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The Impact Of Folate Restriction On Cancer And Aging: A Mechanistic Analysis

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THE IMPACT OF FOLATE RESTRICTION ON CANCER AND AGING: A MECHANISTIC ANALYSIS

by

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DISSERTATION

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Advisor   Date

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DEDICATION

I thank God first and foremost for all of the blessings that He has bestowed on me.

This work is dedicated in loving admiration to my parents Ghassan and Iman. Their love, encouragement, and support are the basis for my success and the reason behind everything I have achieved in life. I would have never reached where I am today without them. I thank you and love you from the bottom of my heart. I thank my wonderful husband, Ali, who has been extremely supportive and patient with me, and constantly pushing me to achieve my potential. It is a blessing having you in my life. I thank my brothers Mohamad and Ali for their unlimited support and encouragement, and constantly going out of their way to give me a helping hand. I thank my in-laws and every family member that constantly encouraged me to excel. I would also like to dedicate this work to my wonderful sons, my joy, and my blessing in life, Yusuf and Yaseen.

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Archana Unnikrishnan! I cannot begin to explain how thankful I am for everything she has done for me. I appreciate her guidance, support and endless giving. It is an honor to have met her and worked with her. I would also like to acknowledge my committee members Dr. Malathy Shekhar, Dr. Diane Cabelof, and Dr. Smiti Gupta for their time and willingness to serve on this committee. My appreciation also goes to the following people who in one way or another helped me with my research: Sukayna Ismail, Ali Fardous, Rawia Khasawanah, Michelle Jones, Hongzhi Ma, Amanda Arrabi, Michael FitzGeral, Aaron Sabal, Nikitha Patel, John Sorge and Tom Prychitko.

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CHAPTER 1: BACKGROUND AND SIGNIFICANCE

Aging

Aging is a multifactorial process that reflects all of the changes that emerge over the progression of life [1]. Aging itself is not a disease [2]. However, with growth, development and maturity comes increased vulnerability to disease and ultimately resulting in mortality [3]. In other terms, aging, as defined by Fedarko is "the decline and deterioration of functional properties at the cellular, tissue and organ level" [4]. Aging is associated with the alteration of several physiological processes, such as loss of homeostasis and loss of functional capacity. In turn, individuals become more susceptible to developing diseases [1]. Morbidities associated with aging include, but are not limited to: cancer, neurodegenerative disorders, autoimmune disease, cardiovascular disease, and type II diabetes mellitus [5]. Since these diseases are highly prevalent in the geriatric population, targeting them individually limits the benefit that could be obtained. As a result, researchers are working to target the aging process as a whole in order to delay the occurrence of age-related diseases, in turn delaying aging.

Lifespan extending strategies

Over 60 years ago, McCay presented a major paradigm; he showed that it was possible to extend the lifespan of laboratory rodents by simply adjusting their diet [6]. Caloric restriction, which is a 40% restriction of diet without malnutrition, is the most extensively studied life extending strategy to date [7]. Caloric restriction is shown to increase both health span and lifespan in many species[2], but this increase is not universal among different strains of the same species. Upon examination of body weight and longevity data, it was shown that the increase in lifespan of certain genotypes due to caloric restriction, is directly related to the gain in body weight under the Ad libitum feeding regimen [7]. In other terms, it may not necessarily be that caloric restriction is

increasing lifespan. On the contrary, overfeeding may be causing the animals to become overweight, and susceptible to early onset of disease and aging. It's also been shown that weight loss as a result of caloric restriction improved glucose tolerance. Also, caloric restriction increased insulin sensitivity in middle aged healthy men and women [8]. Caloric restriction improved these processes (glucose tolerance and insulin sensitivity), which are often found impaired in overweight and obese individuals. The mechanism underlying the beneficial effect of caloric restriction has not yet been determined, but it has been speculated that it's affecting a major nutrient sensing pathway, mTOR.

Over the past 20 years, methionine restriction arose as a promising dietary restriction mimetic [9]. Methionine restriction is a 60-80% restriction in the essential amino acid in the diet. It results in a comparable increase in lifespan as compared to caloric restriction but does not require food restriction[10]. Animals fed methionine restricted diets are much smaller than control but consume more food per body weight [11]. Similar to caloric restriction, methionine restriction increases insulin sensitivity and improves lipid metabolism[10]. Though the mechanism by which methionine restriction increases lifespan was shown to be through activation of autophagy [12]. A human lifestyle consisting of a methionine restricted diet is not easily attainable since it would require developing highly palatable methionine depleted proteins.

Ames dwarf mice surfaced as a valuable model to understand the aging process[13]. These mice have a pituitary deficiency that renders them very small due to a significant reduction in growth hormone, prolactin, and thyroid stimulating hormone. The Ames dwarf mice live significantly longer than their normal siblings. These genetically modified dwarf mice have significantly lower levels of insulin like growth factor-I. ILGF-1 is a mediator of growth hormone action on growth and directly impacts mTOR.

More recently, two major drugs have also been shown to increase lifespan by directly targeting mTOR. Rapamycin, also known as Sirolumus, is a macrolide immunosuppressant drug with anti-proliferative properties[14]. It was initially used as an anti-fungal agent. Rapamycin, a direct inhibitor of the TOR kinase of mTORC1, extends lifespan in various animal models [5]. Rapamycin treatment in some studies results in impaired glucose homeostasis and insulin resistance, which are associated with reduced lifespan [15, 16]. These findings present a paradox of improved survival despite metabolic impairment. Fang et. Al explained this outcome by showing that the duration of rapamycin treatment had a differential effect on metabolism in mice [17].

Metformin, an oral anti-diabetic drug used as first line treatment of type II diabetes mellitus [18], increases lifespan in animal models. Treatment with metformin has been shown to alleviate several human aging-related disorders, such as cancer and non-alcoholic fatty liver disease [19]. Metformin mimics caloric restriction by increasing insulin sensitivity, glucose utilization, and fatty acid oxidation. In mammals, metformin promotes lifespan through activation of a major metabolic fuel gauge (AMPK)[20]. Activation of AMPK increases mitochondrial ROS production and induces stress defense that results in increased longevity; a process known as mitohormesis [19]. Metformin mimics antifolate chemotherapeutic agents such as pemetrexed that directly inhibit multiple folate-dependent targets in the one carbon metabolism, in turn leading to the activation of AMPK and inhibition of mTOR [21].

mTOR

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that regulates cell growth, autophagy, and proliferation by modulating protein synthesis and homeostasis[22]. It is present in two multiprotein complex forms: mTORC1 and mTORC2.

mTORC1 pathway integrates input from various upstream intracellular and extracellular signals that include growth factors, stress, oxygen, energy status, and amino acid levels. The best characterized process downstream of mTORC1 is protein synthesis, via phosphorylation of two downstream target proteins: S6K1 and 4E-BP1[23].

mTOR Signaling
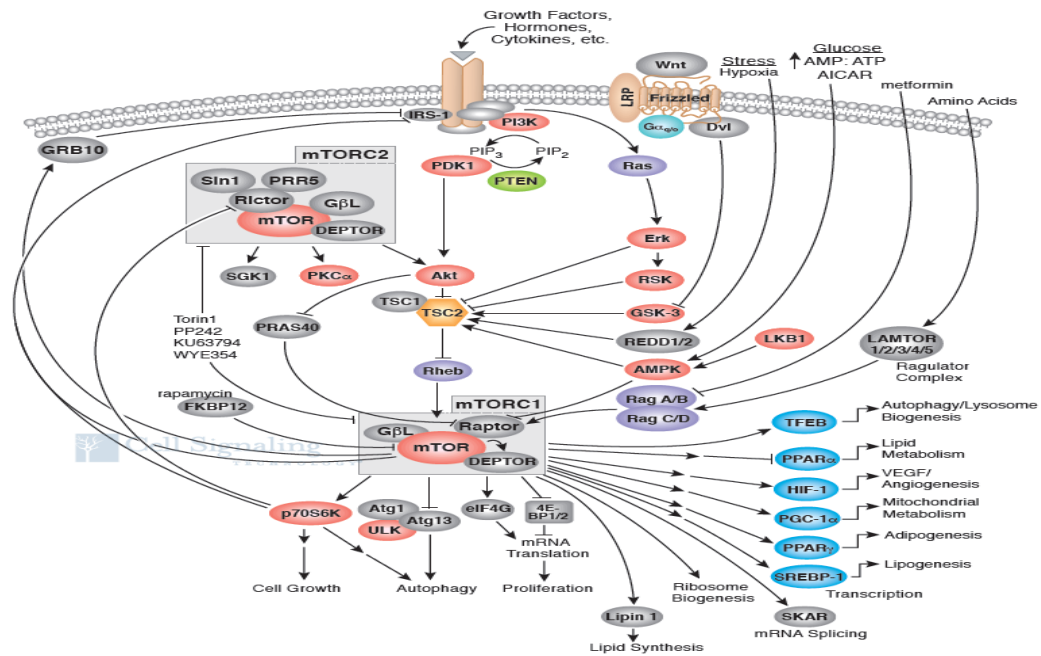
Unlike mTORC1, much less is known about mTORC2. mTORC2 is not sensitive to nutrients but it responds to growth factors in poorly defined mechanisms[23]. Whereas mTORC1 is activated via phosphorylation of AKT at the TH308 residue downstream of the PI3K signaling pathway, mTORC2 phosphorylates and activates AKT at the SER473 residue; the site required for maximal activation[24]. mTORC2 also activates PKCα along with other effectors and regulates the cell's shape by affecting the actin cytoskeleton[25].

Genetic Downregulation of mTOR

The mTOR signaling pathway is altered in many cancers [24]. Genetic down-regulation of TOR signaling delays aging in evolutionary distant organisms from yeast to mammals[26]. Selman et. Al showed that deletion of ribosomal S6 protein kinase 1 (S6K1), a target of mTORC1, extends

median lifespan in female mice by 19%. It also induces a healthy mammalian lifespan by improving insulin sensitivity, and increasing resistance to age-related pathologies, such as bone, immune, and motor dysfunction [27]. In another study, genetic reduction of mTOR expression to 25% of wild type increased mammalian lifespan. They showed that these animals have reduced both mTORC1 and mTORC2 activity, and exhibit a 20% increase in median survival [28].

Folic Acid

A major micro-nutrient that impacts this nutrient sensing (mTOR) pathway is folic acid. Folic acid is an essential water soluble B-vitamin and a cofactor in one-carbon metabolism. It is the synthetic form of the naturally occurring vitamin folate, and is used in supplements and food fortification programs. It has been associated with the etiology of many chronic diseases such as cardiovascular disease, neurological degeneration, and cancer [29].

Folate Metabolism

Folic acid enters through the diet and mediates one-carbon transfer. It is converted to dihydrofolate, then reduced to tetrahydrofolate (THF). In a series of reactions that require MTHFD, THF is then converted to N^5, N^{10} methyleneTHF affecting purine and thymidine synthesis. Folate, in the form of 5-methylTHF, is involved in the remethylation of homocysteine to methionine, a precursor of S-adenosylmethionine (SAM). SAM is the primary methyl group donor for most biological methylations including that of DNA. SAM is then converted into S-adenosylhomosysteine (SAH), a potent inhibitor of most SAM-dependent methyltransferases. Homocysteine is also converted to cysteine and to the anti-oxidant glutathione via the transsulfuration pathway.
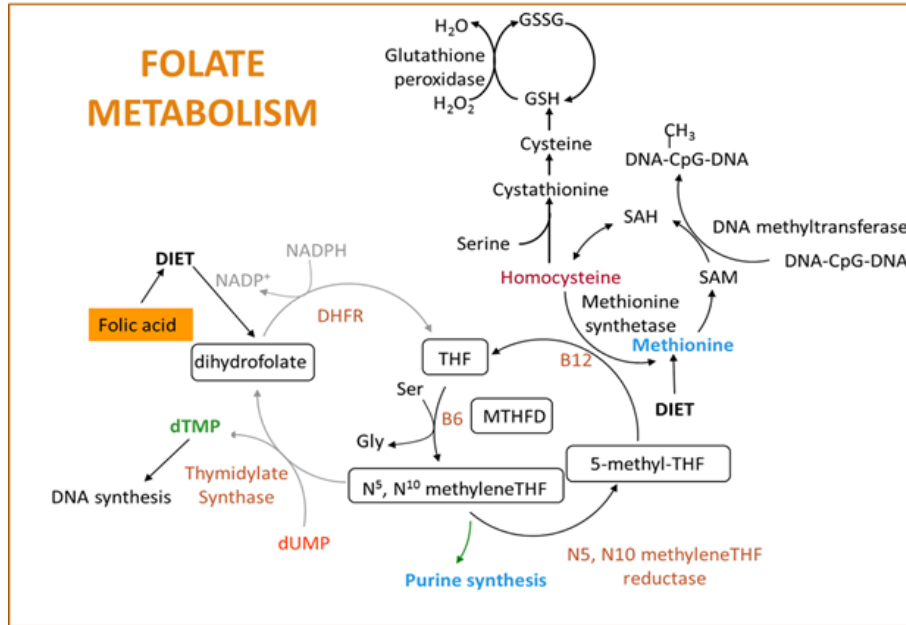
It had been speculated that inadequate folate may lead to uracil misincorporation and deoxynucleotide imbalance, resulting in single strand breaks and DNA damage. Recent evidence suggests that nuclear folate levels were resistant to folate depletion when total cellular folate levels were reduced by more than 50% in the liver. Under folate deficiency (0 mg of Folic acid per Kg diet), MTHFD localizes and becomes enriched in the nucleus to support thymidylate biosynthesis, protecting the DNA by limiting uracil misincorporation. By localizing and enriching in the nucleus, thymidylate biosynthesis is favored over remethylation of homocysteine to methionine [30]. We speculate that the timing and duration of folate restriction result in epigenetic changes in the DNA. Though further studies are required to confirm.

Folate Study

Our lab established an animal model to study the effect of folate in the diet. Data is lacking on folate requirement for mice, and on mice plasma folate levels in the wild. Chow diet was not chosen since the amount of folic acid in it is variable (2 mg-15mg), with an average of 8 mg of folic acid per kg of diet. The diets that we used were a folate adequate (2 mg of FA/kg diet) or a

folate depleted (0 mg FA/kg diet) AIN93G-purified isoenergetic diet. After 12 weeks on the respective diets, plasma folate levels in our mice were assessed to confirm drop of folate in the animals fed a folate depleted diet. There was a 90% decrease in plasma folate in these mice (figure 1.1). We also looked at the effect of a folate depleted diet on plasma folate levels at various time points. Plasma folate levels decreased by the 3rd day of the animals being on a folate depleted diet (figure 1.2). Since no further decrease was observed after the first week on the diet, and the animals did not show any signs of anemia, we termed this condition folate restriction rather than folate deficiency.

Folate Restriction and extension in lifespan

Preliminary data from our lab shows that folate restriction extends median lifespan in our C57BL/6 mouse model. At 850 days of life, 86% of the animals that were on a folate restricted diet were still alive as compared to 60% of the animals that were on a folate adequate diet.

