Long-lived crowded-litter mice have an age-dependent increase in protein synthesis to DNA synthesis ratio and mTORC1 substrate phosphorylation

Joshua C. Drake, Danielle R. Bruns, Frederick F. Peelor 3rd, Laurie M. Biela, Richard A. Miller, Karyn L. Hamilton and Benjamin F. Miller

1Health and Exercise Science Department, Colorado State University, Fort Collins, Colorado; and 2Department of Pathology and Geriatrics Center, University of Michigan, Ann Arbor, Michigan

Submitted 5 June 2014; accepted in final form 4 September 2014

A PROGRESSIVE AGE-DEPENDENT REDUCTION in protein turnover results in an accumulation of damaged proteins and propagates the development of the aging phenotype (36, 37). In tissues that have a limited proliferative capacity, such as 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 homeostasis with age (1, 2). 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 Ref. 16). In tissues with limited ability to proliferate (e.g., skeletal muscle), growth is accomplished by increased protein synthesis, which may be followed by recruitment of new DNA from satellite cell division to maintain the myonuclear domain (44). Since enzymatic capacity for protein repair is low within cells, damaged proteins must be removed and subsequently replaced through the synthesis of new protein (20, 26), a process that does not necessarily involve cell division or DNA recruitment. Therefore, the simultaneous assessment of DNA synthesis and protein synthesis could provide context as to whether the synthesis of new proteins are directed toward new cells or to maintaining existing cellular structures, a phenomenon consistent with slowed aging (11, 22, 23). Our laboratory has shown that, in lifelong caloric restriction (CR) (22, 23) and chronically rapamycin-treated mice (11), DNA synthesis is decreased. However, in long-lived models compared with controls, there are variable responses in protein synthesis based on the protein fraction, tissue, model, and sex. To date, no study, in a model of slowed aging or other, has considered protein synthetic rates in the context of DNA synthesis.

The mechanistic (formerly mammalian) target of rapamycin (mTOR) signaling pathway promotes growth by increasing protein synthesis and cellular cycling via two multiprotein complexes, mTORC1 and mTORC2 (18). CR (17, 46) and chronic rapamycin administration (14, 30, 45) suppress mTORC1 activity and increase lifespan and multiple indexes of health in various species. Although it has been proposed that a decrease in mTOR signaling is a key mechanism of slowed aging (18), it is unclear whether decreased mTOR signaling is necessary for slowed aging.

The nutritional environment of the developing fetus can have a profound impact on gene expression throughout the lifespan of the offspring, affecting health and longevity (8, 13, 40). Furthermore, changing litter size or postnatal maternal protein intake can change offspring lifespan, suggesting that some degree of genomic plasticity also remains during the postnatal suckling period (8, 12, 19, 38, 39). For example, increasing litter size by 50% for the first 3 wk of life, a model termed crowded litter (CL), extends mean and maximal lifespan (39). The increase in litter size presumably imposes a CR period until the pups are weaned and given free access to food (33, 39). How a transient nutrient stress at such a young age extends lifespan is unknown. We hypothesized that, like other long-lived models previously investigated in our laboratory (11, 22, 23), CL mice would have decreased DNA synthesis and mTORC1 signaling but maintained protein synthesis. In addition, we hypothesized that if new protein synthesis was considered in relation to new DNA synthesis, there would be an increase in the ratio within the CL model, indicative of greater preservation of existing protein structures.
METHODS

Animals and Treatments

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 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 C56Bl6F1 female and C3D2F1 male (UM-HET3) mice, which have been well characterized for the development of the CL model (38, 39).

