylation at S380 was not different between groups in heart and liver at 7 mo of age (E, F). We were unable to detect S380 RSK in gastroc complex at 7 mo of age. Data are expressed as a ratio of phosphorylated:total protein. β-tubulin is shown to verify equal loading of protein. Liver mTOR data were log transformed to normalize variance. n=4 per group.

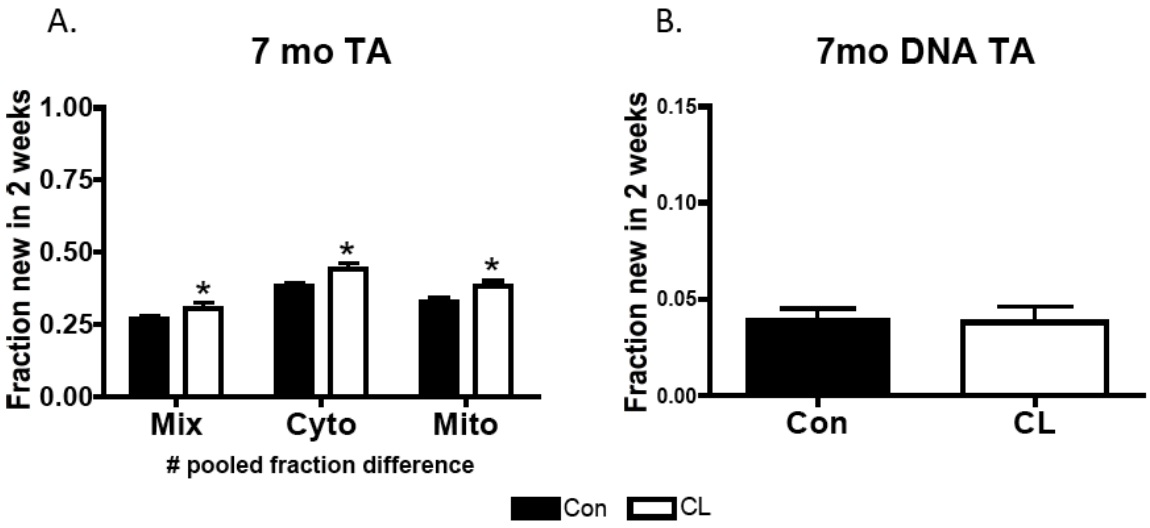

Figure S3.1. Protein and DNA synthesis in the TA muscle of 7 mo CL mice. Protein synthesis was significantly higher in all fraction in TA of CL compared to CON at 7 mo (A). No significant differences in TA DNA synthesis were observed between 7 mo CL and 7 mo CON (B). TA Protein synthesis n=4 per group and fraction. TA DNA synthesis n=3 due to insufficient sample to measure DNA synthesis in one animal. * = $p<0.05$ for CL vs CON. # = $p<0.05$ difference between pooled (CON and CL) fractions.

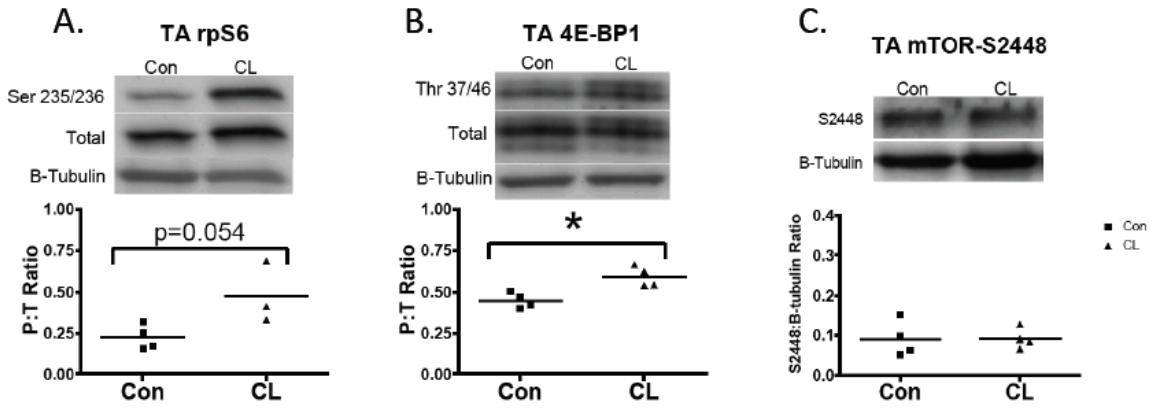

Figure S3.2. Western blotting of rpS6, 4E-BP1, and mTOR in TA of 7 mo CL mice. rpS6 phosphorylation tended to be greater in TA of 7 mo CL compared to 7 mo CON (A). 4E-BP1 phosphorylation was significantly greater in TA of CL compared to CON at 7 mo (B). mTOR phosphorylation at S2448 was not different in TA between 7 mo CL and CON (C). TA rpS6 in CL has an n=3 because one band was not visible to assess and thus was excluded to avoid misrepresenting the data. n=4 per all other groups. Data are expressed as a ratio of phosphorylated:total protein, except for mTOR where S2448 is expressed as a ratio to β-tubulin because total mTOR protein was not visible. β-tubulin is shown for rpS6 and 4E-BP1 blots to verify equal loading of protein. * = $p<0.05$ for CL vs CON.

CHAPTER V – OVERALL CONCLUSIONS

In this current series of studies we identified how changes in protein synthesis, an essential component of proteostasis, are associated with slowed aging. Furthermore, these studies demonstrate the evolution of our thought process; from measuring protein synthesis independently, to considering it in the context of tradeoffs between growth and maintenance, then effectively assessing components of proteostasis. Collectively, we provide evidence to suggest that an increase in proteostatic mechanisms is a shared characteristic of slowed aging.

Past work in our lab on the long-lived caloric restriction model demonstrated that despite reduced cellular proliferation and mTORC1 signaling, the synthesis of mitochondrial proteins was maintained (11). We reasoned that energetic savings from reduced cellular proliferation may have allowed for the maintenance of mitochondria biogenesis (i.e. somatic maintenance). In the first study of the dissertation, we expanded upon our findings in caloric restriction by investigating mice chronically administered the mTORC1 inhibitor, and proposed caloric restriction mimetic, rapamycin. Here we showed that mitochondrial proteins in skeletal muscle were preferentially synthesized despite decreased synthesis in mixed and cytosolic protein fractions, cellular proliferation, and mTORC1 (63). Since reduced protein turnover is thought to be causative to the development of the aging phenotype (1-4), we concluded that reduced cellular proliferation allowed for energy to be diverted to the somatic maintenance of key proteins (15, 16), such as mitochondrial proteins, contributing to slowed aging in both the caloric restriction and rapamycin treated mouse models.

The conclusion of increased somatic maintenance in caloric restriction and Rap models required the assumption that either cellular energetics were stabilized or that the energetic contribution to metabolism was unchanged (11, 15, 16, 63). Further reflections upon our findings in caloric restriction and rapamycin models led us to limit the discussion to proteostasis,

a component of somatic maintenance, rather than of somatic maintenance itself since we could not account for other sources of energetic changes. Protein synthesis is essential for both proliferation/hypertrophy (20-25) and replacing damaged proteins (17-19). By evaluating our data as a ratio of the rate of protein synthesis to the rate of DNA synthesis, we were able to give an estimate of whether new proteins were used for equipping new cells or maintenance of existing cellular structures (Appendix II). Under this new framework, in the Snell dwarf mice, we compared new proteins synthesized to the rate of cellular proliferation and showed that the protein:DNA synthesis ratio was 3-fold higher than it was in control mice across sub-cellular fractions, which we take as evidence of an increase in proteostatic mechanisms.

Finally, we investigated a presumed epigenetic model, the crowded litter mouse, in which a transient intervention of reduced nutrient intake during the suckling period extends lifespan. We demonstrated that in crowded litter, there is a progression from growth at a younger age towards an increase in proteostatic mechanisms later in life. The increase in protein synthesis and mTORC1 within crowded litter later in life was in contrast to our other age-matched investigations in mice undergoing caloric restriction, rapamycin treatment, and Snell dwarf mice. Through comparing the increased protein synthesis to our other finding of unchanged DNA synthesis in crowded litter, we illustrated a similar increase as in our previous models in the new protein to new DNA ratio, indicative of proteostatic mechanisms. These data in crowded litter suggest the novel finding that increases in proteostatic mechanisms can be independent of decreased mTORC1.