Folate Restriction and colon carcinogenesis

Our lab investigated the effect of folate restriction on colon carcinogenesis in β-pol haploinsufficient mice [31]. A β-pol haploinsufficient background has limited base excision repair, thus exposure to a carcinogen results in DNA damage that will not be efficiently repaired. As shown in figure 1.3, there was no ACF (Aberrant crypts per foci) in the colon of control mice fed a folate adequate or a folate depleted diet; indicating that β-pol haploinsufficiency by itself, and β-pol haploinsufficiency in combination with folate restriction do not cause ACF formation.

We treated our mice with DMH (Dimethylhydrazine), a methylating agent that causes colon and liver cancer. Upon examining the data, there was a significant increase in the number of ACF in the colons of folate restricted wild type and folate adequate β-pol haploinsufficient animals. Surprisingly, folate restriction reduced ACF numbers to levels similar to the folate

adequate wild type. Rather than exacerbating the problem, β-pol haploinsufficiency in combination with folate restriction provided a protective effect. This observation went against our expectation. We were interested then to see if this protection is limited to the β-pol haploinsufficient genetic background.

Long term vs. short term Folate restriction

Our lab became interested in investigating the duration that these animals can survive without an essential vitamin. After leaving them for 16 months on a folate depleted diet, the animals were not showing any signs of anemia, and they looked healthier than their folate adequate counterparts. Furthermore, exposing the mice to a carcinogen after having acclimated to a prolonged folate restricted diet resulted in very few ACF. In this small trial, we saw that long term folate restriction did not result in the adverse side effects mentioned in literature. The results also contradicted what we saw in short term folate restriction in our previous study.

In order to confirm these results, we designed a study to look at whether the duration of folate restriction before exposure to a carcinogen has a differential effect on ACF in mice. The animals were fed either a FA (2 mg FA/kg diet) diet, FR (0 mg FA/kg diet), or FA/FR (7 weeks on FA diet then 1 week on FR diet) for a total of 8 weeks. Animals were randomly chosen, exposed to DMH, and later sacrificed.

Upon examining serum folate levels, we saw a significant decrease in serum folate in both short term (FA/FR) and long term (FR) folate restricted groups (figure 1.4). There was a 90% decrease in serum folate levels, similar to what we have seen before. When we examined the ACF data (figure 1.5), we saw that the duration of folate restriction had a differential effect on ACF in our mouse model. Placing the animals on a folate restricted diet a week before DMH treatment

(FA/FR) resulted in a significant increase in ACF as compared to FA. Interestingly, animals on long term folate restriction (FR) had fewer ACF in the colon as compared to FA.

When we compared ACF to serum folate levels, we saw that eventhough folate levels were significantly lower in both folate restricted groups, in one group (FA/FR) it was unfavorable while in another (FR) it was protective; where we saw a decrease in adverse side effects. These results are similar to what we saw in our previous study. Introducing two stressors concurrently without giving the animals time to adapt is disadvantageous. On the other hand, giving the animals time to adapt to folate restriction was protective, where we saw significantly less ACF. (Figure 1.5)

In this study, we first investigate the effect of folate restriction on the mTOR signaling pathway, by examining upstream regulators and downstream targets of mTOR. Data suggests that folate restriction downregulates the mTOR signaling pathway mainly via activation of AMPK. Then we compare the effect of 3 well documented life extending strategies (Rapamycin, crowded litter, caloric restriction, and their control) on the mTOR signaling pathway. Data shows that not all of the strategies downregulate mTOR. Furthermore, the impact seen on upstream regulators of mTOR is differential.

Figure 1.1: *The effect of folate in the diet on plasma folate levels in C57BL/6 mice.* The animals were fed either a folate adequate (FA) or folate depleted (FD) AIN93G-purified isoenergetic diet (Dyets, Inc., Lehigh Valley, PA) as previously described[32]. The FA group received a folate adequate diet containing 2 mg of folic acid/kg diet. The FD group received a folate-deficient diet containing 0 mg of folic acid/kg diet. The animals remained on their respective diets for 12 weeks after which they were sacrificed. Plasma was collected and folate levels were analyzed using the lactobacillus casei microbiological assay[33]. * Significant differences at $P < 0.05$.

Figure 1.1: *The effect of folate in the diet on plasma folate levels in C57BL/6 mice.*

Figure 1.2: *The effect of folate depletion in the diet on plasma folate levels at various time points.* C57BL/6 mice were fed either a folate adequate (FA) or folate depleted (FD) AIN93G-purified isoenergetic diet (Dyets, Inc., Lehigh Valley, PA) as previously described[32]. The FA group received a folate adequate diet containing 2 mg of folic acid/kg diet. The FD group received a folate-deficient diet containing 0 mg of folic acid/kg diet. The animals remained on their respective diets for the duration of the study. Blood was drawn from the capillary of the eye at various time points, and folate levels were analyzed using the lactobacillus casei microbiological assay [33]. Different letters indicate significant differences at $P < 0.05$.

Figure 1.2: *The effect of folate depletion in the diet on plasma folate levels at various time points.*
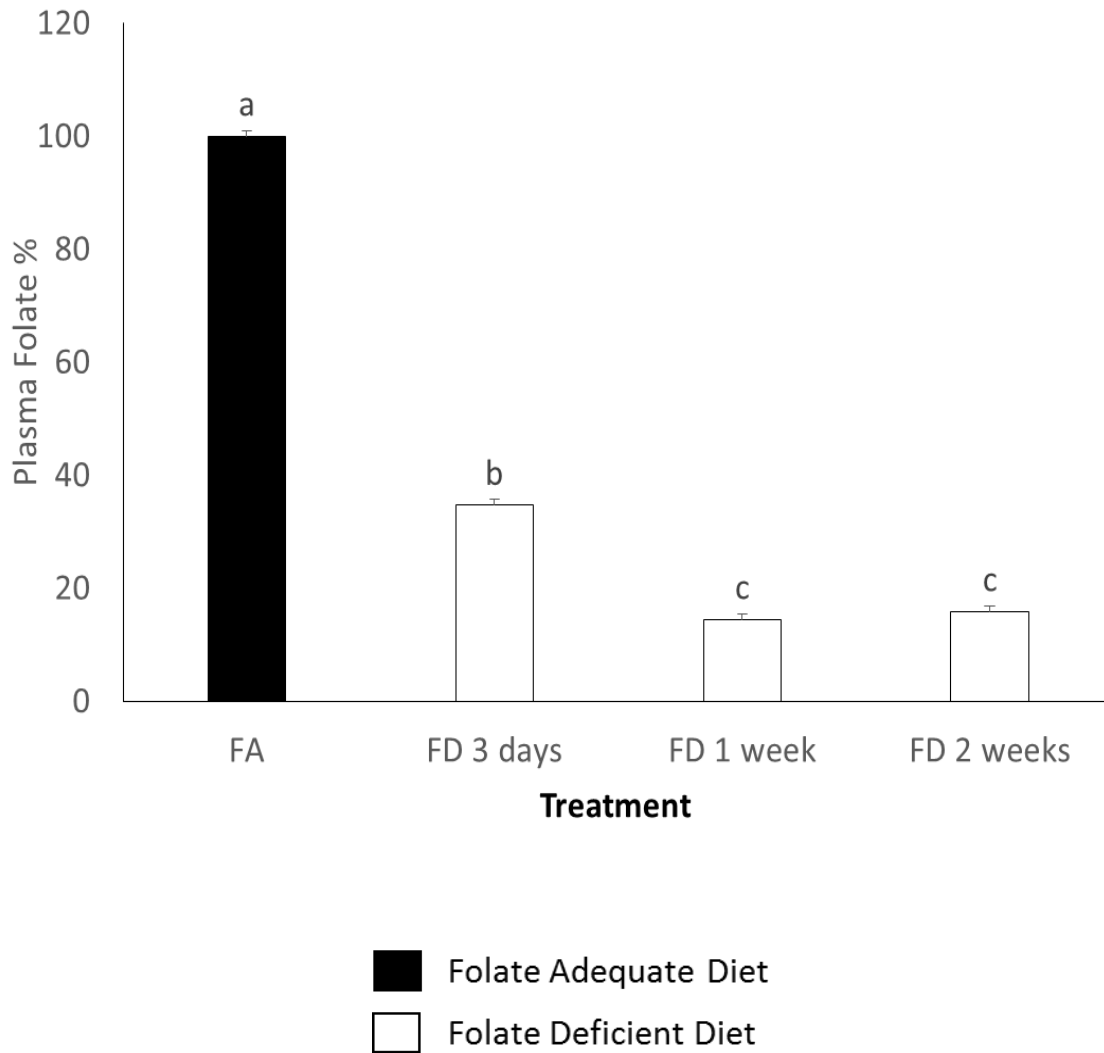
Figure 1.3: *The effect of folate restriction on colon carcinogenesis in β-pol haploinsufficient mice.* WT and β-pol$^{+/-}$ C57BL/6 mice were fed either a folate-adequate (2 mg FA/kg diet) or a folate-deficient (0 mg FA/kg diet) diet. After 1 week on the respective diets, animals were randomly chosen and were injected with 30 mg/kg body weight of DMH for 6 weeks. Six weeks after the final injection, animals were sacrificed by CO_2 asphyxiation. The abdominal cavity was opened and the colon was excised, rinsed with cold phosphate-buffered saline, cut longitudinally, and fixed flat overnight in 10% neutral buffered formalin. On the next day, the colonic crypts were stained with 2 g/liter of methylene blue in phosphate-buffered saline for 5 min. The number of ACF was determined by light microscopy at ×10 magnification in a blinded manner. Different letters indicate significant differences at $P < 0.05$.

Figure 1.3: *The effect of folate restriction on colon carcinogenesis in β-pol haploinsufficient mice.*
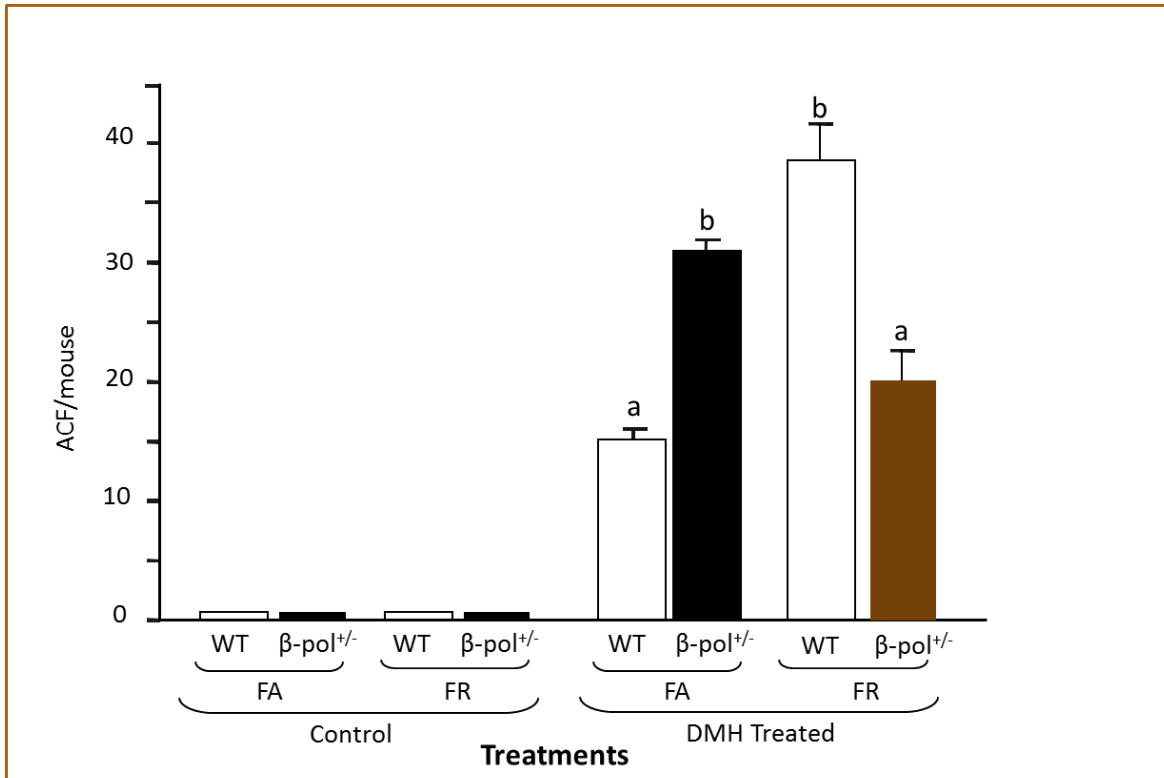
Figure 1.4: *The effect of long term and short term folate restriction with/out antibiotic incorporation in the diet on serum folate levels.* C57BL/6 mice were fed either a folate adequate (FA), long term folate depleted (FR), or short term folate depleted (FA/FR) AIN93G-purified isoenergetic diet (Dyets, Inc., Lehigh Valley, PA) as previously described [32]. 1% succinyl sulfathiazole was added to some of the diets (+AB). The FA group received a folate adequate diet containing 2 mg of folic acid/kg diet. The FR (Long term folate restriction) group received a folate-deficient diet containing 0 mg of folic acid/kg diet. The FA/FR (Short term folate restriction) group received a folate adequate diet for 7 weeks, then switched to a folate deficient diet for one week before animals were randomly chosen and injected with DMH once a week for 6 weeks at 30 mg/kg body weight. The animals remained on their respective diets until they were sacrificed at 21 weeks. Upon sacrifice, serum was collected and folate levels were analyzed using the lactobacillus casei microbiological assay [33]. Different letters indicate significant differences at $P < 0.05$.