Routine veterinary care was provided by the Biomedical Science Research Building staff. UM-HET3 litters were culled to eight, and an additional four mice from separate litters were added, resulting in a 50% increase in litter size (39). Control (Con) UM-HET3 mice were maintained in a litter size of eight pups. After the 3-wk suckling period, female mice were weaned onto a chow diet, and CL and Con were placed in their own respective cages (4 mice per cage per cage) with ad libitum access to food and water. At 4 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, 22, 39)], CL (n = 4, per age group) and Con (n = 4, per age group) mice received deuterium-labeled water (D2O) for 2 wk. Animals received an intraperitoneal injection of 99% enriched D2O to enrich the body water pool (assumed 60% of body wt) to 8% (22, 28). Animals then received 8% D2O in their drinking water with ad libitum access for the next 2 wk. After the labeling period and following an overnight fast (7 mo only), mice were euthanized using a CO2 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 hindlimbs [gastrocnemius, soleus, and plantaris (i.e., mixed skeletal muscle)], heart, liver, bone marrow from the tibia, via flushing the cavity with PBS, 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 being euthanized. Therefore, to assess mTORC1 signaling at a younger age, an additional cohort of ~3-mo CL and Con (n = 4, respectively) mice were euthanized following an overnight fast, and gastroc complex and liver were harvested as already reported.

Protein Isolation

We assessed protein synthesis in three subcellular fractions: mitochondrial enriched (Mito), cytosolic (Cyto), and mixed (Mix). Mix contains nuclei, plasma membranes, and, in the case of skeletal muscle and heart, contractile proteins. Cyto contains all other intracellular components with the exception of mitochondria, which is contained in the Mito fraction. Tissues were fractionated according to our previously published procedures (11, 22, 23, 31). Briefly, tissues (25–60 mg) were homogenized 1:10 in isolation buffer (100 mM KCl, 40 mM Tris-HCl, 10 mM Tris base, 5 mM MgCl2, 1 mM EDTA, 1 mM ATP, pH 7.5) with phosphatase and protease inhibitors (HALT, Thermo Scientific, Rockford, IL) using a bead homogenizer (Next Advance, Averill Park, NY). After homogenization, subcellular fractions were isolated via differential centrifugation as previously described (11, 22, 23). 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.

DNA Isolation

Whole tissue DNA synthesis in skeletal muscle, heart, and liver was assessed according to procedures previously described (5, 11, 22, 23, 28). Approximately 75 ng/μl (heart and gastroc complex) and 400 ng/μl (liver) of total DNA was extracted from ~15 mg tissue (QiAamp DNA mini kit Qiagen, Valencia, CA, USA). DNA for the precursor pool was obtained from bone marrow. DNA from bone marrow was isolated by extracting ~300 mg from the tibial bone marrow suspension which was centrifuged for 10 min at 2,000 g. DNA from the resulting bone marrow pellet was extracted the same as tissue samples already described.

Sample Preparation and Analysis of Analytes Via GC-MS

Protein. Protein was hydrolyzed by incubation for 24 h at 120°C in 6 N HCl. The hydrolysates were iron exchanged, dried under vacuum, and resuspended in 1 ml of molecular biology grade H2O. Suspended samples (500 μl) were derivatized [500 μl acetonitrile, 50 μl 1 M K2HPO4 (pH 11), and 20 μl pentafluorobenzyl bromide (Pierce Scientific, Rockford, IL)], sealed, and incubated at 100°C for 1 h. Derivatives were extracted into ethyl acetate. The organic layer was removed and dried by N2 followed by vacuum centrifugation. Samples were reconstituted in 1 ml of ethyl acetate and then analyzed.

The pentafluorobenzyl-N,N-di(pentafluorobenzyl) derivative of alamine was analyzed on an Agilent 7890A GC coupled to an Agilent 5975C MS, as previously described (11, 22, 23, 32). The newly synthesized fraction (f) of proteins was calculated from the true precursor enrichment (p) using plasma analyzed for 2H2O enrichment and adjusted using mass isotopomer distribution analysis (MIDA) (5). Protein synthesis was calculated as the ratio of 2H2O-labeled to unleveled alanine (5) bound in proteins over the entire labeling period and expressed as fraction new in 2 wk.

Body water. To determine body water enrichment, 125 μl of plasma was placed into the inner well of an o-ring screw-on cap and placed inverted on a heating block overnight. Two microliters of 10 M NaOH and 20 μl of aceton was added to all samples and to 20 μl 0–20% 2H2O 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 of hexane. The organic layer was transferred through anhydrous Na2SO4 into GC vials and analyzed via EI mode using a DB-17MS column.