To summarize, our objective was to understand the role that changes in protein synthesis have in slowed aging (Fig 4.1). We have demonstrated that decreased mTORC1 in some models of long-life does not necessarily equate to decreased protein synthesis across sub-cellular fractions and tissues. Discerning specific proteins and the mechanism(s) by which a subset of proteins can be preferentially synthesized despite decreases in protein synthesis in other fractions and decreased mTORC1, may give further insight into mechanisms of slowed

aging. By considering protein synthesis rates in relation to the synthesis of new DNA, we present novel insight into proteostasis. We demonstrate that increased proteostatic mechanisms is a shared characteristic of multiple unique models of long-life and therefore has potential for future therapeutic development.

Model	Tissue	Fraction	Protein Synthesis	DNA Synthesis	New Pro:DNA Ratio	rpS6	4E-BP1
Rapamycin		Mix	↓	↓	≈	↓	≈
		Cyto	↓				
		Mito	≈				
		Mix	≈	↓	↑	↓	≈
		Cyto	≈				
		Mito	≈				
		Mix	n/a	↓	n/a	↓	≈
		Cyto	n/a				
		Mito	n/a				
Snell Dwarf		Mix	↓	↓	↑	n/a	≈
		Cyto	↓				
		Mito	↓				
		Mix	↓	↓	↑	↓	≈
		Cyto	↑				
		Mito	↓				
		Mix	≈	≈	≈	↓	≈
		Cyto	≈				
		Mito	≈				
Crowded Litter		Mix	↑	≈	↑	≈	↑
		Cyto	↑				
		Mito	↑				
		Mix	↑	↓	↑	↑	↑
		Cyto	↑				
		Mito	↑				
		Mix	↑	≈	≈	↑	≈
		Cyto	↑				
		Mito	↑				

Figure 4.1. Summary of protein and DNA synthesis, Rate of new Pro:DNA ratio, and mTORC1-related signaling in all models and tissues. Arrows indicate the change compared to corresponding control. Findings that were statistically not different from their corresponding control are indicated by (≈). Data that was unable to be assessed is indicated by (n/a).

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APPENDIX I – MANUSCRIPT IV[2]

Calorie restriction does not increase short-term or long-term protein synthesis

Summary

Increased protein synthesis is proposed as a mechanism of lifespan extension during caloric restriction (CR). We hypothesized that CR does not increase protein synthesis in all tissues and protein fractions, and that any increased protein synthesis with CR would be due to an increased anabolic effect of feeding. We used short (4 hour) and long-term (6 weeks) methods to measure in vivo protein synthesis in lifelong ad libitum (AL) and CR mice. We did not detect an acute effect of feeding on protein synthesis in any tissue or fraction, while liver mitochondrial protein synthesis was lower in CR mice versus AL mice. mTOR signaling was repressed in liver and heart from CR mice indicative of energetic stress and suppression of growth. Our main findings were that CR did not increase rates of mixed protein synthesis over the long term or in response to acute feeding, and protein synthesis was maintained despite decreased mTOR signaling.

Introduction

Caloric restriction (CR) without malnutrition is an intervention that consistently increases maximum lifespan across a variety of species. CR can increase mean and maximum lifespan by 40%, and since it is relatively simple and the outcomes robust, it is a good model to study the mechanisms of aging (1). Consistent with evolutionary theories of aging, it is thought that CR induces a state of somatic maintenance, which is anti-aging, rather than the reproductive state

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that is pro-aging (2). Somatic maintenance implies an increase in repair processes, which is supported by evidence that CR inhibits the accumulation of oxidatively damaged proteins (3), increases respiratory control (4), increases mitochondria efficiency (5), and increases autophagy (6).

It has long been recognized that protein and energy metabolism are dependent on each other (7-9). The process of protein synthesis is energetically expensive (10) requiring the greatest proportion (20.5%) of basal respiratory rate in isolated cells (11). In times of energy deficit, either absolute energy deficiency or decreased energetic flux, it is not energetically economical to build up large stores of protein. Given the dependence of protein turnover on energy status, it is surprising that during CR protein turnover is observed to increase in some reports (4, 12-14), and that CR increases transcription of genes associated with increased protein turnover (15).

Protein turnover is tissue specific. In a series of studies on male Sprague-Dawley rats maintained on 50% CR from 3 weeks of age, there was an increase in whole body protein turnover (13), variable results in *tibialis anterior* or *soleus* muscle protein turnover (12) and no change in lung protein turnover (16). Recently, it has been reported that CR does not change actin and myosin turnover in either *tibialis anterior* or *soleus* muscles in male Wistar rats on a 40% restricted diet for 5 months (4) and that short-term (7 day) CR of 40% in male Wistar rats decreases protein turnover in liver and skeletal muscle, but is maintained in heart (17). We (Price et al, Manuscript submitted) used an LC-MS/MS technique, in which in vivo tissue protein synthesis rates are measured by MIDA analysis of D_2O incorporation into peptides fragments, to measure dynamics of the global proteome and found a marked reduction in synthesis rates of almost all proteins in the hepatic proteome, particularly mitochondrial proteins, in rats maintained on chronic CR. In our previous investigation into B6D2F1 mice on lifelong 40% CR, it was found that mitochondrial protein synthesis was maintained with CR compared to AL in heart and skeletal muscle when assessed over 6 weeks (18). Although some have summarized

that CR increases protein turnover (19, 20), the issue does not seem truly resolved, since tissue-specific responses are not consistent.

Much of what we currently know about the rates of protein turnover was obtained by the use of stable or radioactive isotopic tracers. In a series of experiments it was determined that the commonly used method of using flooding doses of essential amino acid tracers were increasing protein synthesis rates (21, 22), an intrinsic limitation based on principles of tracer methodology since the tracer changed the process that was being measured. Subsequent studies have identified leucine-stimulated mTOR signaling and downstream activation of translation initiation factors as the means by which a large amount of essential amino acids stimulated protein synthesis (23). As discussed previously, in general, protein synthesis has been reported to increase with CR although changes are not uniform throughout tissues (4, 12-14, 16, 19, 20, 24). The reports from which these conclusions are drawn (4, 12-14, 16, 24) have all used a flooding dose of an essential amino acid. The flooding doses used in these studies were over twice what is observed to stimulate protein synthesis rates in humans (21, 22, 25). Therefore, the method itself may be simulating feeding, and the measured response is not only the effect of CR, but also reflects the effects of CR on tissue-specific responses to feeding. Also, since some tissues, such as skeletal muscle, are sensitive to a feeding stimulus while others are not, the interpretation of tissue-specific protein turnover measurements over the long-term becomes complicated (26).

An additional method to measure tissue protein turnover rates has been established that circumvents the problem of precursor labeling, namely stable isotopically labeled water (^2H_2O)(27-29). The ^2H_2O equilibrates throughout all tissues within an hour and decays with a half-life of one week (30). The long-term labeling design allows for the determination of average (or cumulative) effects over time, which is an advantage when studying long-term treatments. Importantly, the technique has been shown to be reliable and valid in a variety of tissues (29). Recently others have adapted and validated the ^2H_2O method for acute interventions (17, 31,

32). This method relies on a flooding dose of ^2H_2O administered similarly to a flooding dose of amino acids, but without the simultaneous stimulation of mTOR and translation initiation as with essential amino acids.

In this study, our aims were to understand the effects CR on tissue specific protein turnover. We hypothesized that CR does not increase protein synthesis in all protein fractions and tissues because of the energy requirements of mRNA translation. Further, we hypothesized that if a nutrient sensitive tissue did increase protein synthesis it would be due to increased anabolic responses to acute feeding. To address our hypothesis, we used a combination of ^2H_2O methods to measure in vivo protein synthesis in a variety of tissues in ad libitum (AL) and CR mice. Our use of ^2H_2O methods avoided the independent effect of flooding doses of essential amino acid that confound interpretation of previous studies.

Materials and Methods

Overall study design

Male B6D2F1 mice from the National Institute of Aging (NIA) calorically restricted (CR) colony and age-matched ad lib controls (AL) were used for all aspects of the study. Lifelong CR mice were maintained at the NIA colony at 40% food restricted compared to AL. Mice were purchased at 6, 12, and 21 months of age into both AL and CR groups and were studied as one group. CR animals were maintained on NIH-31/NIA Fortified Diet while AL animals were maintained on NIH-31 diet. Two separate cohorts were used for the study (Fig. 1). The first cohort was used after a one-week acclimatization to housing conditions, while the second cohort was used after a one-week acclimatization and six-week assessment of synthesis (described below). The animals were individually housed and consumed the same absolute quantity of NIA diets while being maintained until experimentation at the CSU Laboratory Animal Resource Center, at 18-26°C (dry bulb), 30-70% humidity, and a 12-hr light/dark cycle. All procedures at the facility meet or exceed the standards for facilities housing animals as

described in the Animal Welfare Act regulations, the Guide for the Care and Use of Laboratory Animals and the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching and were approved by the CSU Animal Care and Use Committee (protocol #09-022A).