Figure 1.4: *The effect of long term and short term folate restriction with/out antibiotic incorporation in the diet on serum folate levels.*
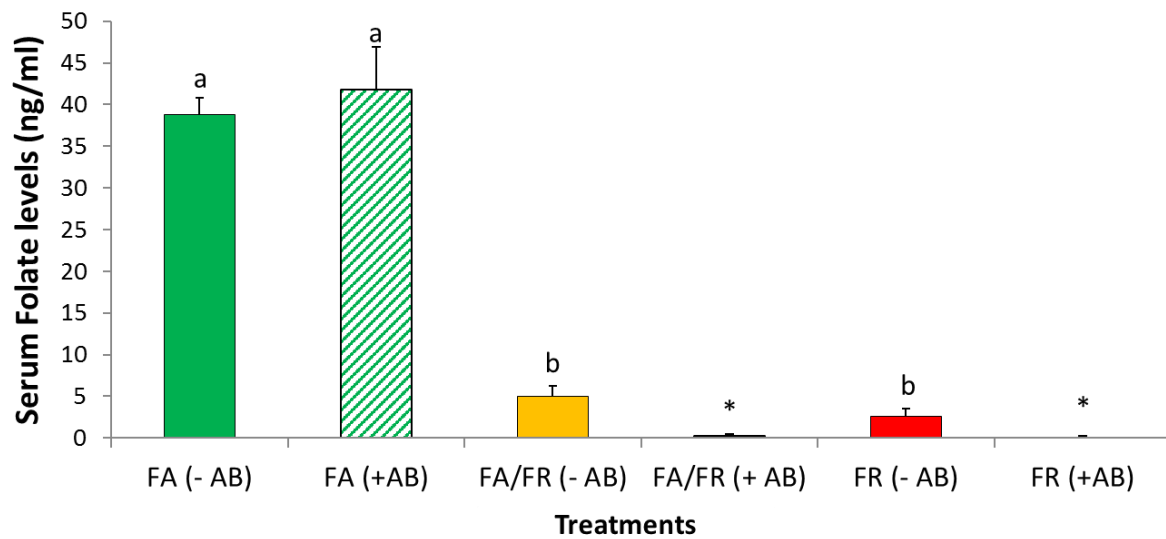
Figure 1.5: *The effect of long term and short term folate restriction in the diet on ACF.* C57BL/6 mice were fed either a folate adequate (FA), long term folate depleted (FR), or short term folate depleted (FA/FR) AIN93G-purified isoenergetic diet (Dyets, Inc., Lehigh Valley, PA) as previously described [32]. 1% succinyl sulfathiazole was added to some of the diets (+AB). The FA group received a folate adequate diet containing 2 mg of folic acid/kg diet. The FR (Long term folate restriction) group received a folate-deficient diet containing 0 mg of folic acid/kg diet. The FA/FR (Short term folate restriction) group received a folate adequate diet for 7 weeks, then switched to a folate deficient diet for one week before animals were randomly chosen and injected with DMH once a week for 6 weeks at 30 mg/kg body weight. The animals remained on their respective diets until they were sacrificed at 21 weeks. After sacrifice, the colon was excised, rinsed with cold phosphate-buffered saline, cut longitudinally, and fixed flat overnight in 10% neutral buffered formalin. On the next day, the colonic crypts were stained with 2 g/liter of methylene blue in phosphate-buffered saline for 5 min. The number of ACF was determined by light microscopy at $\times 10$ magnification in a blinded manner. Different letters indicate significant differences at $P < 0.05$.

Figure 1.5: *The effect of long term and short term folate restriction in the diet on ACF.*
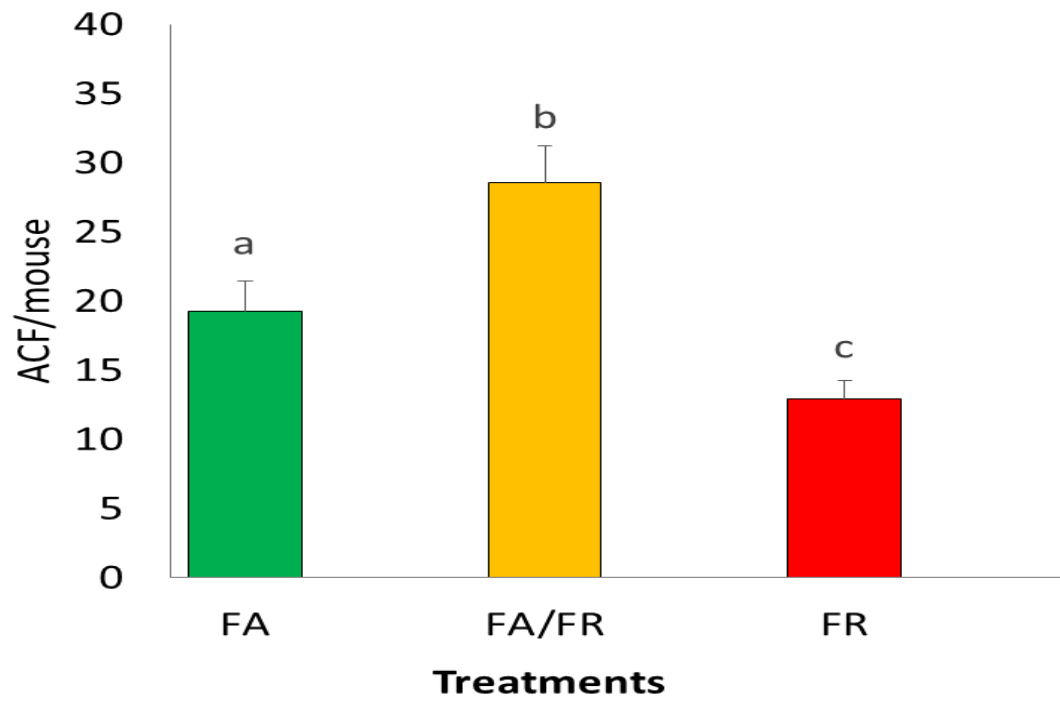
CHAPTER 2: FOLATE RESTRICTION

Introduction

Aging is a multifactorial process associated with alterations in several physiological functions [1]. It increases susceptibility to disease due to loss of functional capacity and loss of homeostasis [2]. Some of the associated diseases include cancer, neurodegenerative disorders, autoimmune disease, cardiovascular disease, and type II diabetes mellitus [5]. Since these diseases are highly prevalent in the geriatric population, targeting the aging process as a whole may provide a better way to delay incidences of many age related diseases [5, 34]. Some of the interventions that have been known to delay aging include: caloric restriction [6], methionine restriction [9], genetic alterations such as Ames dwarf mice [13], rapamycin [35], and metformin [19]. These interventions affect and inhibit a major nutrient sensing pathway, mammalian target of rapamycin (mTOR) [35-39]. The mTOR signaling pathway is altered in many cancers [26] , and down-regulation of TOR signaling delays aging in a number of organisms, ranging from yeast to mammals [27, 28, 38, 40-42].

A major micronutrient that has been associated with the etiology of many of these age related diseases is folate. Folate is an essential water soluble B-vitamin, naturally present in green vegetables, legumes, and citrus fruits. The synthetic form, folic acid, is used in supplements and food fortification programs (1998 FDA mandate) [43]. Folate plays a critical role in one carbon metabolism. It is involved in purine and pyrimidine biosynthesis, amino acid metabolism, regeneration of S-Adenosyl methionine, and glutathione production [44]. Folate is involved in normal development & maintenance of cellular functions, genomic integrity, and regulation of gene expression [45].

Folate deficiency has been associated with many ailments including: megaloblastic anemia, cardiovascular disease, neurodegenerative diseases, and cancer [46-51]. Epidemiological and laboratory rodent based studies show an association between folate deficiency and colorectal, prostate, and breast cancers [52-56]. Although the exact mechanism behind folate deficiency induced carcinogenesis is largely unknown, low folate has been shown to cause genomic instability, DNA uracil misincorporation, alterations in DNA methylation pattern, DNA strand breakage, and chromosomal aberrations [46, 57-60]. Folate deficiency acts synergistically with damaging agents, reducing DNA damage threshold, and increasing mutation frequency [61-64]. A possible mechanism behind folate deficiency induced genomic instability and cancer development, may be impaired DNA repair pathways [65, 66]. To directly test how folate deficiency induces colon cancer, we analyzed the impact of folate deficiency on the onset and progression of cancer in a polymerase β haploinsufficient (β-pol$^{+/-}$) mouse model. β-pol is a rate limiting enzyme in the BER pathway, required for the repair of oxidative DNA damage and uracil misincorporation.

Our lab showed that a folate depleted diet in combination with β-pol haploinsufficiency reduced incidences of preniosplastic lesions [31]. BrdU and TUNEL assays revealed a decrease in the proliferative capacity, and an increase in apoptosis in the colon of FR animals [31]. Others have also shown that reduced dietary folate decreased tumor incidence in various mouse cancer models [67-69]. Kadaveru and colleagues showed that folate deficiency protected against intestinal tumor development in the colon of APC$^{MIN/+}$ mice [68]. MacFarlane and colleagues showed that folate deficiency was negatively correlated with inflammatory bowel disease, a risk factor for developing colon cancer [69]. Bistulfi and colleagues reported that folate deficiency blocked prostate cancer progression in the TRAMP animal model, generally characterized with aggressive tumors [67]. These findings provide insight about the anti-cancer effect of folate restriction.

Inhibition of folate synthesis, extended lifespan in C. elegans [70]. Down regulation of S-adenosyl methionine synthase, a critical enzyme in the one carbon folate metabolism, also extended lifespan in C. elegans [71]. Metformin, an emerging gerosuppressant, impaired one carbon metabolism similar to antifolate drugs in human cancer cells [21]. It's well established that folic acid cofactors are involved in De novo purine synthesis necessary for AMP biosynthesis [72-74]. Stenesen and colleagues showed that a mutation in the AMP biosynthesis pathway increased healthy lifespan [75]. Folate restriction may result in alterations in the De novo purine biosynthesis pathway, impacting aging and cancer. We present folate restriction as a possible nutritional intervention to extend lifespan and improve healthspan, and the objective of this study is to characterize the mechanism.

Experimental Procedures

Animals- All the experiments were performed in male C57BL/6 specific pathogen–free young wildtype mice in accordance with NIH guidelines for the use and care of laboratory animals. The Animal protocol was approved by the Wayne State University Animal Investigation Committee. The animals were fed the standard mouse chow and water ad libitum and were maintained on a 12-hr light/dark cycle.

Diets and Carcinogen treatment- Experiment 1: At 6 weeks of age, mice were randomly assigned to two dietary groups and fed AIN93G-purified isoenergetic diets (Dyets, Inc.,Lehigh Valley,PA) [32]. Diets were stored at -20°C. The control group (FA) received a folate adequate diet (2mg FA/kg diet). The experimental group (FR) received a folate deficient diet (0 mg folic acid/kg diet). Diets were supplemented with 1% succinyl sulfathiazole. The animals were monitored for signs of toxicity and remained on their respective diets for 12 weeks. Mice were anesthetized in a CO_2 chamber and the abdominal cavity was opened up for excising the colon and harvesting the liver

tissue. The harvested liver was flash frozen and stored in liquid nitrogen. Experiment 2: At 4 months of age, mice were randomly assigned to three dietary groups and fed AIN93G-purified isoenergetic diets (Dyets, Inc.,Lehigh Valley,PA) [32]. Diets were stored at -20°C. The control group (FA) received a folate adequate diet (2mg FA/kg diet for 8 weeks). The experimental groups: (FR) received a folate deficient diet (0 mg folic acid/kg diet for 8 weeks) and (FA/FR) received (2mg FA/kg diet for 7 weeks then 0 mg folic acid/kg diet for 1 week for a total of 8 weeks). Diets were supplemented with 1% succinyl sulfathiazole. After 8 weeks on the respective diets, mice were treated IP with 1, 2-dimethylhydrazine HCl (DMH, 30 mg/kg body weight) in 10 mmol/litre of $NaHCO_3$ (Fisher Scientific) once a week for 6 weeks [31]. The animals were monitored for signs of toxicity and remained on their respective diets for a total of 21 weeks. Mice were anesthetized in a CO_2 chamber and the abdominal cavity was opened up for excising the colon and harvesting the liver tissue. The harvested liver was flash frozen and stored in liquid nitrogen

Folate Assay: Serum was collected upon sacrifice. Folate was measured using the Lactobacillus casei microbiological assay of folic acid derivatives as described by Horne et. Al [33]. Briefly, growth response of lactobacillus casei to folate availability was measured at OD 600 nm. A standard curve was used to calculate folate concentrations. Folate levels are expressed as percent folate. The assay was conducted courtesy of Dr. Cabelof's lab.

Amino Acid Levels: Plasma was collected upon sacrifice and sent to MSU and UC-Davis for HPLC analysis. Amino Acid pool and plasma amino acid are expressed as ratio of FR/FA.

Aberrant crypt foci (ACF) analysis- The excised colons were rinsed with cold phosphate-buffered saline, cut longitudinally, and fixed flat overnight in 10% neutral buffered formalin as described previously [31]. The fixed colons were stained with 2g/liter of methylene blue in phosphate-

buffered saline for 5 mins. The number of ACF and aberrant crypts per foci were determined by light microscopy at 10X magnification in a blinded manner as described previously [31].

Isolation of Crude Nuclear extract- Nuclear extracts were isolated using a transfactor extraction kit (Clontech, Mountain View, CA) as previously described [76]. Briefly, a hypotonic buffer is used to lyse the cell allowing for the removal of cytosolic fractions, followed by a hypertonic buffer which helps in the extraction of nuclear proteins. All solutions were made fresh and all samples and tubes were maintained on ice. Low molecular weight contaminants were removed from extracts by dialysis using Slide-A-Lyzer^R mini-dialysis units (Pierce Biotechnology, Rockford, IL) with a molecular weight cut off of 3.5 KD for 3 hours at 4°C. The dialysis buffer has 20mM Tris-HCl, pH 8.0, 100mM NaS2O5, 0.1mM PMSF, and 1mg/ml Pepstatin A. Dialyzed extracts were aliquoted and stored at -80°C for subsequent analysis. Protein concentrations were determined using to Bradford protein assay kit I (Bio-Rad, Hercules, CA).

Isolation of Whole cell extract- Whole cell extracts were isolated using hypotonic and hypertonic salt solutions from transfactor extraction kit (Clontech, Mountain View, CA). Briefly, 100mg of liver tissue was homogenized with the hypotonic salt solution to lyse the cell and further treated with the hypertonic salt solution to release the nuclear contents without any fractionations. The whole cell extract thus obtained was used for various assays.

Western Blot Analysis- Protein expression analysis was performed using western blot technique as previously described [76]. 100 µg nuclear protein was used to run the SDS-PAGE. Upon completion of SDS-PAGE, the region containing the proteins of interest was excised and prepared for western blot analysis while the remaining portion of the gel was stained with Gel Code Blue Stain Reagent (Pierce Biotechnology, Rockford, IL) to ensure equal protein loading. Manufacturer recommended dilutions of anti-sera developed against p-AMPK (Abcam), t-AMPK (Millipore),

p-AKT (Cell Signaling), t-AKT (Cell Signaling), REDD1 (Abcam), p53 (Abcam), PARP (Cell Signaling), IPMK (Abcam), p-S6K1 (Cell Signaling), t-S6K1 (Cell Signaling), p-4EBP1 (Cell Signaling), t-S6K1 (Cell Signaling), PEPCK (Santa Cruz) and G6PASE (Santa Cruz) were used to detect proteins of interest followed by incubation with HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The bands were visualized and quantified using a Molecular Imager System (Bio-Rad, Hercules, CA) after incubation in SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). Data are expressed as the integrated density value (I.D.V.) of the band normalized to β-actin (Sigma Aldrich) or ratio of phosphorylated form of protein to total normalized to β-actin.

Gene expression profiling- The mRNA expression levels of SIRT1 and Gadd45g were quantified using a real-time PCR. Total RNA was extracted from liver tissue using RNeasy extraction kit (Qiagen, Valencia, CA). First strand cDNA was synthesized from 1μg RNA using random primers and purified using QIAquick PCR purification Kit (Qiagen, Valencia, CA). Expression of SIRT1 was quantified using real time PCR with specific primers for the gene (FP: 5'-AACTTCACAGCATCTTCAAT3' and RP 5'-TGACACTGTGGCAGATTGTT3'). Expression of Gadd45g was quantified using real time PCR with specific primers for the gene (FP: 5'-AGTTCCGGAAAGCACAGCCAGGATG-3' and RP: 5'-GCCAGCACGCAAAAGGTCACATTGT-3'). The gene transcript was normalized to RPLO with specific primers for the gene (FP: 5'-AATTTCAATGGTGCCTCTGG-3' and RP: 5'-GATTCGGGATATGCTGTTGG-3'). External standards for all genes were prepared by subcloning the amplicons, synthesized using the specific primers into PGEM-T easy vector.