DNA. Determination of 2H incorporation into purine deoxyribose (DR) of DNA from whole tissue and bone marrow was performed as described previously (5, 11, 22, 32). Briefly, isolated DNA was hydrolyzed overnight at 37°C with nuclease S1 and potato acid phosphatase. DNA was hydrolyzed overnight at 37°C with nuclease S1 and potato acid phosphatase. DNA was hydrolyzed overnight at 37°C with nuclease S1 and potato acid phosphatase.

Fig. 1. Body weight comparison of crowded-litter (CL) and control (Con) mice postweaning, at 4 mo and at 7 mo of age. Postweaning CL mice weighed significantly less than normal-litter-size Con mice. There was no difference in body weight between CL and Con mice at 4 or 7 mo of age; n = 8 per group for postweaning, n = 4 per group in 4-w and 7-mo-old cohorts. *P < 0.05 for CL vs. Con.
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, 22, 32). The fraction new in 2 wk 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, 22, 23).

New Protein-to-New DNA Synthesis Ratio

From the synthesis rates of protein and DNA, we calculated the new protein-to-new DNA synthesis ratio. The rationale is that this ratio illustrates how much protein is made in relation to the rate of new DNA synthesis during the labeling period, giving insight into whether new proteins are made in either existing or new cells.

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). Samples were diluted to the same concentration, boiled with Laemmli buffer, and 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% wt/vol methanol, 0.02% wt/vol 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 Tween 20 (TBST) for 1 h. Antibodies were purchased from Cell Signaling Technologies [Boston, MA; rpS6 phospho-Ser235/236 no. 4858, rpS6 total no. 2217, eukaryotic initiation factor 4E binding protein-1 (4E-BP1) phospho-Thr37/46 no. 9459, 4E-BP1 total no. 9452], or β-tubulin no. sc-5274 (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were incubated overnight with primary antibodies diluted 1:500 (skeletal muscle rpS6), 1:1,000 (all others), 1:2,000 (liver rpS6), or 1:500 (β-tubulin). Blots were washed in TBST and incubated with anti-rabbit or anti-mouse (β-tubulin) HRP-conjugated secondary antibodies diluted 1:5,000 in 5% milk with subsequent chemiluminescence detection (West Dura; Pierce, Rockford, IL). Images were captured and densitometry was analyzed by UVP Bioimaging system.
from that of 4-mo Con (Fig. 3). At 7 mo, CL mice had significantly lower rates of DNA synthesis in the heart but not in gastroc complex and liver (Fig. 3).

**New Protein/New DNA Synthesis Ratio**

In the 4-mo CL, the new protein/new DNA synthesis ratio was significantly decreased compared with 4-mo Con across subcellular fractions in both the gastroc complex and heart (Fig. 4, A and C). At 4 mo, in the liver there was no difference between Con and CL in the new protein/new DNA synthesis ratio (Fig. 4E). However, at 7 mo, the new protein/new DNA synthesis ratio was significantly increased across subcellular fractions in gastroc complex, heart, and liver (Fig. 4, B, D, and F).

**mTORC1 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. 5). At 7 mo, phosphorylation of rpS6 was significantly increased in heart and liver (Fig. 6, B and C). Phosphorylation of 4E-BP1 was significantly increased in gastroc complex (Fig. 6D). Although it did not reach statistical significance, 4E-BP1 in heart (Fig. 4E) was $P = 0.055$.

**DISCUSSION**

**Overview of Primary Findings**

We tested the hypothesis that, compared with Con, CL mice would have decreased DNA synthesis and mTORC1 signaling, and maintained protein synthesis rates. Furthermore, we hypothesized that when the synthesis of new proteins was compared with the synthesis of new DNA there would be changes in the CL model that indicated a greater preservation of existing cellular structures. At 4 mo of age, protein synthesis was not different between CL and Con in any tissue but DNA synthesis was significantly increased in gastroc complex and trended in the heart of CL compared with Con. At 7 mo of age, however, protein synthesis rates were significantly greater in all tissues and fractions, whereas DNA synthesis was signifi-

---

**RESULTS**

**Protein and DNA Synthesis**

Body weight at weaning was significantly reduced in CL compared with Con mice (Fig. 1), suggesting that CL mice were calorically restricted during suckling (33, 39). However, there were no differences in body weight at either 4 or 7 mo of age (Fig. 1). 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 with 4-mo Con (Fig. 2, A, C, and E). By 7 mo of age, there was a dramatic change, in the gastroc complex, heart, and liver (Fig. 2, B, D, and F) all having significantly greater rates of protein synthesis in Mix, Cyto, and Mito fractions compared with 7-mo Con (Fig. 2, B, D, and F). Independent of treatment, protein synthesis was significantly different between subcellular fractions in all tissues and ages (Fig. 2, A–F).