Sixteen hours prior to sacrifice, all food, but not water, was removed from the animals' cages (Fig. 1). Animals were anesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital. Blood was then obtained by cardiac puncture (approximately 1 ml) followed by rapid excision and cryopreservation of the heart, liver, the posterior aspect of both the distal hind limbs (mixed skeletal muscle). All tissues were stored at -80°C until analysis.

Labeled water

The use of heavy water (^2H_2O) allows simultaneous assessment of multiple synthetic processes. In this case we assessed the synthesis of protein in three tissues: heart, liver, and skeletal muscle. Animals in cohort 1 were studied in the short-term by receiving an i.p. injection of 20 µl/gm body weight 99% ^2H_2O followed one hour later by an oral gavage of saline (Fasted) or an amino acid solution (Fed; Aminocyn , 0.05 mg/gm body weight, Aminocyn II 15%, Hospira Inc, Lake Forest, IL). The amino acid dose was roughly equivalent to those used in previous studies of CR (4, 12-14, 16, 24). Both saline and Aminocyn were enriched 4% with ^2H_2O. Three hours after the oral gavage tissues were collected. Animals in cohort 2 were studied over the long-term and received an i.p. injection of 99% enriched ^2H_2O calculated to enrich the body water pool (assumed 60% of body weight) to 5%. Animals were then allowed to drink *ad libitum* water enriched to 4% for the next 6 weeks.

Tissue isolation **-** Because of the heterogeneity of protein synthetic responses within a tissue, heart, liver, and skeletal muscle were fractionated according to our previously published procedures (33) as modified from Butz et al. (34). Tissue (50-70 mg) was homogenized in 1 ml

isolation buffer (100 mM KCl, 40 mM Tris HCl, 10 mM Tris Base, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, pH=7.5) with phosphatase and protease inhibitors (HALT, Thermo Scientific) using a bead homogenizer (Next Advance Inc, Averill Park NY). The homogenate was centrifuged at 800g for 10 minutes to pellet a mixed protein fraction (Mixed). The supernatant from the low-speed spin was carefully removed and centrifuged (9000 x g) for 10 minutes to pellet a mitochondrial enriched fraction (Mito). The crude Mito pellet was washed and suspended in 200 µl of solution 2 (100 mM KCl, 10 mM Tris-HCl, 10 mM Tris base, 1 mM MgSO4, 0.1 mM EDTA, 0.02 mM ATP, and 1.5% BSA, pH 7.4), then centrifuged (8000 x g, 10 minutes, 4°C). The pellet was washed a second time, suspended in 100 µl of solution 2 and centrifuged (6000 x g, 10 minutes, 4°C). The final Mixed and Mito pellets were washed with 500 µl of 100% ethanol, centrifuged (1000 x g, 30 seconds, 4°C), and rinsed with water (repeated twice). Protein pellets were solubilized in 1N NaOH (50°C, 15 minutes) and hydrolyzed into free amino acids (6 M HCl, 120°C, 24 hours).

Preparation of analytes for mass spectrometric analyses - The hydrolysates from the tissue isolations were ion-exchanged, dried under vacuum and then suspended in 1 ml of 50% acetonitrile, 50 mM K_2HPO_4, pH 11. Twenty microliters of pentafluorobenzyl bromide (Pierce Scientific, Rockford, IL, USA) were added, and the sealed mixture was incubated at 100°C for 1 h. Derivatives were extracted into ethyl acetate and the organic layer was removed and dried by addition of solid Na_2SO_4 followed by vacuum centrifugation.

GC-MS analysis of derivatized amino acids - Using negative chemical ionization (NCI), derivatized amino acids (AA) were analyzed on a DB225 gas chromatograph column. The starting temperature was 100°C, increasing 10°C per minute to 220°C. The mass spectrometry used NCI with helium as the carrier gas and methane as the reagent gas. The mass-to-charge ratios of 448, 449, and 450 were monitored for the pentafluorobenzyl-*N*,*N*-

di(pentafluorobenzyl)alaninate derivative. In all cases, these mass-to-charge ratios represented the primary daughter ions that included all of the original hydrocarbon bonds from the given amino acid. ^{2}H enrichment was calculated as described previously **(35).** The newly synthesized fraction (f) of muscle proteins was calculated from the true precursor enrichment (p) using mass isotopomer distribution analysis [MIDA (36, 37)]. Protein synthesis was calculated as the change in enrichment of deuterium labeled alanine (37) bound in muscle proteins over the entire labeling period and expressed as the common unit for protein synthesis rates (%/hr).

Western blot analyses

From Animals in Cohort 1, approximately 30 mg of frozen tissue was homogenized (Next Advance Inc, Averill Park NY) in 500 µl of ice-cold buffer (100 mM KCl, 40 mM Tris HCl, 10 mM Tris Base, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, pH 7.4) and commercial protease with phosphate inhibitor (Halt, Thermo Fisher, Rockford IL). Samples were centrifuged (10 minutes, 10000g, 4°C), then the supernatant was removed and protein concentration determined using a bicinchoninic acid assay (Thermo Fisher, Rockford IL). Samples were diluted to the same concentration, boiled with Laemmlli buffer, then 20 µg (heart and skeletal muscle) or 30 µg (liver) were separated using 12% SDS-PAGE at 200V. Proteins were transferred at 4°C (100 V for 60 minutes in 20% w/v methanol, 0.02 % w/v SDS, 25 mM Tris, 192 mM glycine, pH 8.3) to nitrocellulose paper and incubated in Superblock (Thermo Fisher, Rockford IL) for one hour at room temperature. Antibodies were purchased from Cell Signaling Technologies (Boston, MA, USA; RpS6 #2217, RpS6 phospho-Ser[240/244] #2215, 4E-BP-1 #9452, phospho-4E-BP-1[Thr37/46] #9459). Blots were incubated overnight with primary antibodies diluted 1:250 in Superblock reagent. Blots were washed in tris-buffered saline with Tween and incubated with anti-rabbit HRP conjugated secondary antibody diluted 1:2000 in Superblock with subsequent chemiluminescence detection (West Dura, Pierce, Rockford, IL, USA). Images were captured and densitometry analyzed using a UVP Bioimaging system (Upland CA, USA). Blots were

probed for phosphorylated proteins first, then stripped and re-probed for total protein. Equal loading was verified using ponceau-s staining as well as actin antibodies (sc-8432, Santa Cruz Biotechnology, Santa Cruz CA).

Statistics

Statistical analysis was performed using SPSS (V19, IBM SPSS, Somers, NY). Our initial goal was to also examine whether there were changes in protein turnover at three different ages. We did not detect a difference with age in our primary outcomes and therefore collapsed the ages into AL and CR groups to increase the number of animals in each comparison and increase statistical power. The effects of treatment (AL vs. CR), and condition (Fasted vs. Fed) were examined by two-by-two analysis of variance (ANOVA) with *a priori* linear contrasts. In conditions where data were unequally variable, appropriate transformations were performed. For comparing AL and CR without respect to feeding (long-term and collapsed short-term data), a two-tailed unpaired t-test was used. Significance was set at $p \leq 0.05$. Data are presented as means ± SEM.

Results

Liver, heart and skeletal muscle mixed protein synthesis were assessed in the four-hour period after feeding an amino acid (or saline) solution, and over a six-week period. In the short-term cohort there were no significant increases in mixed protein synthesis in all three tissues with feeding and no differences between AL and CR animals (Figure 2A). In liver, mitochondrial protein synthesis was lower in CR animals when compared to AL animals (Figure 2B) in both the fasted and fed states. There were no differences between AL and CR animals for heart or skeletal muscle mitochondrial protein synthesis. When all three tissues were assessed over the long-term, there were no significant differences between AL and CR animals for mixed protein

synthesis (Figure 2C), although liver mixed protein was 100% newly replaced in this time period and therefore it is not possible to differentiate rates of synthesis between treatments.

All three tissues were assessed for activation of the mTOR pathway by Western blotting for phospho and total 4E-BP-1 and RpS6. In liver, phospho-4E-BP-1 (Figure 3A) and phospho:total (Figure 3B) were decreased with CR as was phospho-RpS6 (Figure 3D) and total (Figure 3E). For heart phospho-RpS6 (Figure 3D) and phospho:total (Figure 3F) were decreased with CR.

Discussion

We hypothesized that CR does not increase protein synthesis in all protein fractions and tissues because of the energy requirements of mRNA translation. Further, we hypothesized that if a nutrient sensitive tissue did increase protein synthesis it would occur in the context of an increased anabolic effect to an acute feeding. Our main findings were that CR did not increase rates of mixed protein synthesis in response to an acute feeding or over the long term. Moreover, short-term responses indicate a potential decrease in mitochondrial protein synthesis in the liver during CR.