Caspase Assay- Caspase-3 activity was measured using Enzchek Caspase-3 Assay Kit No.1 (Molecular probes, Eugene, OR) as described previously [76]. Briefly, Liver tissues were

homogenized, and cytosolic extracts were isolated using Transfactor Extraction Kit (Clonetech, Mountain View, CA). The extracts (250 mg protein) were incubated for 2hr at room temperature in the working solution (25mM PIPES, pH 7.4, 5mM EDTA and 2.25% CHAPS) containing synthetic caspase-3 substrate, Z-DEVD-AMC. Caspase mediated proteolytic cleavage of the substrate yields a bright blue-fluorescent product. An additional control assay was performed using reversible aldehyde inhibitor Ac-DEVD-CHO to confirm that the fluorescence observed in the sample assay was due to caspase activity. The fluorescence was measured using a fluorescence microplate reader (Genios plus, Tecan) at excitation: 342nm, emission: 441nm. The caspase activity was determined using an AMC (7-amino-4-methylcoumarin) standard curve (0-100mM), and reported as fluorescence per mg of protein.

ATP Assay: Total ATP was extracted from 10 mg of liver using Abcam's ATP Assay kit. This is a calorimetric assay that utilizes the phosphorylation of glycerol to generate product that is easily quantified at OD 570 nm. Total amount (cellular and mitochondrial) of active ATP is detected. ATP levels are expressed as (nmoles/ml).

NAD/NADH Assay: Total NAD (NAD and NADH) was extracted from 20 mg of liver using Abcam's NAD/NADH Assay kit. This is a calorimetric assay that detects intracellular nucleotides at OD 570 nm. Total NAD is extracted and detected. Then NAD^+ is decomposed to detect NADH. The values are used to calculate for NAD^+. NAD^+ levels are expressed as (uM). The ratio of NAD^+ to NADH is also calculated.

Statistical Analysis- Statistical significance between means was determined using t-test and analysis of variance followed by post tukey test wherever appropriate. P-values less than 0.05 were considered statistically significant.

Results

Analysis of folate status in the diet on plasma folate levels in C57Bl/6 mice:

We established an animal model to study the effect of dietary folate. Data is lacking on folate requirement for mice, and on mice plasma folate levels in the wild. Chow diet was not chosen since the amount of folic acid in it is variable (2 mg-15 mg), with an average of 8 mg of folic acid per kg of diet. The diets that were used were a folate adequate diet (2 mg of folic acid per kg of diet) or a folate depleted diet (0 mg of folic acid per kg of diet). Both diets were supplemented with 1% succinyl sulfathiazole, an antibiotic known to alter flora in the colon limiting the number of bacteria that can generate folate. We randomly assigned 8 C57BL/6 mice at 6 weeks of age into two groups. One group was fed a folate adequate diet, while the other group was fed a folate depleted diet. The animals remained on their respective diets for 12 weeks and then sacrificed. Plasma was collected, colon was excised, and tissues were harvested and flash frozen in liquid nitrogen for further analysis. Similar to what we have shown before [31], the amount of food consumed and body weight did not significantly differ between groups (Data not shown).

Plasma folate levels were assessed at various time points throughout the study. There was 60% reduction in plasma folate by the 3rd day on the folate depleted diet, and 90% reduction within the first and second weeks Figure 2.1. The 90% reduction in plasma folate was maintained for the duration of the 12 week study Figure 2.2. Since no further decrease was observed after the first week on the diet, and the animals did not show any signs of anemia, this condition was termed folate restriction rather than folate deficiency.

Analysis of the development of preneoplastic lesions during folate restriction in C57Bl/6 mice:

Low folate status has been associated with the development and progression of various types of cancer, specifically colorectal cancer [52-54]. However, recent studies show that low folate may be protective against spontaneous [77], and chemically induced cancer [78, 79]. Inconsistencies in these studies may be due to differences in experimental design, dosages, timing and duration of folate deficiency. In the first experiment, we investigated the effect of low folate status on the development of spontaneous preoplastic lesions in the colon of C57BL/6 mice. We looked at aberrant crypt foci (ACF), which are clusters of preoplastic legions, and are the earliest changes in the colon that may lead to cancer [80-82]. Aberrant crypts develop as single crypts, and grow over time in a process known as crypt fission. They appear as a foci with multiple aberrant crypts, and are homogenous in their genetic make-up. Animals were placed on a folate adequate (FA) or folate restricted (FR) diet for 12 weeks, after which they were sacrificed for analysis. The numbers of ACF and aberrant crypts per foci were determined using light microscopy. As shown in Figure 2.3, the colons from FA and FR groups appeared morphologically normal, with no signs of aberrant crypts. Folate restriction by itself proves to be a low penetrance event. It does not independently give rise to spontaneous preoplastic lesions.

In the second experiment, we investigated the effect of low folate status on the development of chemically induced preoplastic lesions in the colon of C57BL/6 mice. 1,2-dimethylhydrazine (DMH), a potent colon and liver carcinogen, was used to induce ACF. The DMH-mouse model of colon carcinogenesis is extensively studied [83-87]. Cancer is induced in a manner histologically similar to human colon cancer [88-90]. DMH is a known alkylating agent that is activated in the liver [91, 92]. Upon activation, DMH produces metabolic intermediates that are transported to the colon via bile and blood [93, 94]. The colon is also capable of converting DMH into mutagenic products without involving the liver[95]. We randomly assigned C57Bl/6 mice into 3 groups.

Animals were fed a FA (2 mg FA/kg diet) diet, FA/FR (7 weeks on FA diet then 1 week on FR diet), or FR (0 mg FA/kg diet) for a total of 8 weeks; after which we began their carcinogen treatment. All three groups were injected with 30 mg/kg body weight of DMH, once a week for 6 weeks. Seven weeks after the final DMH injection, the mice were sacrificed and the colons were scored for aberrant crypt foci. As shown in Figure 2.4, the FR animals which were acclimated to folate depletion for 8 weeks prior to DMH treatment, showed significantly less ACF than FA. On the contrary, the FA/FR animals which were treated with DMH one week after folate depletion, showed significantly higher number of ACF as compared to both FA and FR. Also, ACF did not correlate with plasma folate levels (Data not shown). Eventhough folate levels were significantly lower in both folate restricted groups, there was a differential effect on ACF. There was significantly more ACF in the short term folate restricted group (FA/FR), but significantly less ACF in the long term folate restricted group (FR). Giving the animals time to adapt to folate restriction before introducing a carcinogen was protective, in terms of seeing significantly less ACF (Figure 2.4).

Aberrant crypt foci, once formed, can regress with time. Persistence of these aberrant crypts, and occurrence of foci with multiple aberrant crypts, correlates with tumor aggressiveness. A foci with more than two aberrant crypts can potentially develop and progress into an adenoma and adenocarcinoma. Upon evaluation of crypt multiplicity, the acclimated FR animals displayed reduced crypt multiplicity (1.49) when compared to FA (1.87) and FA/FR (2.33). Long term acclimation to folate restriction reduces susceptibility to environmental insults, and can potentially reduce development of colon cancer. Our study shows that an acclimation period on a folate depleted diet is essential to detect the beneficial effect of FR, and can explain the inconsistencies surrounding the pro-carcinogenic impact of folate deficiency.

Analysis of one-carbon cycle, amino acid metabolism and energy metabolism during folate restriction:

Folate is an essential water soluble B-vitamin that plays a vital role in one carbon metabolism (Figure 2.5). In the first segment of the pathway, dietary folate is converted to dihydrofolate, then reduced to tetrahydrofolate (THF). THF is then converted to N5, N10 methyleneTHF affecting purine and thymidine synthesis. The reduced folates act as cofactors for the biosynthesis of amino acids such as glycine. The second segment of the one carbon cycle is the trans-methylation pathway. Folate, in the form of 5-methylTHF, is involved in the remethylation of homocysteine to methionine, an essential amino acid and a precursor of the universal methyl donor S-adenosylmethionine (SAM). The third segment of the folate cycle consists of the trans-sulfuration pathway. This results in the conversion of homocysteine into cystathionine, then to cysteine and then to the antioxidant glutathione. Therefore, folate plays a central role in nucleic acid biosynthesis, DNA methylation and epigenetics, amino acid metabolism, and redox regulation (Figure 2.5).

We analyzed the effect of a 12 week FR regimen on plasma amino acid pools, nucleotide biosynthesis, and energy metabolism. As shown in Table 2.1, there was an overall reduction in plasma amino acid levels in FR. Of importance were methionine levels, which were reduced by ~60% in FR as compared to FA. This revealed a physiological methionine restriction, created by the folate depleted diet. Despite the decrease in methionine, homocysteine levels were also reduced in FR. This observation was contrary to common belief that folate deficiency increases homocysteine levels [96]. In addition, branched chain amino acids (BCAA), shown to have regulatory effects on cellular proliferation and growth, such as valine, leucine and isoleucine [97], were also significantly reduced in FR (Table 2.1). However, FR significantly increased β-alanine

levels (~40%), suggesting uracil catabolism. Uracil pools are generally catabolized to β-alanine by the sequential action of the enzymes dihydropyrimidine dehydrogenase, dihydropyrimidinase and β-alanine synthase [98]. Folate restriction, although may increase uracil accumulation, it also increases uracil breakdown possibly to protect the DNA by limiting uracil misincorporation. Field et. Al showed that a long term folate deficient diet did not affect the uracil content in liver genomic DNA [30].

Tryptophan levels were also lower in FR as compared to FA (Table 2.1). Tryptophan is the precursor of the water soluble vitamin Niacin. Niacin is the generic name for nicotinic acid and nicotinamide, the precursors for coenzyme Nicotinamide adenine dinucleotide (NAD) [99]. NAD cycles between the oxidized (NAD^+) and reduced (NADH) forms, partaking a central role in cellular metabolism and energy production [100]. To determine the impact of FR on energy metabolism, we analyzed NAD^+ levels, and NAD^+/NADH ratio in the liver. Using a calorimetric assay, we detected intracellular nucleotides (NAD total and NADH). There was a significant increase in NAD^+ levels (~ 85%) (Figure 2.6) and in NAD^+/NADH ratio (~78 %) (Figure 2.7) in FR as compared to FA. In addition, SIRT1, also known as NAD-dependent deacetylase sirtuin-1, was significantly upregulated in FR (Figure 2.8). To directly test the impact of FR on energy metabolism, we determined total ATP levels in the liver using a colorimetric assay. Total ATP levels were significantly reduced (~60%) in FR as compared to FA (Figure 2.9). Our data suggests that folate restriction impacts and modifies the one carbon metabolism, potentially modulating downstream nutrient sensing pathways.

Analysis of the nutrient sensing pathway - mTOR in the liver of C57Bl/6 mice during folate restriction:

Our lab has previously shown that folate deficiency in combination with β-pol haploinsufficiency confers protection against the development of chemically induced preneoplastic lesions [31]. BrdU and TUNEL assays confirmed a decrease in the proliferative capacity, and an increase in apoptosis in the colon of FR mice [31]. Microarray analysis of the folate deficient β-pol$^{+/-}$ animals showed a significant reduction in the expression of DNA repair genes, and significant upregulation in the expression of the pro-apoptotic genes [31]. In line with our previous data, our acclimated FR animals displayed reduced proliferation (~30%) when compared to FA. Colons were immunostained for Ki-67, an indicator of proliferation in the colon (data not shown). Microarray analysis of the colon showed a significant reduction in the expression of mTOR in FR as compared to FA (Table 2.2). To further elucidate the molecular mechanism behind the anti-proliferative effect of folate restriction, we looked at the signaling pathway central to proliferation and cell growth, the mTOR pathway.

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that regulates cell growth, autophagy and proliferation [23]. It integrates signals from upstream factors, and is modulates between growth and starvation [101]. mTOR dysregulation is implicated in aging and in many age-related diseases [24]. mTOR exists in two multiprotein complexes: mTORC1 and mTORC2. mTORC1 pathway integrates input from various upstream intracellular and extracellular signals that include: growth factors (ILGF), stress, hypoxia, energy status (AMP/ATP, NAD$^+$ /NADH), purine metabolism, and amino acid levels [101]. The best characterized process downstream of mTORC1 is protein synthesis, via phosphorylation of two downstream target proteins: S6K1 and 4E-BP1[23]. mTORC1 phosphorylates and activates 4EBP1, relieving inhibition off of protein synthesis [102, 103]. On the other hand, S6K1 phosphorylation and activation by mTORC1, promotes ribosome biogenesis and protein synthesis

[103, 104]. Therefore, mTORC1 is a crucial regulator of protein synthesis, cellular proliferation and metabolism [103-105].

mTORC2 is not sensitive to nutrients but responds to growth factors by poorly defined mechanisms[23]. Whereas mTORC1 is activated via phosphorylation of AKT at the TH308 residue downstream of the PI3K signaling pathway, mTORC2 phosphorylates and activates AKT at the SER473 residue; the site required for maximal activation[24]. mTORC2 also activates PKCα along with other effectors and regulates the cell's shape by affecting the actin cytoskeleton[25].

mTORC1 is regulated through intermediary effectors that relay intracellular and extracellular upstream signals; such as IPMK, REDD1, AMPK, and AKT. IPMK mediates the activation of mTORC1 in response to amino acids. This regulation is independent of its catalytic function, instead it binds to mTOR and raptor maintaining the mTOR-raptor association [106]. IPMK also physiologically binds AMPK, and inhibits its activity in a glucose dependent manner [107], in turn activating mTORC1. Folate restriction reduced the protein levels of IPMK, but there was no significant difference (Figure 2.10). REDD1 levels increased following exposure to hypoxia or DNA damage, leading to activation of TSC1/TSC2 complex, and inhibition of mTOR signaling. There was a significant decrease in REDD1 expression in FR as compared to FA (Figure 2.11). This indicates that the downregulation of mTORC1 may not necessarily be mediated by IPMK or REDD1.

Folate restriction significantly increases phosphorylation of AMPK (pAMPK) (Figure 2.12), an inhibitor of mTORC1. AMPK is a metabolic fuel gauge that detects changes in intracellular AMP/ATP ratio [108]. AMPK is activated in response to nutrient depletion, and it acts to maintain energy stores by switching on pathways that produce ATP, and switching off pathways that consume ATP [108]. AMPK directly inhibits mTORC1, and neutralizes PI3K

activation of mTORC1. AMPK enhances SIRT1 activity (Figure 2.8) by increasing cellular levels of NAD^+ (Figure 2.6), both of which were seen upregulated in our FR animals. Accumulation of AICAR, an intermediate formed during De novo purine biosynthesis [109], and a potent activator of AMPK [110], can occur during folate restriction. Furthermore, Low ATP levels activate AMPK and inhibit hepatic gluconeogenesis in an insulin independent manner [111]. This effect of hepatic AMPK inhibition of gluconeogenesis, overrides the inductive signal elicited through glucagon. Folate restriction resulted in reduced expression of PEPCK (rate limiting enzyme in gluconeogenesis) (Figure 2.13), but there was increased expression of G6PASE (Figure 2.14), possibly as a feedback mechanism due to decreased glucose and gluconeogenesis. Interestingly, FR increased phosphorylation of AKT S473, a target of insulin-like growth factors (Figure 2.15). AKT S473 phosphorylation is mTORC2 dependent.