DNA synthesis was significantly greater in the gastroc complex of 4-mo CL mice compared with Con (Fig. 2A). DNA synthesis in heart trended greater in 4-mo CL compared with 4-mo Con but did not reach statistical significance ($P = 0.070$; Fig. 3A). Liver DNA synthesis in 4-mo CL was not different from that of 4-mo Con (Fig. 3A). At 7 mo, CL mice had significantly lower rates of DNA synthesis in the heart but not in gastroc complex and liver (Fig. 3).

**New Protein/New DNA Synthesis Ratio**

In the 4-mo CL, the new protein/new DNA synthesis ratio was significantly decreased compared with 4-mo Con across subcellular fractions in both the gastroc complex and heart (Fig. 4, A and C). At 4 mo, in the liver there was no difference between Con and CL in the new protein/new DNA synthesis ratio (Fig. 4E). However, at 7 mo, the new protein/new DNA synthesis ratio was significantly increased across subcellular fractions in gastroc complex, heart, and liver (Fig. 4, B, D, and F).

**mTORC1 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. 5). At 7 mo, phosphorylation of rpS6 was significantly increased in heart and liver (Fig. 6, B and C). Phosphorylation of 4E-BP1 was significantly increased in gastroc complex (Fig. 6D). Although it did not reach statistical significance, 4E-BP1 in heart (Fig. 4E) was $P = 0.055$.

**DISCUSSION**

**Overview of Primary Findings**

We tested the hypothesis that, compared with Con, CL mice would have decreased DNA synthesis and mTORC1 signaling, and maintained protein synthesis rates. Furthermore, we hypothesized that when the synthesis of new proteins was compared with the synthesis of new DNA there would be changes in the CL model that indicated a greater preservation of existing cellular structures. At 4 mo of age, protein synthesis was not different between CL and Con in any tissue but DNA synthesis was significantly increased in gastroc complex and trended in the heart of CL compared with Con. At 7 mo of age, however, protein synthesis rates were significantly greater in all tissues and fractions, whereas DNA synthesis was signifi-

---

![Fig. 3. DNA synthesis in gastroc complex, heart, and liver over 2 wk in 4-mo and 7-mo CL and Con.](http://ajpendo.physiology.org/)

A: gastroc complex DNA synthesis was significantly greater in 4-mo CL than in 4-mo Con, heart DNA synthesis in 4-mo CL tended to be greater ($P = 0.070$) than 4-mo Con, and there were no differences in liver DNA synthesis between 4-mo animals. B: in 7-mo animals, DNA synthesis was significantly less in CL heart than in Con, and no significant differences were observed between 7-mo CL and 7-mo Con in gastroc complex or liver. DNA synthesis rates in heart tissues from 4-mo mice were log transformed to normalize variance; $n = 4$ in both ages and groups except that in 7-mo Con gastroc complex and 7-mo CL gastroc complex and heart $n = 3$ due to one data point being excluded as an outlier. *$P < 0.05$ for CL vs. Con.
cantly less in the heart of CL than in Con. By comparing the synthesis of new protein to the synthesis of new DNA, we present novel insight into the potential destination of new proteins in long-lived CL mice. We demonstrate for the first time that, compared with normal litter sized controls, the transient nutrient stress of a 50% increase in litter size leads to alterations in the new protein/new DNA ratio later in life, suggesting a switch in which fewer of the new proteins synthesized are directed toward new cells and more are directed toward the maintenance of existing cellular structures. Finally, we report the novel finding of tissue-dependent increases in mTORC1 activity at 7 mo compared with no difference at 3 mo in the long-lived CL mice, suggesting that a decrease in mTORC1 may not be requisite for lifespan extension in CL.