Short and long-term protein synthesis.

Prior to investigation, we hypothesized that measurements of increased rates of protein synthesis with CR could have been due to an increased anabolic response to a flooding dose of essential amino acids. Others have recently adapted the labeled water method to assess short-term changes in protein synthesis (17, 31, 32), which allowed us to measure changes in protein synthesis to an amino acid bolus over a period of 4 hours. There was no detectable effect of feeding in any tissue and this did not differ between lifelong AL and CR groups. This finding is consistent with what others have found in heart (17, 38). In liver, however, rates of protein synthesis seem to differ based on the amino acid quantity infused (38) indicating a threshold of amino acid bolus. We were surprised not to find an anabolic effect of feeding in skeletal muscle,

which is contrary to what others have previously reported (17). There are two potential reasons for the lack of a feeding response in the skeletal muscle. First, the mode of "feeding" is different among comparable experiments. In the current study an oral gavage with an amino acid solution was used for the acute feeding. In other studies, free access to food over 20 hours (17), or intravenous infusion (21, 22, 39) was used. Although we modeled our feeding dose off the flooding dose of Smith et al., (21), it is possible that the mode of delivery and the necessity of crossing the gut could change metabolic responses to a given comparable amino acid dose. Second, in our hands, the incorporation of label over the 4-hour period into skeletal muscle was very low due to the slower rate (as compared to liver and heart) of protein synthesis in skeletal muscle thus increasing variability around a limit of detection. Therefore the number of samples that we could obtain usable data for were low. In the future, studies using the acute assessment techniques should be undertaken for longer periods of time (5 hours or more) to assess skeletal muscle protein synthesis.

The ability to measure long-term protein synthesis (e.g. days to weeks) is a particular advantage of the labeled water approach. Here we report that over the long-term there was no effect of CR on mixed protein synthesis in three different tissues. Long-term synthesis rates were greatest in liver, in which protein was already replaced within the 6-week period, followed by heart and skeletal muscle. Previously we assessed the synthesis rates of just the mitochondrial protein fraction (mitochondrial biogenesis) over the long-term in liver, heart, and skeletal muscle (40). In that report, liver mitochondrial proteins were 100% new, making these data uninterpretable kinetically, with slower rates of synthesis for heart and skeletal muscle. The relative synthesis rates between tissues in both the current and our previous publication are consistent with those published in neonatal pigs (38). In the current study we assessed short-term mitochondrial protein synthesis and found slower rates of synthesis in CR compared to AL mice. This finding substantiates that there may be decreased mitochondrial protein synthesis during CR in the liver as others have reported. The current findings, in combination with our

previously published report (40) illustrate the importance of assessing multiple tissues when exploring the systemic effects of CR since responses seem to vary by tissue.

Methodological considerations

Recently we proposed that the assessment of rates of protein synthesis is important when determining age-related changes (18). It is therefore important to discuss how well our short-term and long-term labeled water methods qualitatively and quantitatively support each other. With our short-term measurement liver protein synthesis was greater than heart protein synthesis, which was greater than skeletal muscle. Qualitatively, these rates are supported by our long-term outcomes and are in line with what we expect for synthetic rates relative to each other in these tissues (38). Quantitatively, the results of short-term and long-term studies do not necessarily match. As an example, we can use data from short-term and long-term skeletal muscle proteins. Long-term measurements calculate to 0.055%/hr while short-term measurements average 0.28%/hr. It is therefore apparent that values measured over 4 hours do not quantitatively predict the long-term values. It is worth noting that values calculated from the long-term experiment are reflective of what others have found with short-term incorporation of labeled amino acids (33, 41, 42). Further, when others (32) simultaneously measured protein synthesis with a radioactive phenylalanine tracer and $^{2}H_2O$ methods similar to ours, they also found that there were quantitative differences between the methods, but not qualitative differences.

The differences between short and long-term measurements could be explained by differences in turnover between the protein pools of the tissue. It could be that one pool, representing 75% of the proteins, is rapidly turned over. In the current study the short-term value of 0.28 %/hr would equate to 100% new protein in approximately 14 days. A second pool representing 25% of the proteins could be synthesized more slowly with minimal turnover in 6 weeks (e.g. extracellular matrix). If the 75% pool is at a plateau of 100% for 4 weeks, and a

second pool's synthesis is minimal, a 6-week integrated value could equate to 0.055 %/hr for the entire period.

Finally, the observation of higher synthesis rates over the short-term is not unique to labeled water and may be a result of flooding doses in general. In rats given a flooding dose of [$^{13}C_6$]phenylalanine, skeletal muscle mitochondrial protein synthesis was about 0.6 %/hr (43) a value even higher than our values. Other studies have found similar values in rat mixed muscle (32, 44, 45) and all have used some form of a flooding dose. It has been well documented that in skeletal muscle the flooding dose of amino acid was likely causing a feeding response (21, 22). However, that should not be the case when using ^2H_2O (31). Future studies would need to consider multiple time points or further tissue fractionation to determine whether this is true. This variation of synthesis rates over time reiterates the importance of both short-term and long-term measurements of protein turnover when investigating CR or other anti-aging interventions.

Translation regulation of protein synthesis.

In our previous publication using long-term CR mice we reported a general suppression of mTOR signaling in CR animals (40). We confirm that finding in the present study since, although tissue specific, there is a general decrease in 4E-BP-1 and rpS6 phosphorylation. In this report, as well as our previous, we chose to present phospho, total, and phospho:total ratios since it is unclear which is the driving factor in the degree of mTOR activation. For example, in liver, there is a decrease in 4E-BP-1 phosphorylation and no change in total 4E-BP-1 resulting in a decreased phospho:total ratio. However, in RpS6, there is a decrease in phosphorylation and a concomitant decrease in total protein resulting in no change in the ratio. We are unsure whether the phosphorylation or the ratio of phosphorylated to total ultimately dictates mTOR regulation. However, responses in liver and heart are consistent with decreased activation of the mTOR pathway since any changes are consistently in the direction of decreased activation. The observed decrease in mTOR activation with CR, at least in liver and heart, is consistent

with a stress response in which energetic constraints, either total energy or energy flux, leads to decreased translation initiation (46, 47). It is therefore interesting that protein synthesis rates are maintained in these tissues despite the energetic constraints. In our previous report (40) we demonstrated that cellular proliferation in the liver and heart of CR mice were decreased during lifelong CR. It is therefore apparent that in times of energetic constraints, there are tradeoffs between energy consuming cellular processes such as protein synthesis and cellular proliferation (as measured by DNA synthesis on our series of studies).

Perspectives and conclusions

The current report along with our previous report (40) collectively illustrate the antagonistic pleiotropy between growth and somatic maintenance (2). At the center of these decisions is the mTOR pathway due to its role in cell growth and survival. In skeletal muscle strategies such as amino acid feeding serve to increase protein synthesis by activation of the mTOR pathway (48) and have been strongly advocated for an older population to stimulate growth and slow sarcopenia. However, suppressed mTOR signaling seems to be a common feature of longevity models (49), and chronic rapamycin feeding, which inhibits mTOR signaling, decrease cellular proliferation (40) and increase longevity (50). Because of differences between a cells propensity to replicate, it is likely that tissue specific responses to energetic constraints or other cellular stresses will play a large role in determining the success of anti-aging treatments. We therefore suggest approaches that consider multiple tissues for systemic anti-aging treatments.

Author Contributions

M.M.R., F.F.P, M.D.B, and M.K.L. developed the methods and collected and analyzed the data D.J.R, J.C.D, collected, analyzed, and interpreted the data. K.L.H, and B.F.M wrote the

manuscript, designed the study, directed the study, and analyzed and interpreted the data. All authors contributed critical feedback to the manuscript.