These factors converge on downregulation of mTORC1 but not mTORC2 during folate restriction. mTORC1 dependent phosphorylation of 4EBP1 (Figure 2.16) and S6K1 (Figure 2.17) were reduced in FR as compared to FA. FR downregulates mTORC1, reducing protein synthesis and cellular proliferation. Folate restriction specifically reduces mTORC1 expression, while it induces mTORC2, possibly as a compensatory mechanism to promote survival [5]. Lamming et al. demonstrated that mTORC2 expression is required for maintaining insulin sensitivity, and was important for the insulin-mediated suppression of hepatic gluconeogenesis [5]. Folate restriction alters the one-carbon metabolism, and the downstream mTOR nutrient sensing network, possibly explaining the anti-proliferative and anti-cancer effects observed.

Analysis of apoptosis in the liver of C57Bl/6 mice during folate restriction:

Apoptosis acts as a protective mechanism during stress, and helps to eliminate damaged DNA and eventually damaged cells [112]. It is an essential process for maintaining homeostasis

and survival in multi-cellular organisms [113]. To characterize the effect of folate restriction on cell cycle arrest and apoptosis, we analyzed four markers of apoptosis: Gadd45γ (growth arrest and DNA damage-inducible gene γ), p53, caspase 3, and parp-1 cleavage. Using real-time PCR we analyzed the mRNA expression of gadd45γ, a genotoxic stress-inducible gene associated with cell cycle arrest and apoptosis. We saw decreased expression of Gadd45γ (~38%) in FR as compared to FA (Figure 2.18). Since Gadd45γ is a p53 responsive gene, and p53 is a well-established regulator of cell cycle arrest and apoptosis [114, 115], we next evaluated the stabilization of p53 protein. P53 stabilization is dependent on S6K1 phosphorylation and inhibition of the E3 ubiquitin ligase MDM2 [116]. Since we saw reduced p-S6K1 in FR (Figure 2.17), we presumed that MDM2 may be free to inhibit p53. As expected, we saw decreased nuclear p53 protein in FR as compared to FA (Figure 2.19). We also determined the activity of caspase-3. Upon activation, caspases initiate cell death and drive the process of apoptosis [117]. Caspase-3 activity was significantly reduced (~26%) in FR (Figure 2.20). We further determined the cleavage of parp-1, an early indicator of apoptosis. PARP-1 cleavage by caspases is a hallmark of apoptosis [118]. We saw decreased PARP-1 cleavage in FR as compared to FA (Figure 2.21). Folate restriction reduced apoptotic activity in our animals. This reduction in apoptosis may be an adaptation to modulate between reduced damage and reduce proliferation, possibly slowing down the aging process.

Analysis of folate restriction as a potential anti-aging dietary intervention:

Aging is a multifactorial process that increases vulnerability to disease and eventually leads to mortality. Interventions that delay aging include: nutritional manipulations (caloric restriction [6] and methionine restriction [9]), genetic models (Ames dwarf mice [13]), and drug treatments (rapamycin [35] and metformin [19]). These interventions either impact cellular energy stores

(NAD$^+$/NADH ratio and ATP production), or directly modulate the nutrient sensing pathway mTOR. Rapamycin, an immunosuppressant drug, is a potent inhibitor of mTOR signaling [14]. It extends lifespan of yeast, flies and mice by directly inhibiting the TOR kinase of mTORC1 [40, 42, 119]. Caloric restriction, the most established life extending strategy, also inhibits the mTOR pathway [36]. Metformin, an oral antidiabetic drug, extends lifespan in C. elegans by activating AMPK [39]. Increased activation of AMPK, a sensor of AMP levels and an inhibitor of mTORC1, also increases lifespan in flies [120]. Human breast cancer cells treated with Metformin showed impaired one-carbon metabolism similar to the effect of anti-folate chemotherapeutic drugs [21]. Treatment with metformin resulted in accumulation of a folate variant essential for de novo purine and pyrimidine synthesis [21].

Interestingly, metformin suppressed angiogenesis and proliferation of DMH induced colon cancer in diabetic and non-diabetic mice [121]. We had previously shown that a folate deficient diet in combination with β-pol haploinsufficiency reduced incidences of DMH induced preneoplastic lesions [31]. We have also shown that acclimated folate restriction modulates the onset and progression of cancer (Figure 2.4). Since cancer is one of the most important age related diseases, and since FR modulates one-carbon metabolism, energy metabolism, and mTOR, we hypothesized that folate restriction can potentially extend lifespan. We maintained 4 week old mice on a FR diet to study the impact of the diet on their lifespan. Plasma folate levels were monitored periodically and showed 90% reduction in folate, similar to what we have shown before (Figure 2.2). FR mice did not show any signs of anemia, or development of spontaneous cancers. Their food consumption and body weight did not significantly differ from their folate adequate counterparts. At 32 months of age, FR animals appeared physiologically younger than their folate fed counterparts. Based on our ongoing survival study, FR modulates survival independent of

caloric restriction. Our aging colony shows an 86% survival rate for FR mice as compared to 60% survival for FA at 850 days of life (Figure 2.22). In this study, we have identified a direct link between the folate cycle and the nutrient sensing mTOR pathway, both of which converge on target genes impacting aging and cancer.

Discussion

Folate deficiency has been associated with many age-related diseases. Birth defects, specifically neural tube defects (NTD's), have also been linked to dietary folate deficiency. NTD is a complex disorder that is caused by various genetic, nutritional, and environmental factors. NTD is congenital malformation with genetic etiology that includes trisomy 13, trisomy 18, specific chromosome rearrangements, and Meckel Gruber syndrome [122]. Apart from genetic susceptibility, pregnant mothers who are obese, have poorly controlled diabetes, take anti-seizure medications, or take anti-folate drugs, are more pre-disposed to having babies with NTD's [123]. Over the years, research showed that adequate folic acid intake, before and during pregnancy can greatly reduce the prevalence of NTD's [124]. These findings led to the 1998 FDA mandate to fortify all staple food items with folic acid. Although there has been roughly a 19% drop in NTD's since the fortification, it is not clear whether this drop is actually due to fortification, increased awareness, improved nutrition or increased prenatal diagnosis[125].

Quinlivan and Gregory show that there is a linear relationship between folate intake and plasma folate levels [126]. However, not many studies have been conducted to directly check the adverse effects of elevated folic acid intake [127]. Emerging studies show an increased risk of many types of cancer since the fortification, specifically colorectal and prostate cancer [128] . Apart from cancer, increased folate levels have been shown to mask Vitamin B-12 deficiency, and aggravate neurologic impairment [129]. Furthermore, excess folic acid (supplements or fortified

foods) can suppress Natural Killer cells that are vital for normal immune functioning in postmenopausal women[130]. There was a significant increase in the unmetabolized folic acid levels in the fasting plasma of 78% of subjects. Unmetabolized or oxidized forms of folic acid are not naturally present is food sources. Dihydrofolate reductase (DHFR) is the rate limiting enzyme that is responsible for physiologically converting folic acid to the reduced form of folate. In humans, increased folic acid intake can lead to accumulation of unmeatbolized folic acid in the plasma, due to the slow activity of DHFR in the liver[131]. This warrants the need for further studies to characterize the toxicity of unmetabolized folic acid in the plasma, and its potential long term effect on immune function and health[130].

Earlier studies showing the association of folate hypo-insufficiency with various cancers are inconsistent. These studies vary in the intervention implemented, and in the dosage and timing of the dietary folate regimens [58]. While many epidemiological and animal studies show a strong correlation between low folate and cancer [52, 54], other studies show the association of folate supplementation and cancer [78, 132]. Therefore, the effect of folate on the manifestation of many diseases, specifically cancer, remains inconclusive. Folic acid supplementation promotes the progression of aberrant crypt foci into colorectal cancer in AOM treated rats[132]. Folic acid supplementation also has tumor-promoting effect on pre-existing preneoplastic lesions [133]. NHANES data show that 32-38% of individuals aged over 60 years have high serum folate concentrations [134, 135], and 25-50% of people develop asymptomatic colorectal adenomas by 50 years of age [136]. These figures indicate that folic acid supplementation is potentially a risk factor for cancer development and progression[132]. Furthermore, rats kept on a folate depleted diet for 4 weeks before AOM treatment showed reduced incidences of intestinal tumors, and

numbers of malignant tumors [137]. It's proposed that the decrease in tumor numbers may be due to folate's role in cell multiplication or proliferation.

Our lab had previously demonstrated that a combination of folate deficiency and β-pol haploinsufficiency reduced the development of preneoplastic lesions in the colon [31]. This combination also reduced crypt multiplicity in our model. We also observed that an acclimation period on the FR diet is an important factor to detect the beneficial impact of FR. Wild-type C57Bl/6 mice were fed a long term folate-depleted diet (0 mg FA/kg diet for 8 weeks), a folate adequate diet (2mg FA/kg diet for 8 weeks) or a short term folate depleted diet (2 mg FA/kg diet for 7 weeks, then 0 mg FA/kg diet for 1 week) prior to the start of DMH treatment. Long term folate depletion before exposure to a carcinogen resulted in significantly less ACF. Interestingly, we find that methionine restriction (MR), a regimen shown to extend lifespan [138], requires a period of adaptation to exhibit its anti-cancer effect, similar to FR (Manuscript in preparation). Our data imply that long-term FR results in a folate restricted microenvironment that is protective against spontaneous and chemically induced cancer.

To explain the protective effect of FR, we analyzed its impact on amino acid pools, energy metabolism, and nucleotide biosynthesis in mice. These factors are important for cellular survival and proliferation, as they impact a major nutrient sensing pathway, mTOR. We demonstrated that a 12 week FR regimen reduced plasma amino acid levels, specifically methionine, isoleucine, and leucine. Despite the decrease in methionine, FR did not increase levels of homocysteine. FR increased β-alanine levels, suggesting that uracil is being catabolized. This finding is supported by Field et. Al, indicating that a long term FD diet did not affect the uracil content in liver genomic DNA [30]. We also demonstrated that FR significantly decreased total ATP levels in the liver. FR increased NAD^+ levels and NAD^+/NADH ratio. An increase in NAD^+ has been associated with

elevation of SIRT1 expression [139]. We saw a significant increase in SIRT1 expression in FR. FR also increased phosphorylation of AMPK, a metabolic fuel gauge that senses changes in AMP/ATP ratio, and modulates mTORC1. FR downregulates mTORC1 as observed by the decreased activation of two downstream targets: S6K1 and 4E-BP.

Furthermore, FR significantly increased AKT S473 phosphorylation. This site is phosphorylated by mTORC2. FR increased phosphorylation of AKT at the S473 residue possibly as a compensatory mechanism to maintain insulin sensitivity [5, 140]. Interestingly, we also observed a significant decrease in the expression of the gluconeogenesis enzyme PEPCK.

mTOR signaling is also impacted by REDD1 expression in response to hypoxia and DNA damage [141]. REDD1 is a transcriptional target gene of p53 [142]. In response to DNA damage, REDD1 expression is upregulated, inhibiting mTORC1. We show that FR decreased expression of REDD1. This suggests that the effect seen on the downregulation of mTORC1 in our model, may not be mediated through REDD1. Downstream of mTOR, phosphorylation of S6K1, phosphorylates and inactivates MDM2, in turn stabilizing p53 in the nucleus [116]. FR reduced S6K1 phosphorylation, so we expected MDM2 inhibition on p53 translocation to the nucleus. We observed reduced nuclear p53, reduced expression of the p53 responsive cell cycle arrest gene Gadd45g, and consequently decreased caspases 3 activity. FR imposes a low level of stress that is insufficient to promote apoptosis, but creates a balance between reduced proliferation and apoptosis. We suggest that keeping apoptosis down is beneficial in the absence of DNA damage. Elevated apoptosis, while anti-cancerous, accelerates aging.

mTOR is a major nutrient sensing pathway that impacts aging, and many age-related diseases. Inhibition of mTOR extends life-span in many laboratory models including yeast, worms, flies and rodents [35, 119, 143, 144]. Wu et. Al demonstrated that downregulation of mTOR to

25% of wild type increased median lifespan by 20% in mice, and slowed down tissue-specific aging [28]. We propose that folate restriction, a nutritional intervention that impacts the mTOR pathway, potentially extends lifespan. Our FR animals showed significantly improved survival rate when compared to FA at 1250 days of life. Upon gross analysis at sacrifice, aged FR animals did not show signs of macroscopic tumors. FR significantly extends the life-span of long-lived flies fed media containing 0.3M folate versus 1M folate (data not shown). Observations made in C. elegans showed that reduction in the folate pathway increased life-span [70, 71]. We show that FR extends life span of our experimental mouse model, without developing spontaneous tumors. The pro-longevity and anti-cancer effects of folate restriction appear to be due to modulation of the folate pathway, purine biosynthesis, energy metabolism, and converging on the nutrient sensing pathway, mTOR.

Two major life extending strategies, caloric restriction and rapamycin have been shown to inhibit mTOR [35-37]. Folate restriction, also an inhibitor of mTOR, is a novel anti-aging/anti-cancer nutritional intervention. It combines the beneficial effects of rapamycin and caloric restriction, while avoiding their drawbacks. Unlike caloric restriction, FR is an attainable nutritional intervention that would be more feasible to implement within the human population. FR impacts the nutrient sensing pathway as a whole, avoiding the resistance seen in rapamycin, a direct inhibitor of mTORC1. Moreover, chronic rapamycin treatment inhibits both mTORC1 and mTORC2 [15]. This in turn compromises insulin sensitivity and impairs glucose tolerance. Unlike rapamycin, FR enhances mTORC2 activity, possibly maintaining insulin sensitivity.

Folate restriction infers protection against the onset and progression of cancer in mice, and extends their median lifespan. The innovative nature of our findings, is that they contradict the current understanding of the role of folate status on the etiology of cancer. These results may

represent a new paradigm in our current understanding of folate supplementation and the ramifications on the health and cancer risk of various subsets of the population.

Figure 2.1: *The effect of folate depletion in the diet on plasma folate levels at various time points.* C57BL/6 mice were fed either a folate adequate (FA) or folate depleted (FD) AIN93G-purified isoenergetic diet (Dyets, Inc., Lehigh Valley, PA) as previously described [32]. The FA group received a folate adequate diet containing 2 mg of folic acid/kg diet. The FD group received a folate-deficient diet containing 0 mg of folic acid/kg diet. The animals remained on their respective diets for the duration of the study. Blood was drawn from the capillary of the eye at various time points, and folate levels were analyzed using the lactobacillus casei microbiological assay [33]. Values represent an average [S.E.M] for data obtained from 4 mice in each group and are representative of separate identical experiments. Different letters indicate significant differences at P < 0.05.