Postnatal Feeding and Growth

Gene expression during in utero development can be profoundly affected by maternal nutrition, having lifelong effects on the healthspan and longevity of offspring (8, 13, 40). Changes in lifespan and healthspan through litter reduction in rats (3–4 pups/litter) (12) as well as litter enlargement (i.e., CL) experiments in mice (38, 39) suggest that nutritional status during early development can also have long-lasting effects. For example, reducing litter size increases pups’ caloric intake, leading to a vast array of pathologies associated with poor health (12). Alternatively, CL extends mean and maximal lifespan (39) and upregulates the transcription of some phase I xenobiotic enzymes (38), which are protective against environmental toxins, and improves multiple markers of metabolic health (33). However, it is not known how an increase in litter size affects the synthetic processes that contribute to growth and increased lifespan and healthspan.

The use of $^2$H$_2$O to measure DNA synthesis is reflective of S-phase synthesis, not DNA repair or random incorporation (6, 28). In our previous studies using models of slowed aging (11, 22, 23) and human exercise (32), we repeatedly demonstrated synthesis of new DNA in tissues comprised predominantly of postmitotic cells (i.e., heart and skeletal muscle). In the present
study, we found changes in DNA synthesis within tissues comprised predominantly of postmitotic cells that is consistent with our previous investigations (11, 22, 23, 32). Therefore, we propose two possibilities for measurable changes in DNA synthesis in postmitotic 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, cardiomyocytes have some ability to replicate their own DNA (4, 35) or incorporate DNA from other neighboring cell types (3, 29). Furthermore, regulation of cardiac DNA synthesis may vary by stage of development (42), which is supportive of the varying age-dependent DNA synthesis rates in CL heart. Similarly, skeletal muscle is primarily made up of postmitotic, multinucleated myofibers but also contains resident proliferative satellite cells, pericytes, interstitial cells, and myoendothelial cells (10, 25, 47). When myofiber size increases, requiring an increase in protein synthesis, there is an increase in satellite cell recruitment (i.e., new DNA) to stabilize the myonuclear domain (32, 41), which isotopic labeling reflects (27). Previous work in exercising humans from our laboratory suggest that it is unlikely that mitochondrial DNA synthesis contributes significantly to our measurement of total DNA synthesis in skeletal muscle using $^{2}$H$_{2}$O (32), although there could be a species difference in mice. Therefore, the presence of new DNA synthesis in skeletal muscle of 4-mo CL may indicate a period of catch-up growth. Importantly, we assessed DNA synthesis by extracting DNA from whole tissue. Thus, the age- and tissue-dependent changes we present here are likely the summation of the various cell types within multiple, primarily postmitotic, tissues of the long-lived CL mouse model. Collectively, our current data in CL mice provide novel

![Western blotting of mTOR complex 1 (mTORC1) substrates rpS6 and 4E-BP1 in fasted 3-mo CL mice. There were no differences between 3-mo CL and Con in rpS6 or 4E-BP1 phosphorylation in gastroc complex (A and C) or liver (B and D). Data are expressed as a ratio of phosphorylated to total protein. β-Tubulin is shown to verify equal loading of protein.](image-url)
insights into growth regulation across whole tissue at different ages.

**Insights from Simultaneous Assessment of Protein and DNA Synthesis**

Low enzymatic capacity for protein repair within cells means that damaged proteins must be removed and replaced through synthesis of new proteins (20, 26). In light of our previous investigations in long-lived models, we posit that maintaining rates of protein synthesis while DNA synthesis is decreased suggests that newly synthesized proteins are made primarily in existing cells, which may promote the maintenance of existing cellular structures (i.e., proteostasis), contributing to increased lifespan and healthspan (2, 11, 22, 23). In contrast to our investigations into long-lived CR and chronic rapamycin-fed mouse models of the same age (11, 22), the 7-mo long-lived CL mice had increased protein synthesis in all tissues and subcellular fractions in gastroc complex, heart, and liver. 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 (11); thus, strain differences cannot account for their differing response in protein synthesis. The elevated rates of mitochondrial protein synthesis in 7-mo CL, indicative of mitochondrial biogenesis (21), is consistent with our previous investigations and hypothesized to be key to slowed aging (11, 22, 24). Therefore, determining whether the increase in protein synthesis in 7-mo CL was occurring in new cells or existing cells may provide insight into mechanism(s) responsible for increased lifespan in CL.