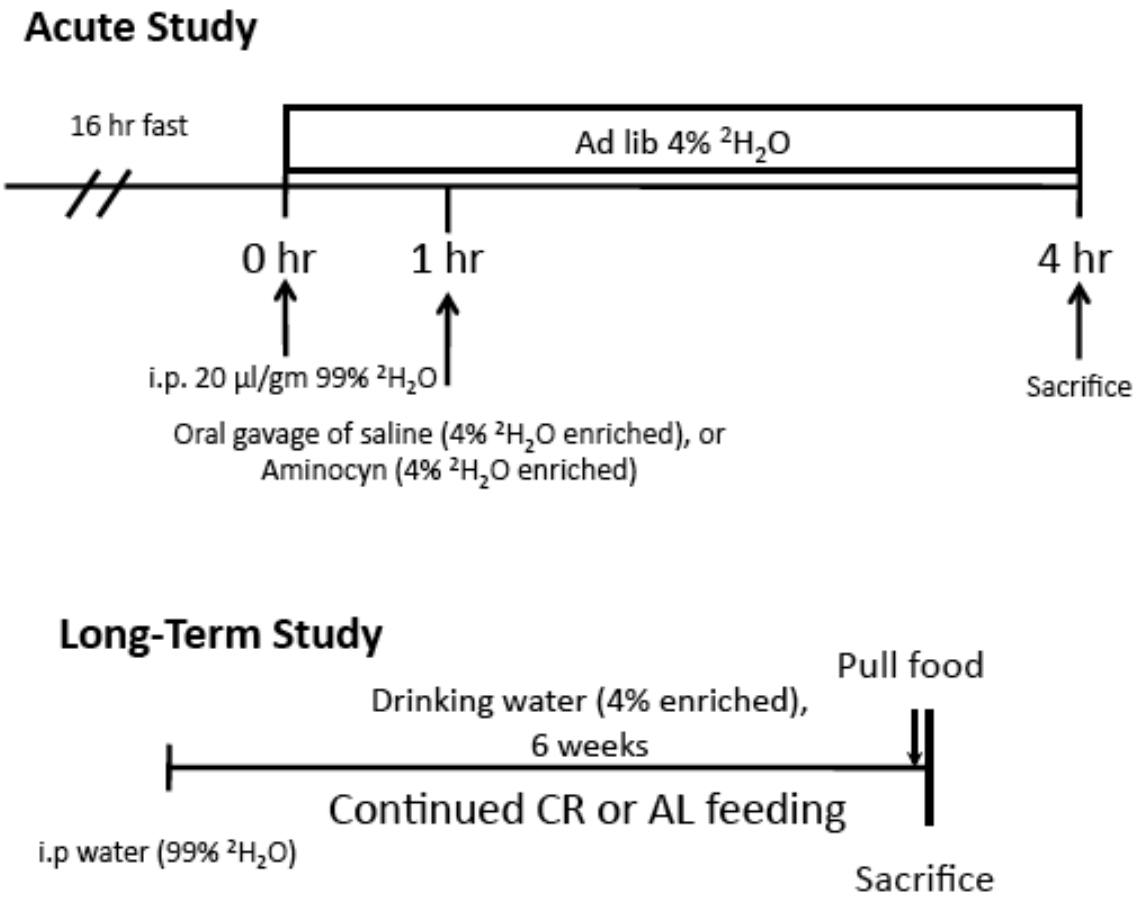

Figure A.1. Experimental design of short and long-term studies.

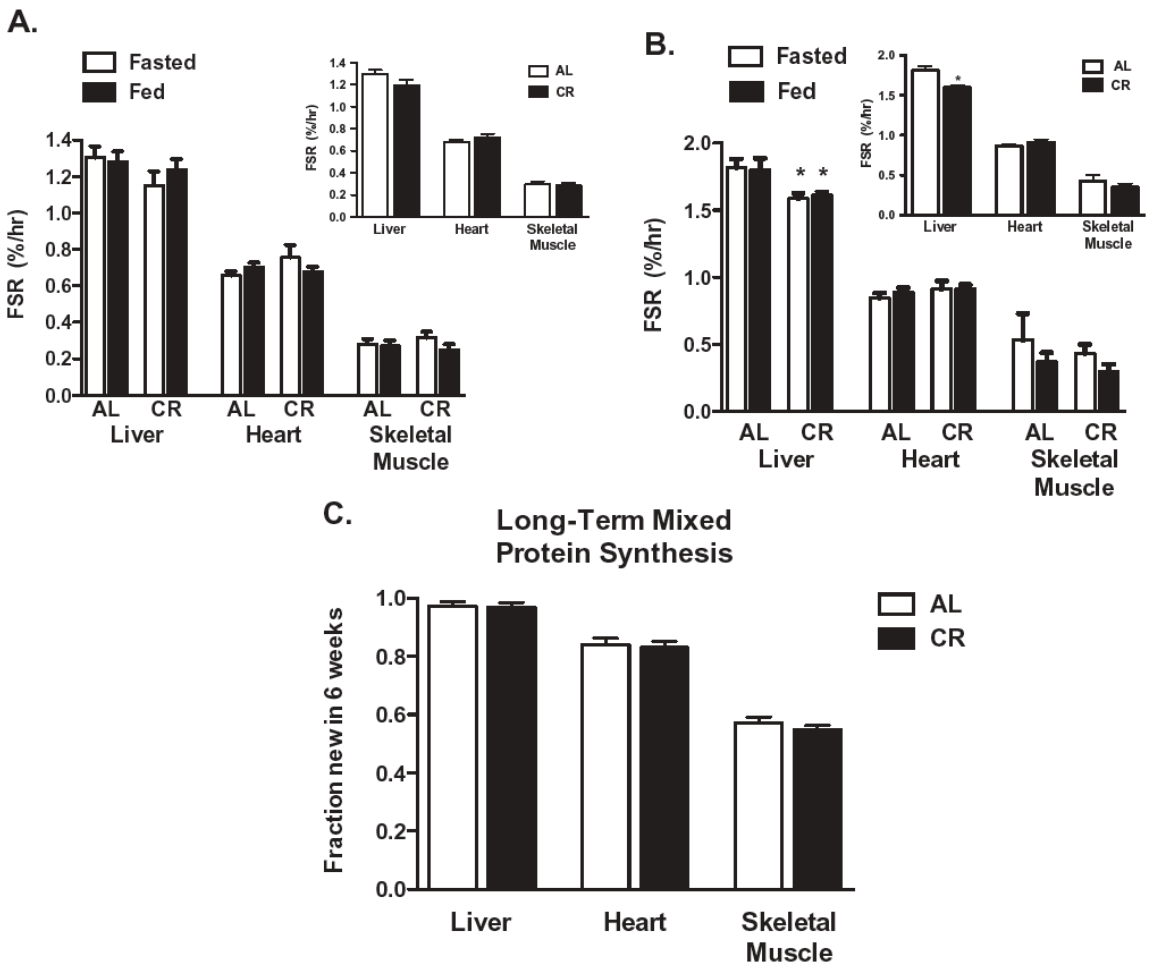

Figure A.2. Rates of protein synthesis assessed over the short term (4 hours) (A and B) and long term (6 weeks) in liver, heart, and skeletal muscle of lifelong CR or AL male B6D2F1 mice. Acute feeding of an amino acid solution did not increase mixed protein synthesis in any tissue and responses did not differ between AL and CR mice (A). Because no differences were noted with feeding, Fed and Fasted groups were collapsed into one group and compared (panel insets). Acute feeding did not increase mitochondrial protein synthesis (B), but the liver of CR mice had decreased rates of mitochondrial protein synthesis compared with AL mice in the fasted and fed conditions (B). Again, the only difference was slower rates of mitochondrial protein synthesis in the liver of CR animals. When animals were compared over the long term, there were no differences in mixed protein synthesis, although the liver protein was 100% replaced by this time point and therefore cannot be interpreted kinetically. FSR = fractional synthesis rate. *Significantly different from AL, $p<.05$. Mice were purchased at 6, 12, and 21 months of age into both AL and CR groups and were studies as one group. For short-term study, n = 15-17 for heart and liver (both fractions), 9-13 for skeletal muscle mixed, and 2-5 for skeletal muscle mitochondria. For long-term study, n = 23-24 for all groups.