Figure 2.1: *The effect of folate depletion in the diet on plasma folate levels at various time points.*

Figure 2.2: *The effect of folate in the diet on plasma folate levels in C57BL/6 mice.* The animals were fed either a folate adequate (FA) or folate depleted (FD) AIN93G-purified isoenergetic diet (Dyets, Inc., Lehigh Valley, PA) as previously described[32]. The FA group received a folate adequate diet containing 2 mg of folic acid/kg diet. The FD group received a folate-deficient diet containing 0 mg of folic acid/kg diet. The animals remained on their respective diets for 12 weeks after which they were sacrificed. Plasma was collected and folate levels were analyzed using the lactobacillus casei microbiological assay[33].Values represent an average [S.E.M] for data obtained from 4 mice in each group and are representative of separate identical experiments. * Significant differences at $P < 0.05$.
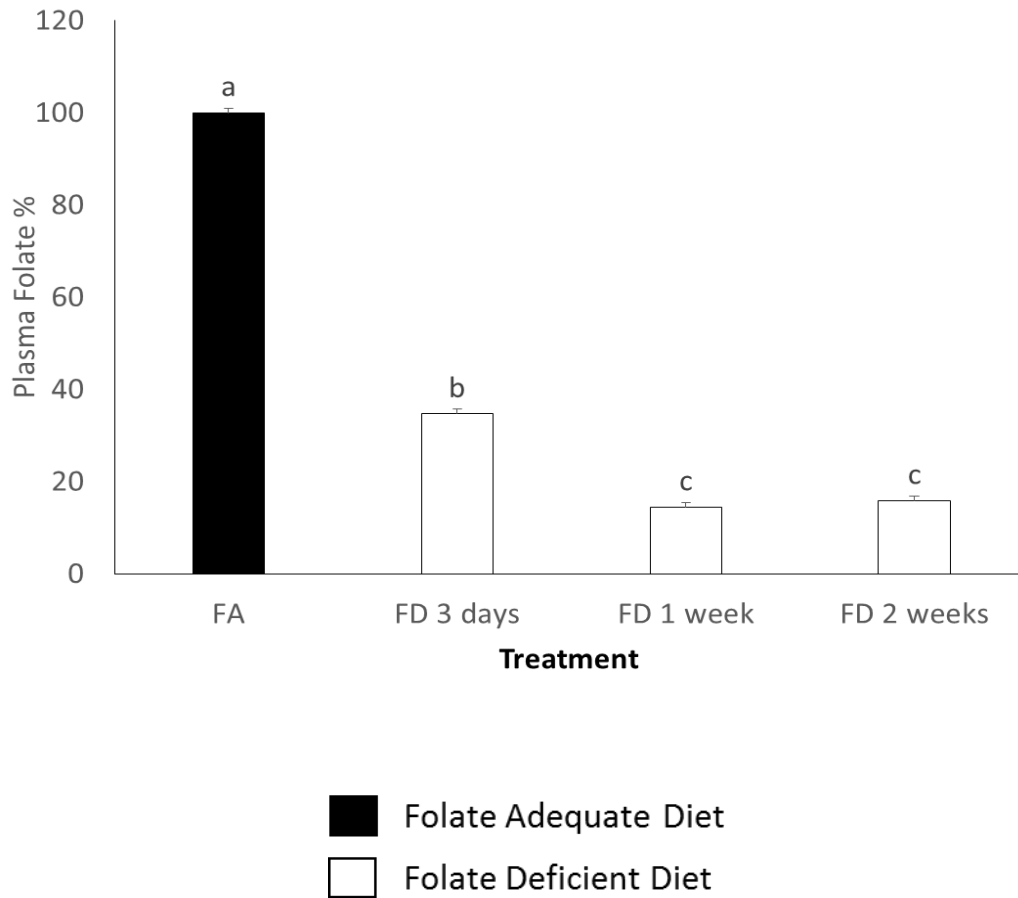
Figure 2.2: *The effect of folate in the diet on plasma folate levels in C57BL/6 mice.*

Treatment

■ Folate Adequate Diet

□ Folate Deficient Diet
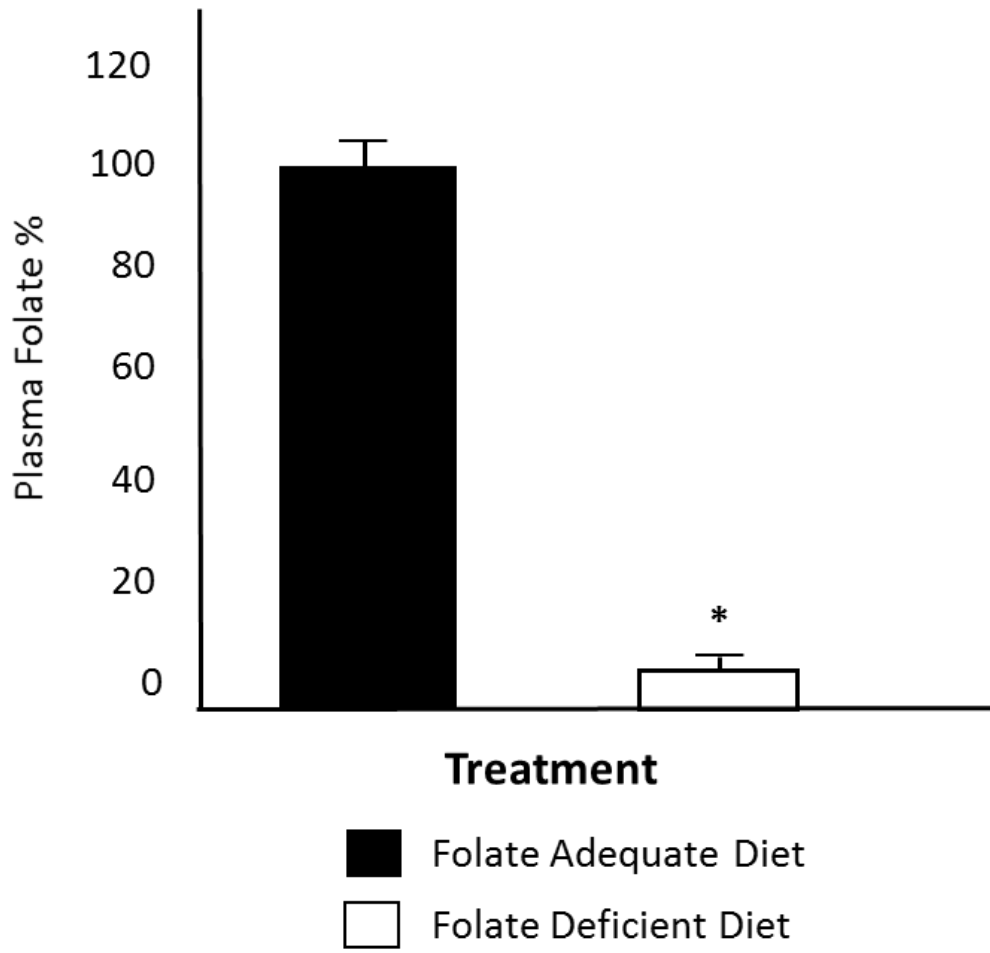
Figure 2.3: *The effect of folate restriction on development of spontaneous preneoplastic lesions.* The animals were fed either a folate adequate (FA) or folate depleted (FD) AIN93G-purified isoenergetic diet (Dyets, Inc., Lehigh Valley, PA) as previously described [32]. The FA group received a folate adequate diet containing 2 mg of folic acid/kg diet. The FD group received a folate-deficient diet containing 0 mg of folic acid/kg diet. The animals remained on their respective diets for 12 weeks after which they were sacrificed. After sacrifice, the colon was excised, rinsed with cold phosphate-buffered saline, cut longitudinally, and fixed flat overnight in 10% neutral buffered formalin. On the next day, the colonic crypts were stained with 2 g/liter of methylene blue in phosphate-buffered saline for 5 min. The number of ACF was determined by light microscopy at ×10 magnification in a blinded manner. A representative colon image of a mouse exposed to folate restriction.

Figure 2.4: *The effect of long term and short term folate restriction on ACF.* C57BL/6 mice were fed either a folate adequate (FA), long term folate depleted (FR), or short term folate depleted (FA/FR) AIN93G-purified isoenergetic diet (Dyets, Inc., Lehigh Valley, PA) as previously described[32]. 1% succinyl sulfathiazole was added to some of the diets (+AB). The FA group received a folate adequate diet containing 2 mg of folic acid/kg diet. The FR (Long term folate restriction) group received a folate-deficient diet containing 0 mg of folic acid/kg diet. The FA/FR (Short term folate restriction) group received a folate adequate diet for 7 weeks, then switched to a folate deficient diet for one week before animals were randomly chosen and injected with DMH once a week for 6 weeks at 30 mg/kg body weight. The animals remained on their respective diets until they were sacrificed at 21 weeks. After sacrifice, the colon was excised, rinsed with cold phosphate-buffered saline, cut longitudinally, and fixed flat overnight in 10% neutral buffered formalin. On the next day, the colonic crypts were stained with 2 g/liter of methylene blue in phosphate-buffered saline for 5 min. The number of ACF was determined by light microscopy at $\times 10$ magnification in a blinded manner. Values represent an average [S.E.M] for data obtained from 14 mice in each group and are representative of separate identical experiments. Different letters indicate significant differences at $P < 0.05$.

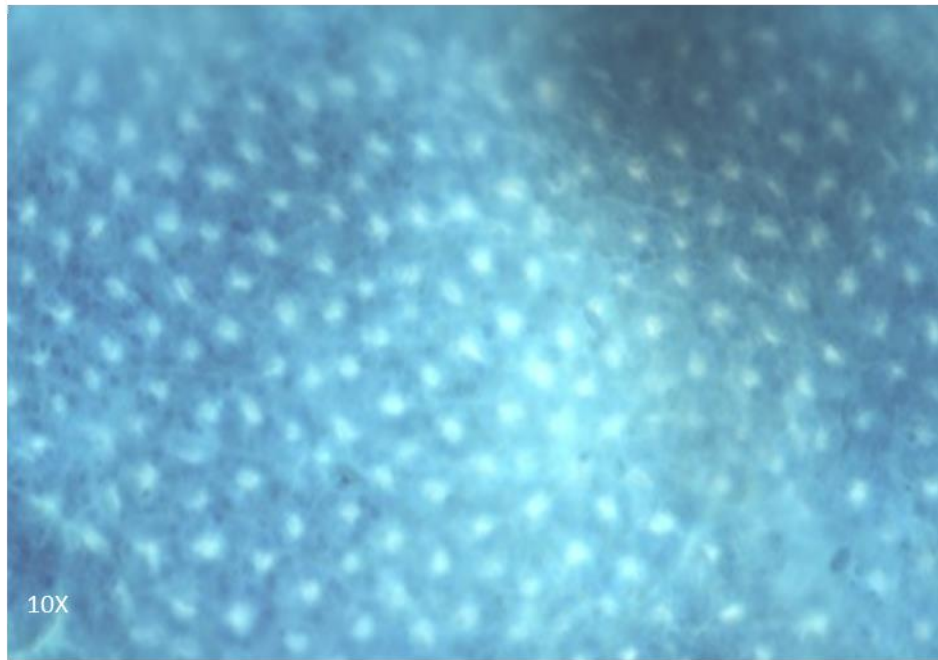Figure 2.4: *The effect of long term and short term folate restriction on ACF.*
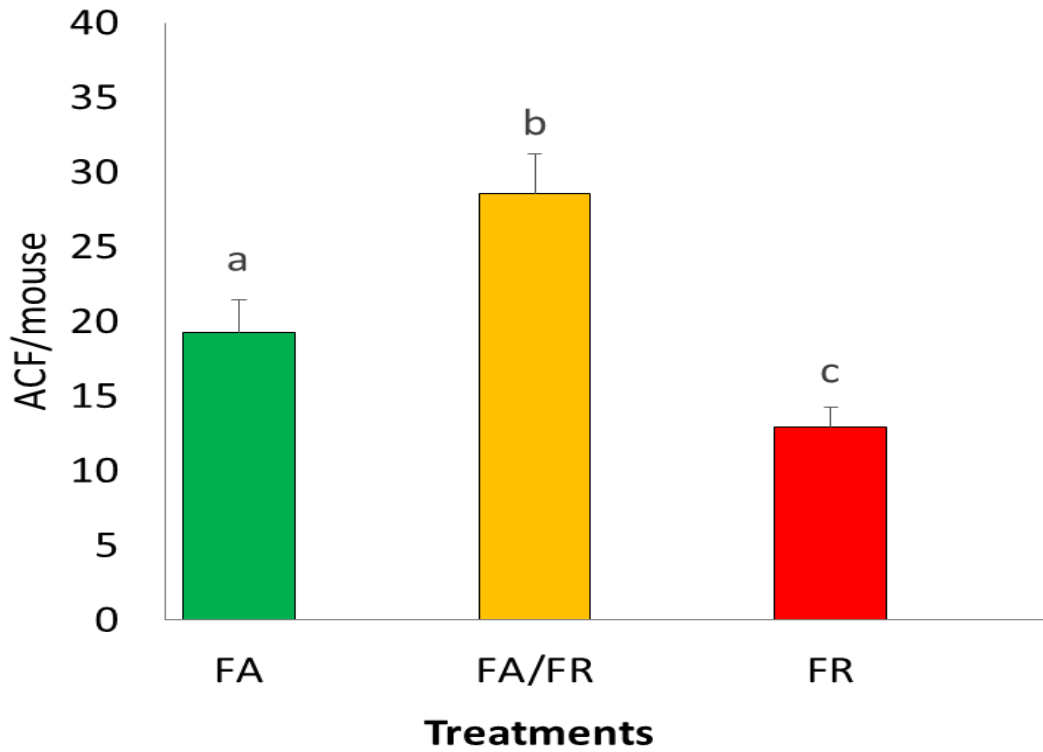
Figure 2.5: **Folate Cycle "One Carborn Metabolism".**

Figure 2.6: *Effect of folate restriction on NAD$^+$ levels.* Total NAD (NAD$^+$ and NADH) was extracted from the liver of C57BL/6 mice fed either FA or FR diet, using Abcam's NAD/NADH Assay kit. Intracellular nucleotides are detected using a plate reader at OD 570 nm. Total NAD is extracted and detected. Then NAD$^+$ is decomposed to detect NADH. The values are then used to calculate for NAD$^+$. NAD$^+$ levels are expressed as (uM). Values represent an average [S.E.M] for data obtained from 4 mice in each group and are representative of separate identical experiments. * Significant differences at P < 0.05. FA: Folate adequate; FR: Folate restricted.

Figure 2.6: *Effect of folate restriction on NAD$^+$ levels.*

Figure 2.7: *Effect of folate restriction on NAD$^+$/NADH ratio.* Total NAD (NAD$^+$ and NADH) was extracted from the liver of C57BL/6 mice fed either FA or FR diet, using Abcam's NAD/NADH Assay kit. Intracellular nucleotides are detected using a plate reader at OD 570 nm. First, total NAD is extracted and detected. Then NAD$^+$ is decomposed to detect NADH. The values are then used to calculate for NAD$^+$. NAD$^+$/NADH ratio calculated. Values represent an average [S.E.M] for data obtained from 4 mice in each group and are representative of separate identical experiments. * Significant differences at $P < 0.05$. FA: Folate adequate; FR: Folate restricted.