To gain insight into what proportion of newly synthesized proteins are going to new cells vs. existing cellular structures, we characterized protein synthesis at the level of mTORC1 substrates rpS6 and 4E-BP1 in 7-mo CL mice. Western blotting revealed that rpS6 phosphorylation was significantly greater in heart and liver in 7-mo CL than in Con (B and C). 4E-BP1 phosphorylation was significantly greater in gastroc complex and tended to be greater in heart (P = 0.055) in 7-mo CL vs. 7-mo Con (D and E). Data are expressed as ratio of phosphorylated to total protein. B-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.
we compared the synthesis of new protein to the respective synthesis of new DNA. Comparing 4-mo CL to 4-mo Con, it appears that a greater proportion of protein synthesis is directed to cellular expansion. In contrast, in 7-mo CL mice compared with 7-mo Con, new proteins are synthesized primarily in existing cells, as illustrated by a significant increase in new proteins synthesized in relation to new DNA, indicative of new cells added to the population, across tissues. In summary, we speculate that in CL mice a tradeoff from growth to proteostasis occurs between 4 mo and 7 mo of age that is characterized by an increase in the synthesis of new protein/new DNA ratio across subcellular fractions. Therefore, maintained proteostasis may be a critical determinant in the extended lifespan and healthspan of CL mice.

**CL Growth Signaling**

mTORC1 is a central regulator of protein turnover, cell cycle, and mRNA translation (18). Inhibiting or downregulating mTORC1 signaling is suggested to be integral to the lifespan extension imparted by CR and chronic rapamycin treatment (14, 15, 22, 23). Expanding upon our protein synthesis findings in long-lived CL mice, we assessed two mTORC1 substrates, rpS6 and 4E-BP1, which are involved in rRNA transcription and translation initiation, respectively (7, 43). In young fasted (~3 mo of age) mice, phosphorylation of rpS6 and 4E-BP1 was not different between CL and Con mice in any tissue, corroborating the 2-wk protein synthesis measurements in 4-mo CL. In contrast, 7-mo CL mice had greater rpS6 phosphorylation in heart and liver, whereas 4E-BP1 phosphorylation was greater in the gastroc complex compared with 7-mo Con, consistent with the increased rates of protein synthesis observed over 2 wk in CL at 7 mo. An increase in phosphorylation of mTORC1 substrates is in contrast to the reduced mTORC1 activity seen in age-matched calorie-restricted, rapamycin-treated (11, 22), and S6K1−/− mice (34) as well as the unaltered mTORC1 activity in methionine-restricted mice (39). However, branched-chain amino acid (BCAA) supplementation extends lifespan while subsequently increasing mTORC1 activity (9). Together with our current data in the CL model, this suggests that although decreasing mTORC1 activity is sufficient to extend lifespan, it may not be necessary. We propose that increases in proteostatic mechanisms may be more important factors in lifespan and healthspan than decreased mTORC1 (39).

**Summary and Conclusion**

Here, we demonstrate in long-lived CL mice that a transient nutrient 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, compared with normal-litter-size controls, there is a greater proportion of protein synthesis directed to new cells at 4 mo, but it switches to a greater proportion of protein synthesis in existing cells, thus being directed to the maintenance of existing cellular structures, at 7 mo. We also present novel data indicating that, in CL mice, lifespan extension may be accomplished independently of reduced mTORC1 activity in primarily postmitotic tissues. These data highlight the importance of assessing pathways thought to be integral to slowed aging, such as mTORC1, as well as the metabolic outcomes, at multiple time points across the lifespan. Collectively, we expand on the body of evidence establishing CL as a novel model of longevity that provides valuable insight into protein synthesis and its role in lifespan and healthspan that could aid in the development of novel strategies to promote slowed aging.

**ACKNOWLEDGMENTS**

We thank Kathryn Baeverstad for assistance with Western blotting and Sabrina Van Roekel for technical assistance.

**GRANTS**

This project was funded by National Institutes of Health grants 1K01 AG-031829-01 to B. F. Miller, NIH R01 AG-042569 to B. F. Miller and K. L. Hamilton, and R01 AG-019899 to R. A. Miller.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**