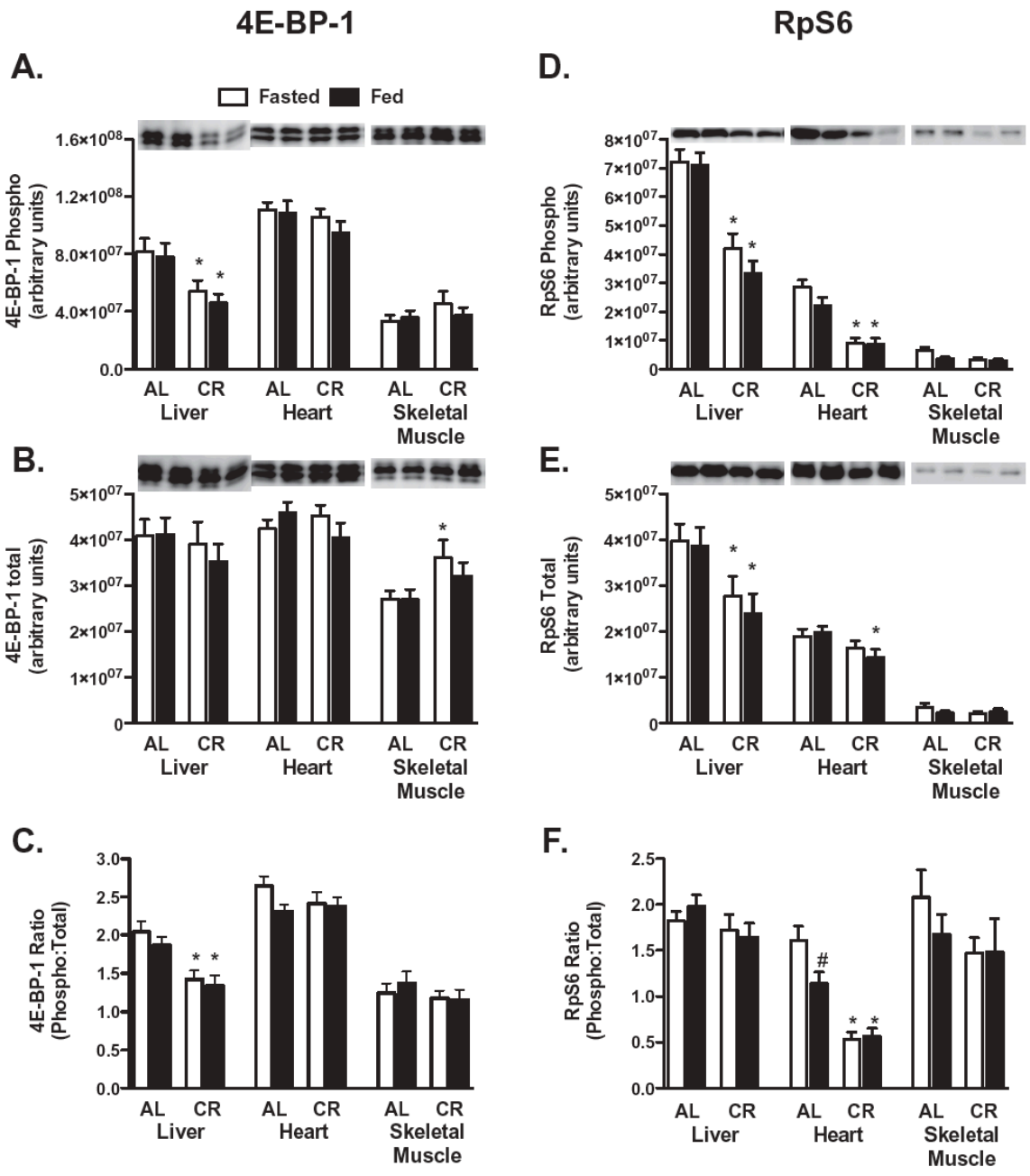

Figure A.3. 4E-BP-1, phospho-4E-BP-1[Thr37/46], RpS6, and phospho-RpS6 [Ser240/244] were determined by Western blot for phospho (A and D), total (B and E), and phospho:total (C and F) in liver, heart, and skeletal muscle of AL and CR mice in the fasted and fed conditions. *Significantly different from AL, $p < .05$. #Significantly different from Fasted, $p < .05$. n = 7-10 for skeletal muscle RpS6 and 14-15 for all other quantifications.

APPENDIX II – MANUSCRIPT V

Assessing proteostasis for studies of slowed aging

Summary

Slowing the aging process can reduce the risk for multiple chronic diseases simultaneously. It is increasingly recognized that maintaining protein homeostasis (or proteostasis) is important for slowing the aging process. Since proteostasis is multi-faceted, assessing it is not a simple task and requires use of methods capable of capturing dynamic information. This review will introduce the concept of proteostasis, how to assess protein and DNA synthesis using deuterium oxide (D_2O), and how protein and DNA synthesis outcomes provide insight into proteostatic mechanisms. Finally, we provide a discussion on how these assessments of protein and DNA synthesis are "mechanistic" investigations and provide an appropriate framework for the further development of slowed-aging treatments.

Introduction

Long-lived models are ideal for studying slowed aging because slowed aging, in the absence of trauma or infectious disease, is presumably what imparts long life. Two potential approaches for studying slowed aging are to examine unique characteristics or manipulations that lead to slowed aging in a given model, or to examine shared characteristics of a variety of long-lived models. This mini-review will present a strategy for the latter, because we believe that shared characteristics are potentially more meaningful than unique characteristics when developing strategies for slowing the aging process.

Protein homeostasis (i.e. proteostasis) refers to controlling the concentration, location, and conformation of proteins in cells (Balch et al., 2008). In addition to protein folding, trafficking, and degradation, a change in protein synthesis is considered one of the primary

"proteostasis regulators" that can restore protein homeostasis (Balch et al., 2008). The ability to maintain proteostasis has become a key outcome in aging research (Austad, 2010; Balch et al., 2008; Perez et al., 2009; Salmon et al., 2009). One characteristic of slowed aging is the ability to maintain healthy, functional proteins (proteostasis) since proteins play an important role in cellular stability and repair (Treaster et al., 2013). Whether or not increased proteostasis is an underlying mechanism of slowed aging is yet to be definitively determined, but investigations into proteostasis provide a reasonable contextual framework for studying the aging process. Of course if one wants to study proteostasis, appropriate methods to do so are necessary. Since proteostasis is a dynamic process, capturing the process is not a simple task. In this review, we will present one strategy for the assessment of proteostasis and suggest reasons why this approach provides valuable insight into the aging process and how to slow its progression.

Discussion

Distinguishing cell growth, adaptation and repair to understand proteostasis

When a proliferative cell replicates it doubles its mass to have two equal sized daughter cells (Grebien et al., 2005). When a post-mitotic cell, such as a skeletal muscle cell, hypertrophies it recruits DNA from resident stem cells (Collins et al., 2005) to maintain a constant DNA to cytoplasm ratio (Allen et al., 1999; Pavlath et al., 1989). Therefore, in both proliferative and post-mitotic cells, new DNA, either from true DNA replication or DNA recruitment, is associated with increases in protein synthesis.

The capacity for cells to enzymatically repair proteins is low (Mary et al., 2004; Mortimore and Pösö, 1987; Poppek and Grune, 2005). Therefore to maintain proteostasis, damaged proteins are removed and replaced with new proteins (Poppek and Grune, 2005). To cope with environmental stresses, cells constantly integrate multiple signals that relay information about the intra- and extracellular environments and synthesize new proteins to adapt to those environments. Therefore, protein synthesis to replace damaged proteins and to

adapt to the environment maintains cellular protein homeostasis. These processes are counter to the previously described phenomenon in which protein synthesis increases in parallel with an increase in cell proliferation. In one case, there are adaptive responses to prevent or replace protein damage to maintain proteostasis, whereas in the other, protein synthesis increases to equip the newly made cells. By this rationale, when examining protein synthesis in the context of increasing proteostatic mechanisms, one should consider both the rate of protein synthesis and the rate of cellular proliferation in order to account for how much protein is directed toward cell doubling versus how much is directed toward maintaining existing cellular structures.

Using D_2O for assessing synthesis of DNA and protein

Here we propose the novel idea that when measuring protein synthesis as a proteostatic mechanism, it is also necessary to consider cell proliferation. The assessments of rates of synthesis are especially important in this context. During periods of cellular stress, there are several post-transcriptional mechanisms such as selective translation (Zid et al., 2009), stress granules (Kimball et al., 2003), and micro-RNA (Adeli, 2011), that determine which mRNAs are translated to protein. Therefore, measurements of cellular signaling and mRNA content are not appropriate for assessing protein synthesis because they fail to account for post-transcriptional mechanisms, a concept we have previously discussed (Miller and Hamilton, 2012). Assessing rates of synthesis of new protein and DNA is critical because simply measuring content or number masks ongoing cellular processes. For example, if protein synthesis increased by one hundred-fold and was matched by a one hundred-fold increase in breakdown, there would be no detectable change in protein content even though there were positive outcomes related to proteostasis.