Figure 2.7: *Effect of folate restriction on NAD⁺/NADH ratio.*

Figure 2.8: *Effect of folate restriction on SIRT1 mRNA expression.* SIRT1 mRNA levels in the liver tissue of C57BL/6 mice fed FA or FR diets were quantified using real-time PCR. Values represent an average [S.E.M] for data obtained from 4 mice in each group and are representative of separate identical experiments. * Significant differences at $P < 0.05$. FA: Folate adequate; FR: Folate Restricted.
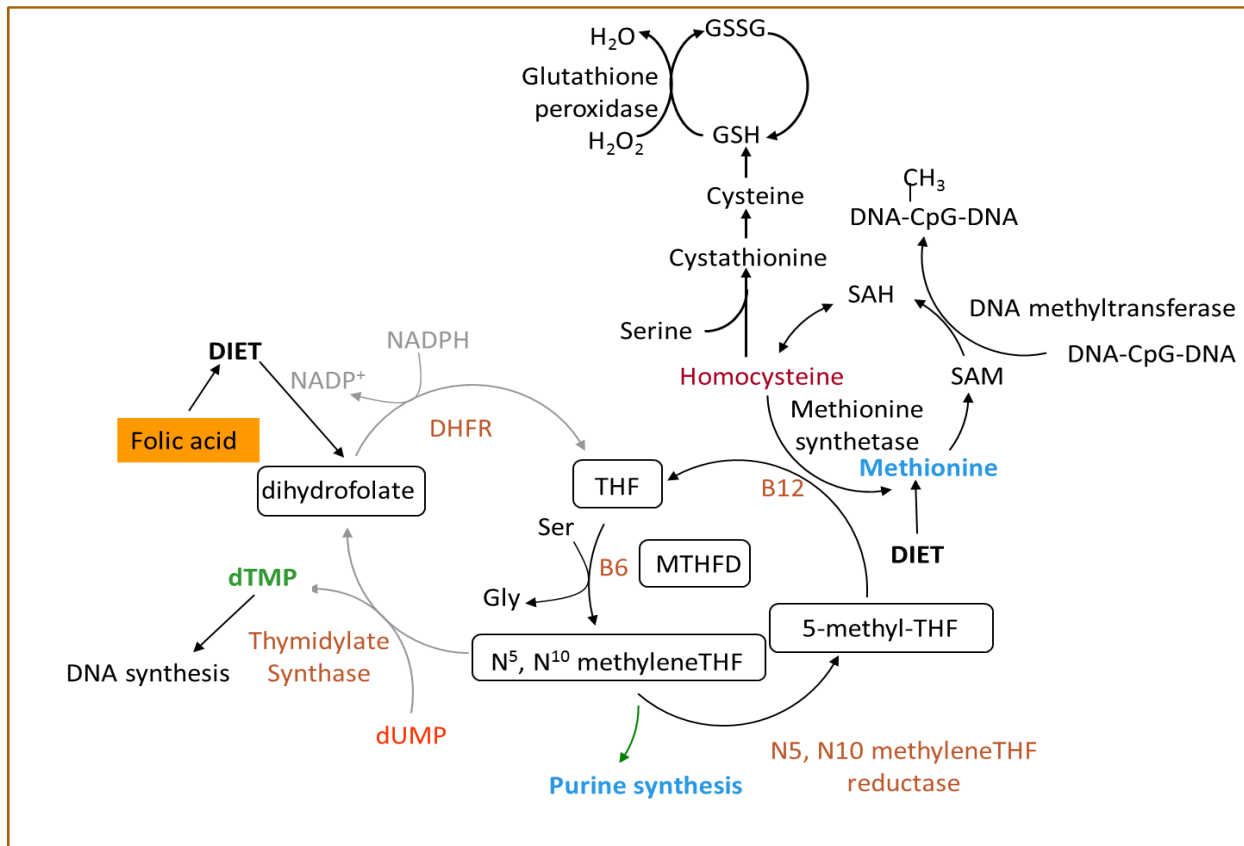
Figure 2.9: *Effect of folate restriction on ATP levels.* Total ATP was extracted from the liver of C57BL/6 mice fed FA or FR diets using Abcam's ATP Assay kit. Active ATP was measured using a plate reader at OD 570 nm. ATP levels are expressed as nmoles/ml. Values represent an average [S.E.M] for data obtained from 4 mice in each group and are representative of separate identical experiments. * Significant differences at $P < 0.05$. FA: Folate adequate; FR: Folate restricted.

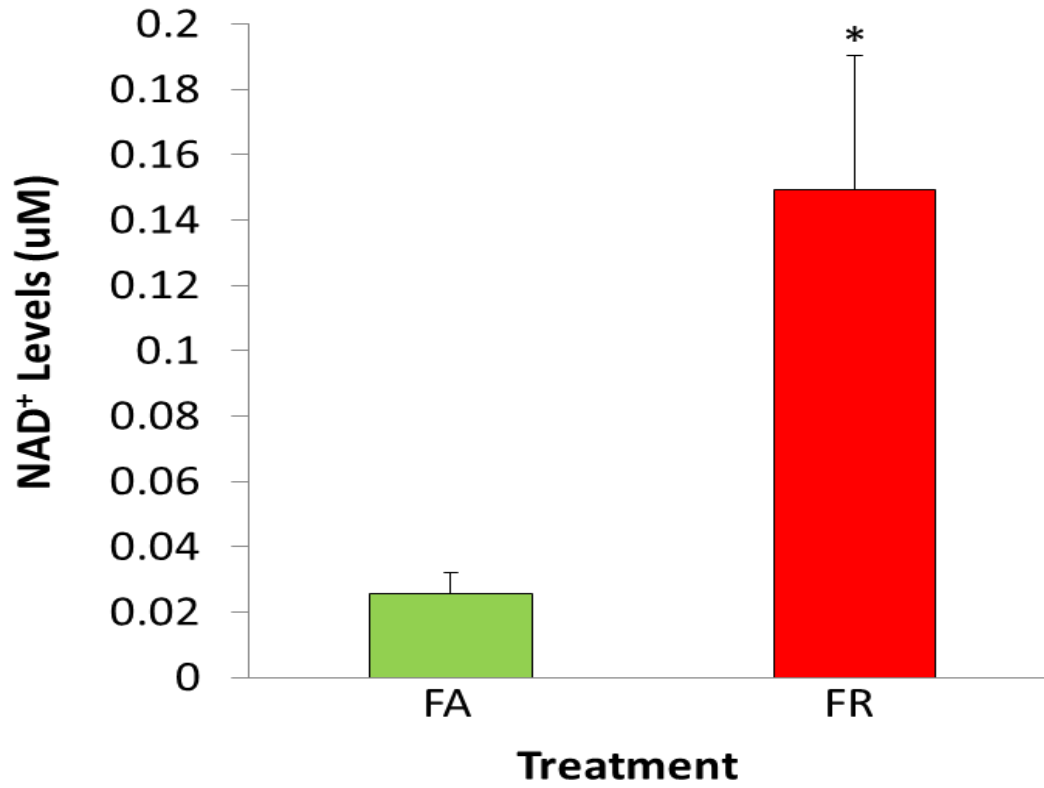Figure 2.9: *Effect of folate restriction on ATP levels.*

Figure 2.10: *Effect of folate restriction on expression of IPMK protein*. The level of IPMK protein in 100 ug of liver whole cell extract from C57BL/6 mice fed FA or FR diets was determined by western blot analysis. The level of IPMK was normalized based on the amount of protein loaded. The I.D.V (integrated density value) corresponding to the level of IPMK protein as quantified by the BioRad Molecular Imager® System, normalized to β-actin. Values represent an average [S.E.M] for data obtained from 4 mice in each group and are representative of separate identical experiments. * Significant differences at $P < 0.05$. FA: Folate adequate; FR: Folate Restricted.

Figure 2.10: *Effect of folate restriction on expression of IPMK protein*
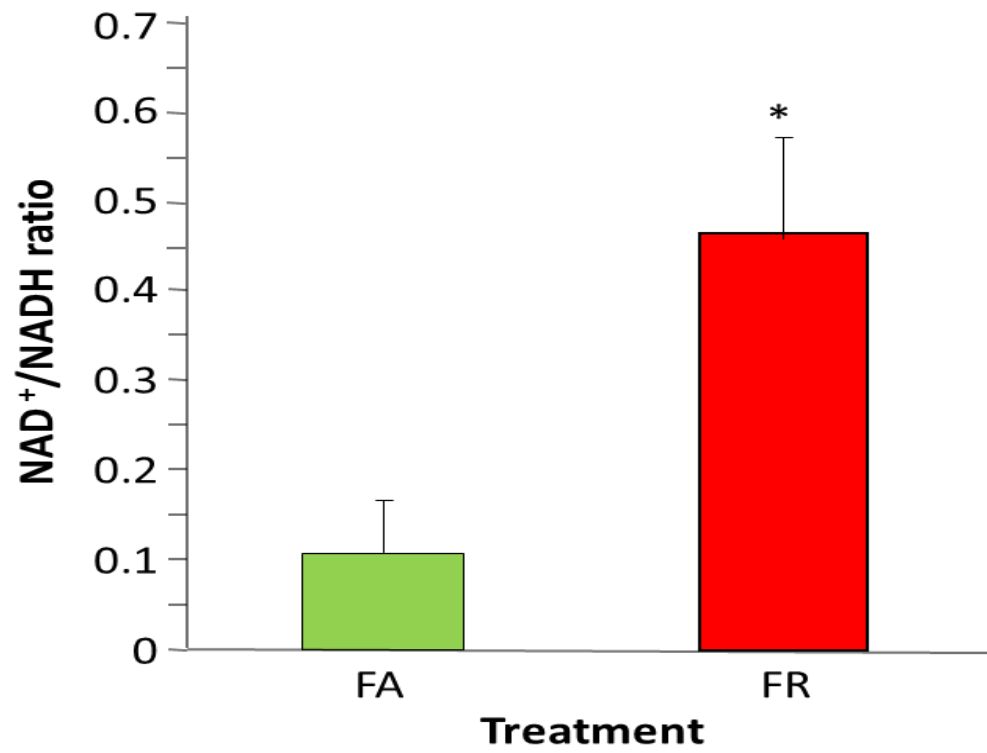
Figure 2.11: *Effect of folate restriction on expression of REDD1 protein.* The level of REDD1 protein in 100 ug of liver whole cell extract from C57BL/6 mice fed FA or FR diets was determined by western blot analysis. The level of REDD1 was normalized based on the amount of protein loaded. The I.D.V (integrated density value) corresponding to the level of REDD1 protein as quantified by the BioRad Molecular Imager® System, normalized to β-actin. Values represent an average [S.E.M] for data obtained from 4 mice in each group and are representative of separate identical experiments. * Significant differences at P < 0.05. FA: Folate adequate; FR: Folate Restricted.
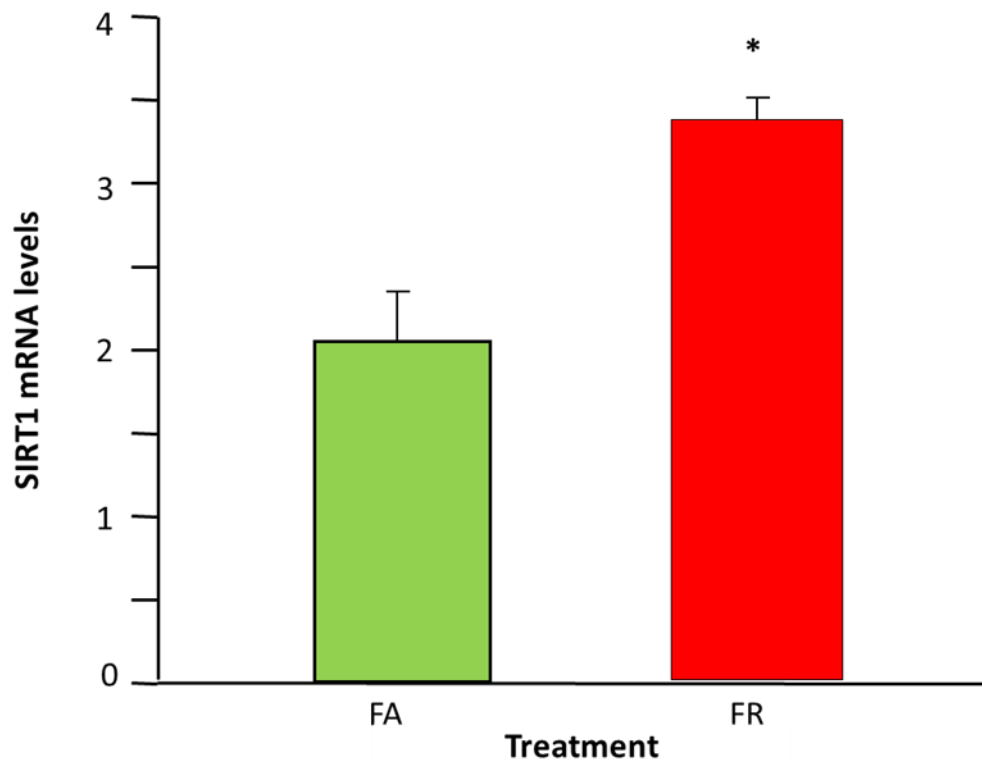
Figure 2.11: *Effect of folate restriction on expression of REDD1 protein.*