The use of deuterium oxide (D_2O) in vivo allows for the simultaneous measurement of both protein (Busch et al., 2006; Drake et al., 2013; Miller et al., 2012; 2013; Robinson et al., 2011) and DNA synthesis (Drake et al., 2013; Miller et al., 2013; 2012; Neese et al., 2002;

Robinson et al., 2011). To perform a study, D_2O is introduced as an intraperitoneal injection (Gasier et al., 2009; Miller et al., 2013; Yuan et al., 2008) or as drinking water (Drake et al., 2013; Miller et al., 2013; 2012) to enrich the body water pool with deuterium (**Figure 1**). Equilibrium is rapidly established between the body water deuterium enrichment and the enrichment of metabolites that contain labile hydrogens. Those labeled metabolites (the precursors) are then incorporated into synthetic products (ex. deoxyribose of DNA and alanine within proteins) and can then be used to calculate synthesis rates by standard precursor/product calculations (**Figure 1**). A particular advantage of using D_2O, compared to traditional approaches, is that it can be used over prolonged periods to capture the synthesis of slowly synthesized species while an organism is free living. This ability to measure outcomes over the long-term is advantageous because the sum of physiological effects over time, not just short snapshots that vary based on the prevailing metabolic state, are assessed. When studying processes associated with aging and slowed aging in laboratory models and humans, the ability to capture data during prolonged free living removes uncertainty associated with more traditional isotope approaches reflective of only a 4-6 hour time frame.

What measuring proteostasis tells us about slowed aging

Long-lived models (i.e. models of slowed aging) are ideally suited for use in studies to identify the mechanisms responsible for aging and age-related declines. Calorically restricted (CR) (Masoro, 2005) and rapamycin treated mice (Wilkinson et al., 2012) are long-lived and demonstrate characteristics consistent with prolonged healthspan. These models also share the characteristic of decreased mechanistic (formerly mammalian) target of rapamycin (mTOR) activity (Drake et al., 2013; Harrison et al., 2009; Miller et al., 2012), a key modulator of protein synthesis and cell cycling (Sengupta et al., 2010). However, as discussed below, decreased mTOR activity is not universal among long-lived models.

Caloric restriction - Our first investigation into proteostasis began with CR mice because of the abundance of studies investigating longevity in this model. We were particularly interested in this model because some evidence suggests that mitochondrial biogenesis is maintained or increased during CR (Nisoli, 2005), while others have questioned whether CR increases mitochondrial biogenesis (Hancock et al., 2011). There had also been speculation that decreased protein synthesis is a key mechanism leading to lifespan extension (Kapahi, 2010; Tavernarakis, 2008). Given the background provided above regarding post-transcriptional mechanisms, especially those employed during activation of pathways associated with energetic stress, we thought that it was possible that protein synthetic responses were not being truly captured with the methods previously employed. Using D_2O we measured protein synthesis, including mitochondrial protein synthesis, both acutely (4 hours) and over the long-term (6 weeks) in liver, heart, and skeletal muscle of CR animals compared to ad libitum fed controls (Miller et al., 2013; 2012). We found that almost uniformly among the three tissues, protein synthesis was maintained in CR mice, including in the mitochondrial fraction (Miller et al., 2013; 2012) (data from skeletal muscle show in **Figure 2A**). Although the finding that there is no difference in protein synthesis between CR and controls (CON) initially appears fairly unremarkable, when considered in relation to DNA synthesis, a different picture emerges (**Figure 2B**). Because DNA synthesis rates were lower in CR animals compared to CON, the ratio of protein synthesis to DNA synthesis is greater in CR compared to CON. Therefore, it appears that a greater proportion of protein synthesis is dedicated to proteostasis rather than cell proliferation. That the CR mice maintained rates of protein synthesis is counter to the idea that a decrease in protein synthesis is a mechanism of slowed aging. However, by also measuring DNA synthesis, we found that maintenance of protein synthetic rates happened in the face of decreased DNA synthesis, indicating that an increase in proteostatic mechanisms is associated with slowed aging in this model.

Rapamycin treatment - Inhibition of mTOR increases lifespan in a variety of non-mammalian species (Jia et al., 2004; Kapahi et al., 2010; Powers et al., 2006) while chronic administration of the mTOR inhibitor rapamycin has been shown to extend mean and maximal life span in heterogeneous mice (Harrison et al., 2009). Given that rapamycin inhibits mTOR, it was surprising to us that no one had previously measured protein synthesis and cell replication in long-lived rapamycin treated mice. In our study, we measured protein synthesis and DNA synthesis over a 4-week period in heterogeneous mice treated for 12 weeks with rapamycin (Drake et al., 2013). In heart tissue we found no differences between rapamycin treated mice (Rap) or controls (CON) for protein synthesis rates of mixed, cytosolic, or mitochondrial proteins (Drake et al., 2013) (**Figure 2C**). However, in the heart (as well as skeletal muscle), there was a decrease in DNA synthesis so that when the protein synthesis to DNA synthesis ratio was calculated, it became apparent that like CR, there is an increase in proteostatic mechanisms in Rap mice compared to CON (Fig. 2D).

Crowded litter - The crowded litter model (CL) is a relatively newly described long-lived model (Steinbaugh et al., 2012; Sun et al., 2009). The model is an extension of the developmental origins of health and disease hypothesis (Hales and Barker, 2001) in that the critical developmental period spans beyond the in utero environment to the immediate post-natal environment. In the CL model, litter size is increased by 50% and this increase in litter size presumably imposes a transient caloric restriction period until the pups are weaned and subsequently given free access to food. Even though the CL mice are given ad libitum access to food for the remainder of their lives, mean and maximal lifespan are increased (Sun et al., 2009).

Recently, we characterized protein and DNA synthetic rates in long-lived CL mice and our findings took us quite by surprise (Drake et al., 2014). We found that compared to normal litter sized controls (CON), rates of protein synthesis in CL mice were uniformly greater in heart,

liver, and skeletal muscle in all sub-fractions examined (Drake et al., 2014) (skeletal muscle shown in Fig. 2E). In addition, as opposed to previously examined long-lived models, DNA synthesis was relatively unchanged in the same tissues examined in CR and rapamycin models. Although synthesis rates were seemingly divergent from previously explored long-lived models, when the protein synthesis to DNA synthesis ratio was calculated, it again appeared that there was greater activation of proteostatic mechanisms in the CL mice compared to CON (skeletal muscle shown in Fig. 2F). Finally, activation of ribosomal protein subunit S6 (rpS6), a marker of mTOR activation, was greater in CL compared to CON (Drake et al., 2014). Therefore, although a decrease in mTOR activity is thought to be important for lifespan extension, and is indeed decreased in both the CR and Rap mice, the CL model illustrates that the resultant improvement in proteostasis is likely a more important outcome than decreased mTOR activity itself.

Why are studies of proteostasis mechanistic?

It is easy to dismiss the measurement of protein and DNA synthesis as largely descriptive and not mechanistic. However, when investigating the end result of cellular processes, the measurement of synthesis rates is perhaps one of the more insightful mechanistic outcomes. For example, aerobic exercise, a known modulator of the aging process (Short et al., 2005), activates both the transcriptional coactivator peroxisome proliferator-activated receptor-coactivator-1α (PGC-1α) (Pilegaard et al., 2003) and AMP-activated protein kinase (AMPK) (Winder and Hardie, 1996). Whereas PGC-1α activation triggers increases in the transcription of nuclear and mitochondrial coded mRNA for mitochondrial protein synthesis, AMPK inhibits protein translation through its interactions with mTOR (Mounier et al., 2011). Therefore, without a measurement of protein synthesis, it is difficult to determine whether the newly transcribed mRNA was actually translated into new protein. Many models of lifespan extension in lower organisms are the result of single gene mutations. Since the manipulation of

gene targets can have multiple downstream effects (pleiotropy), many of which are unanticipated or uninvestigated, the synthetic outcomes actually provide exceptional insight into why and how changes in lifespan occur. The present review proposes that considering protein synthesis rates in the context of DNA synthesis rates provides novel insight into proteostatic mechanisms or outcomes. These measurements are perhaps even more discerning when using in vivo models of slowed aging since long-lived models have complex systemic changes that might not be captured during analysis of signaling pathways or protein contents selected a priori.

To date, our assessments strongly support the concept that slowed aging is associated with improved proteostasis. Importantly, they also illustrate the importance of using the assessment of synthetic rates as a mechanistic outcome. The inhibition of mTOR has been the focus of much research, and rightly so, because of its potential to slow the aging process (Flynn et al., 2013; Wilkinson et al., 2012; Wu et al., 2013; Ye et al., 2013). However, our findings in the CL slowed aging model indicate that lifespan extension can happen independently of a decrease in mTOR activity (Drake et al., 2014). This lifespan extension independent of a decrease in mTOR activity is seen in other longevity treatments such as branched chain amino acid supplementation (D'Antona et al., 2010). Therefore, future studies could be enhanced by focusing on proteostasis as a key regulator, or mechanism, of lifespan extension.

Conclusions

By recognizing that proteostasis is a key feature of slowed aging, promotion of proteostatic mechanisms can then be a mechanistic target to slow the aging process. In addition to protein folding, trafficking, and degradation, a change in protein synthesis is considered one of the primary “proteostasis regulators” that can restore protein homeostasis and is thus a potential target for slowed aging treatments. The assessment of protein synthesis is important because it represents the sum of the highly regulated transcriptional and post-

transcriptional processes. However, to properly assess the increase in protein synthesis to proteostasis, one must also consider the making of new cells to determine how much protein synthesis is dedicated to new cells versus replacing of existing proteins. It is our goal to further characterize protein synthetic responses in long-lived models in order to improve proteostasis to slow the aging process.

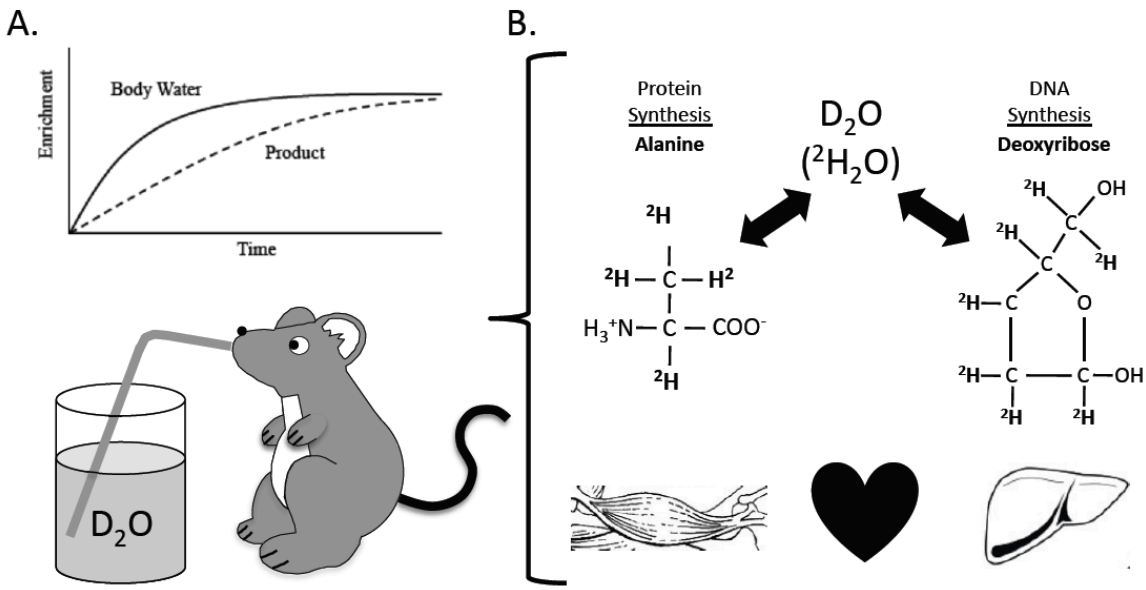

Figure A.4. Using D_2O for assessing synthesis of DNA and protein. Following an intraperitoneal injection, D_2O (^2H_2O) is administered via the animal's drinking water where it equilibrates with the body water pool (A). Deuterium in the body water pool exchanges with labile hydrogen in metabolites that are incorporated into synthetic products (B). For example, deuterium is incorporated into the deoxyribose of DNA and the amino acid alanine, which are then incorporated into DNA and protein, respectively (B). DNA and protein synthesis can be simultaneously assessed across multiple tissues, such as skeletal muscle, heart, and liver as we have done.

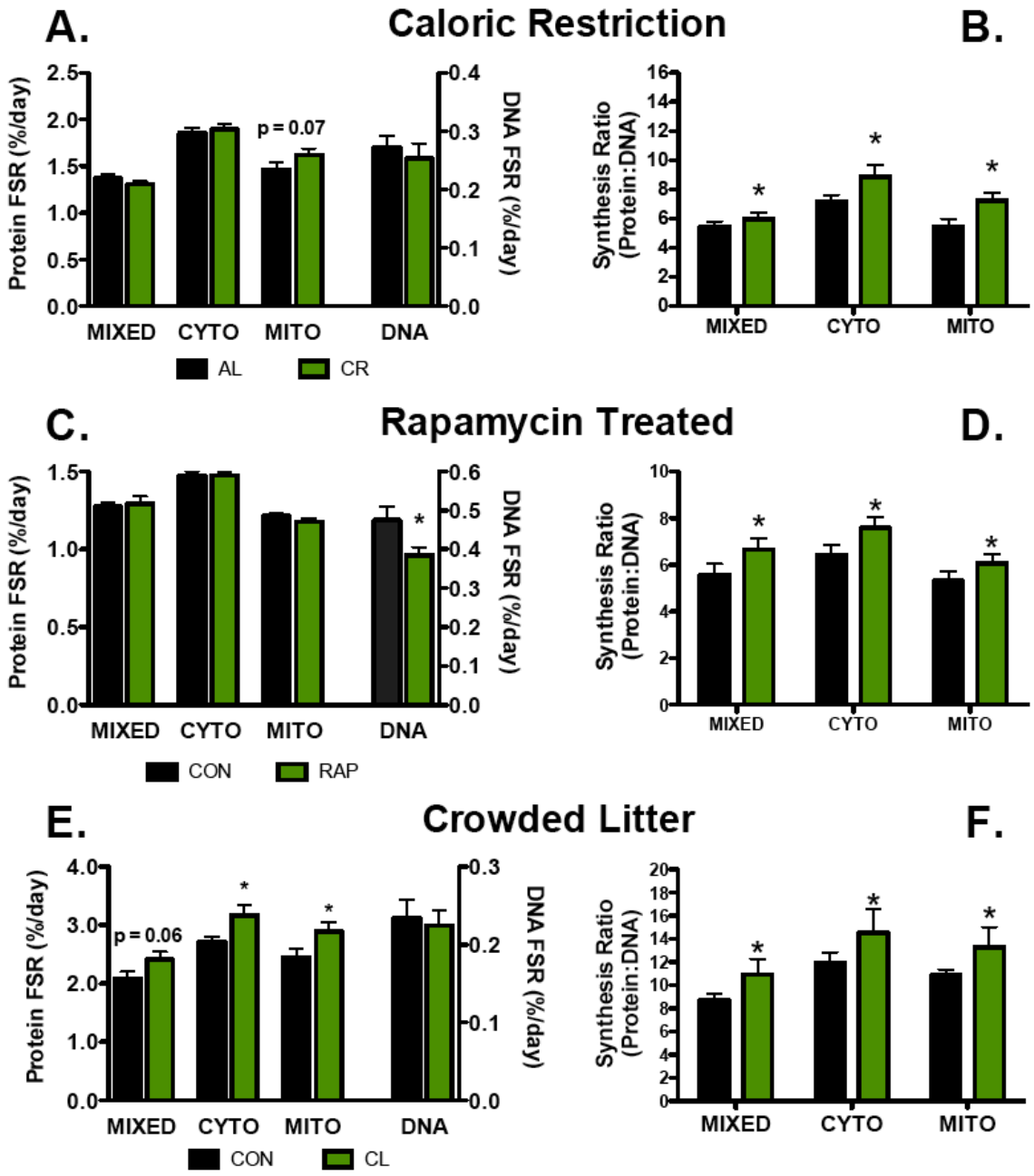

Figure A.5. Representative protein synthesis, DNA synthesis and protein synthesis:DNA synthesis in three models of slowed aging. A) Synthetic rates of DNA and three fraction of skeletal muscle (Mixed = myofibrillar, CYTO = cytoplasmic, MITO = Mitochondrial) in lifelong calorically restricted mice (CR) and ad lib fed controls (AL). Although there is a trend for greater mitochondrial protein synthesis in CR, protein synthesis rates are largely similar between CR and AL (data reproduced from (Miller et al., 2013; 2012)). B) When the data from skeletal muscle are expressed as a synthesis ratio, it is clear that there is increase protein synthesis dedicated to proteostasis in CR animals as compared to AL. C) Synthetic rates of DNA and three fraction of heart in mice chronically fed rapamycin (RAP) and age matched controls (CON). There are no differences between RAP and CON for protein synthetic rates, although DNA synthesis is lower in RAP compared to CON. D) When the data are expressed as a

synthesis ratio, there is increase protein synthesis dedicated to proteostasis in RAP compared to CON (data reproduced from (Drake et al., 2013)). E) Synthetic rates of DNA and three fractions of skeletal muscle in crowded litter mice (CL) and normal litter sized controls (CON). As opposed to CR and RAP models, there is greater protein synthesis CL compared to CON and no difference in DNA synthesis (data reproduced from Drake et al., 2014)). F) Despite qualitative differences in synthesis rates between CL compared to CR and RAP, the ratio of synthesis rates still implies improved proteostasis. Data are means ± SEM, n = 4-12/group. *$p<0.05$.

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