Figure 2.12: *Effect of folate restriction on AMPK phosphorylation.* The level of AMPK protein in 100 ug of liver whole cell extract from C57BL/6 mice fed FA or FR diets was determined by western blot analysis. The level of the phosphorylated form of AMPK was determined first. The membrane was then stripped and tested for Total AMPK. The I.D.V (integrated density value) corresponding to the level of AMPK protein (phosphorylated and total) was quantified by the BioRad Molecular Imager® System. Phosphorylated AMPK was normalized to total and expressed as p-AMPK protein expression ratio. Values represent an average [S.E.M] for data obtained from 4 mice in each group and are representative of separate identical experiments. * Significant differences at $P < 0.05$. FA: Folate adequate; FR: Folate Restricted.
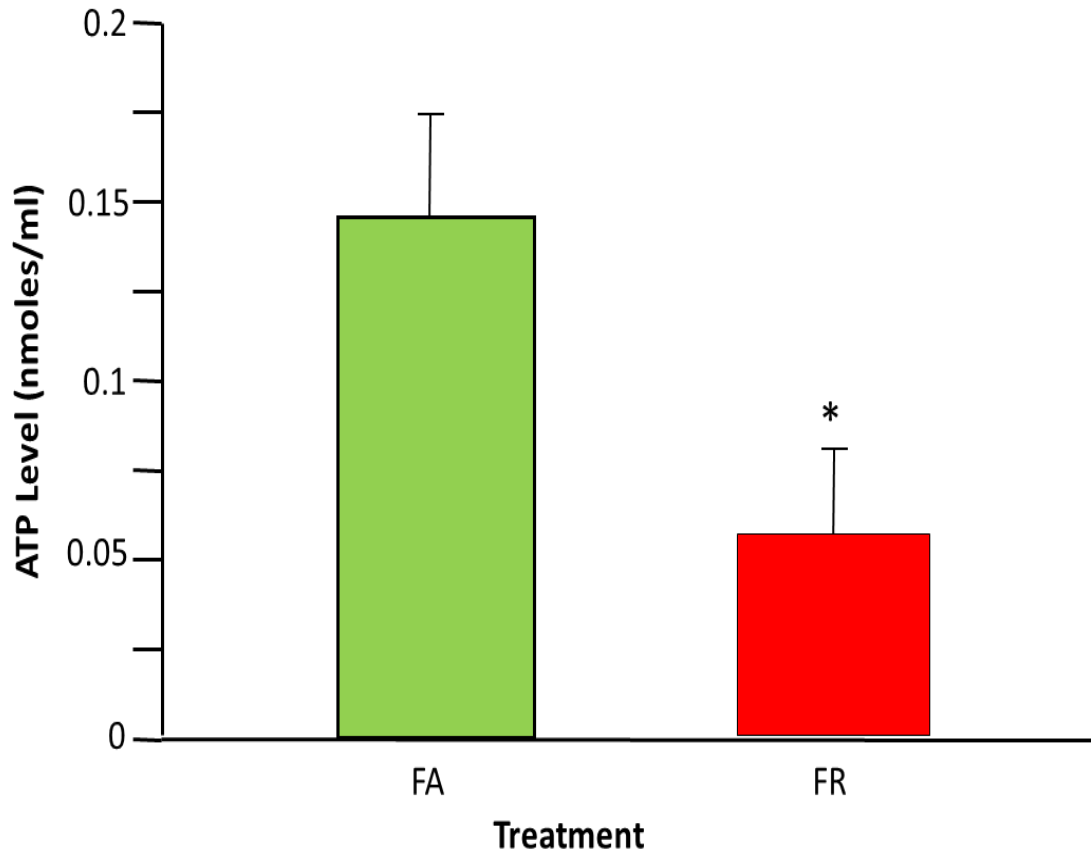
Figure 2.12: *Effect of folate restriction on AMPK phosphorylation.*

Figure 2.13: *Effect of folate restriction on gluconeogenesis enzyme PEPCK.* The levels of PEPCK protein in 100 ug of liver whole cell extract from C57BL/6 mice fed FA or FR diets were determined by western blot analysis. The level of PEPCK was normalized based on the amount of protein loaded. The I.D.V (integrated density value) corresponding to the level of PEPCK protein as quantified by the BioRad Molecular Imager® System, normalized to β-actin. Values represent an average [S.E.M] for data obtained from 4 mice in each group and are representative of separate identical experiments. * Significant differences at $P < 0.05$. FA: Folate adequate; FR: Folate Restricted.
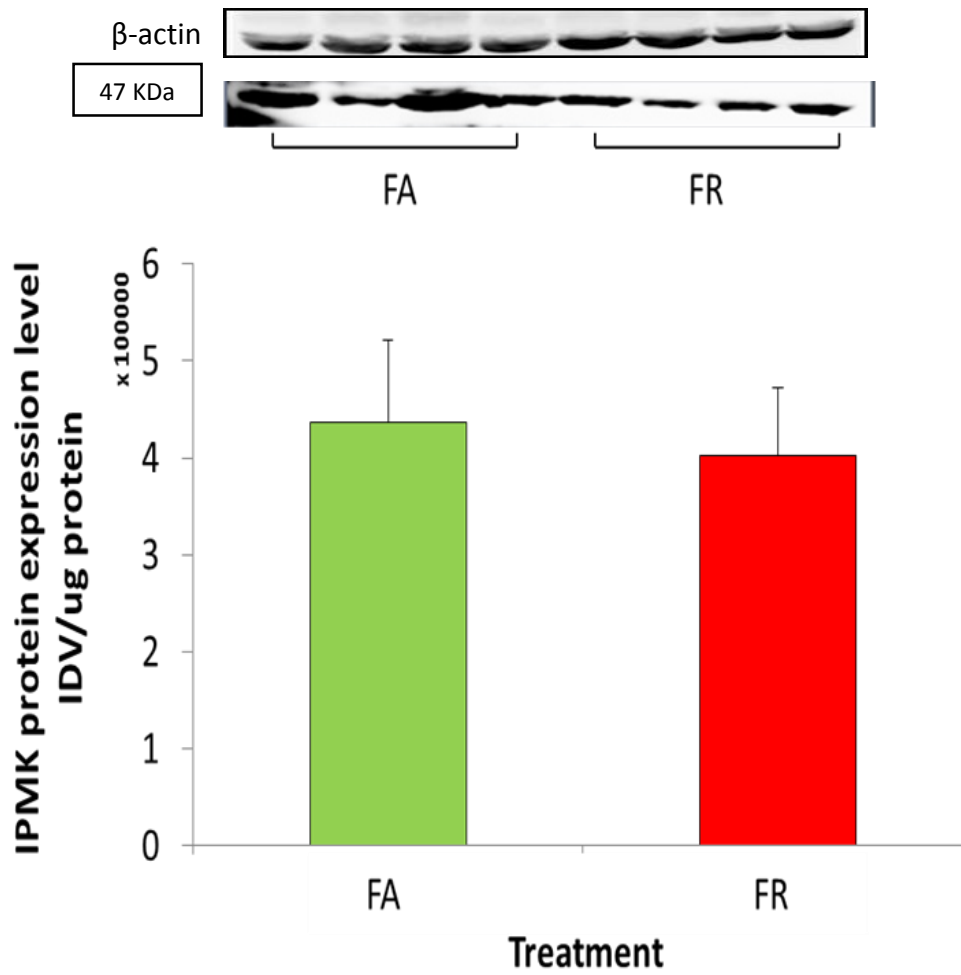
Figure 2.13: *Effect of folate restriction on gluconeogenesis enzyme PEPCK.*

Figure 2.14: *Effect of folate restriction on gluconeogenesis enzyme G6PASE*. The levels of G6PASE protein in 100 ug of liver whole cell extract from C57BL/6 mice fed FA or FR diets were determined by western blot analysis. The level of G6PASE was normalized based on the amount of protein loaded. The I.D.V (integrated density value) corresponding to the level of G6PASE protein as quantified by the BioRad Molecular Imager® System, normalized to β-actin. Values represent an average [S.E.M] for data obtained from 4 mice in each group and are representative of separate identical experiments. * Significant differences at $P < 0.05$. FA: Folate adequate; FR: Folate Restricted.
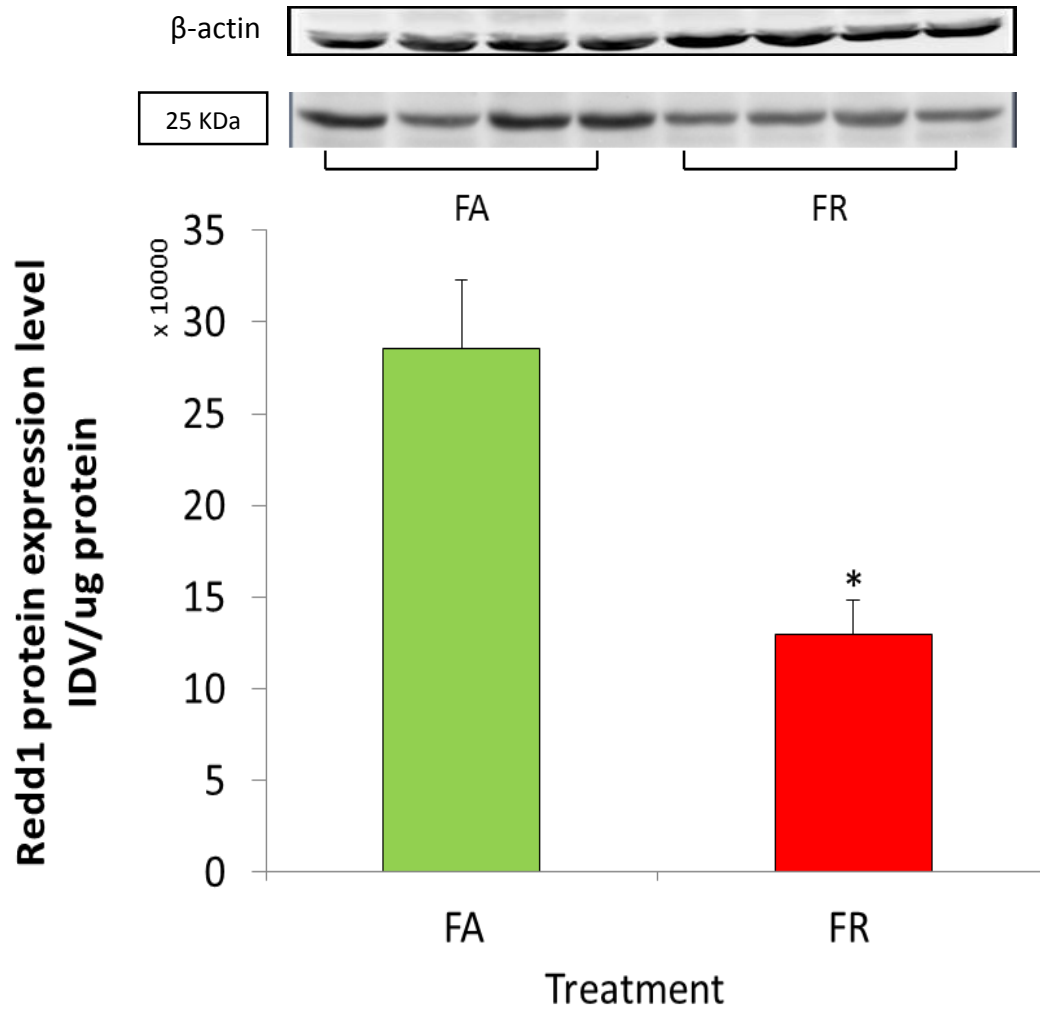
Figure 2.14: *Effect of folate restriction on gluconeogenesis enzyme G6PASE*

Figure 2.15: *Effect of folate restriction on AKT SER473 phosphorylation.* The level of AKT protein in 100 ug of liver whole cell extract from C57BL/6 mice fed FA or FR diets was determined by western blot analysis. The level of the phosphorylated form of AKT at SER473 residue was determined first. The membrane was then stripped and tested for Total AKT. The I.D.V (integrated density value) corresponding to the level of AKT protein (phosphorylated and total) was quantified by the BioRad Molecular Imager® System. P-AKT (ser473) was normalized to total AKT and expressed as p-AKT ser473 protein expression ratio. Values represent an average [S.E.M] for data obtained from 4 mice in each group and are representative of separate identical experiments. * Significant differences at $P < 0.05$. FA: Folate adequate; FR: Folate Restricted.
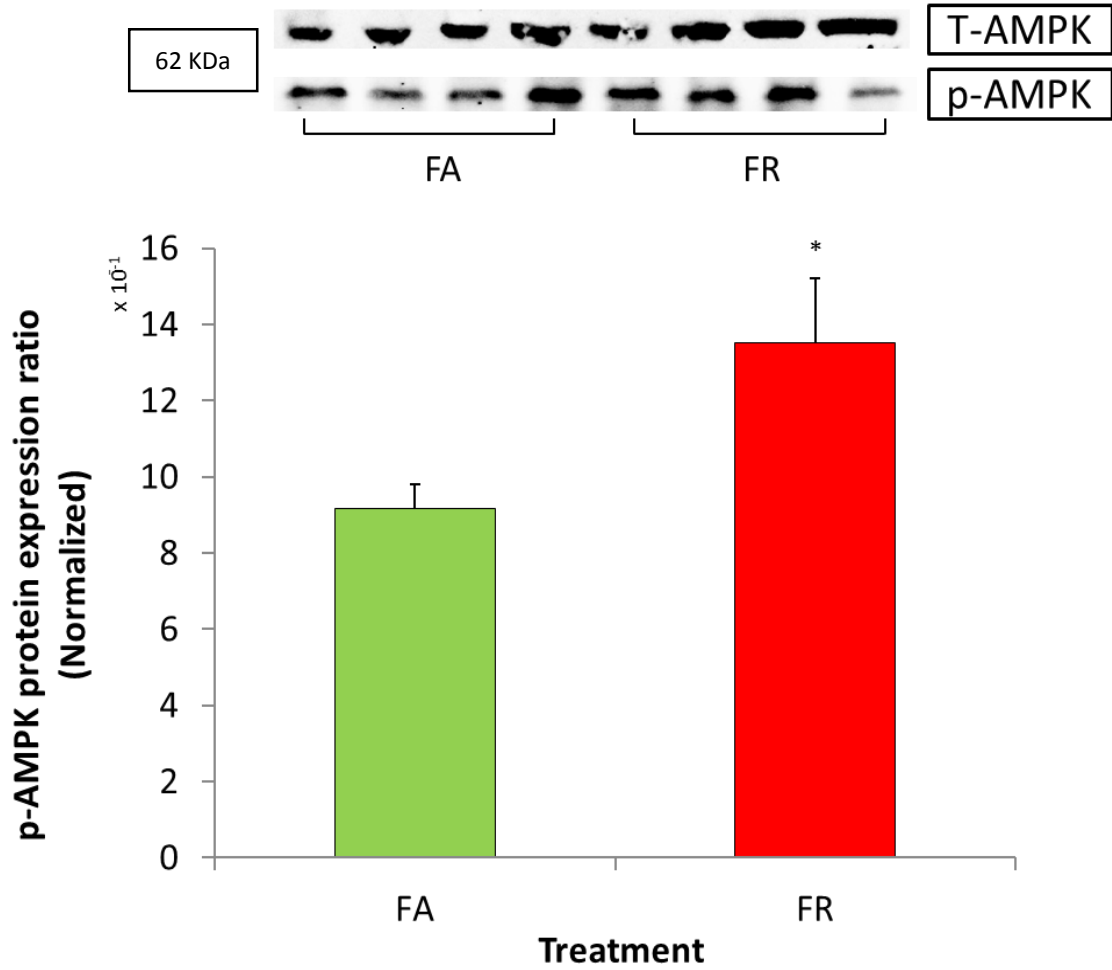
Figure 2.15: *Effect of folate restriction on AKT SER473 phosphorylation.*

Figure 2.16: *Effect of folate restriction on 4EBP-1 phosphorylation.* The level of 4EBP-1 protein in 100 ug of liver whole cell extract from C57BL/6 mice fed FA or FR diets was determined by western blot analysis. The level of the phosphorylated form of 4EBP-1 was determined first. The membrane was then stripped and tested for Total 4EBP-1. The I.D.V (integrated density value) corresponding to the level of 4EBP-1 protein (phosphorylated and total) was quantified by the BioRad Molecular Imager® System. Phosphorylated 4EBP-1 was normalized to total and expressed as p-4EBP-1 protein expression ratio. Values represent an average [S.E.M] for data obtained from 4 mice in each group and are representative of separate identical experiments. * Significant differences at $P < 0.05$. FA: Folate adequate; FR: Folate Restricted.
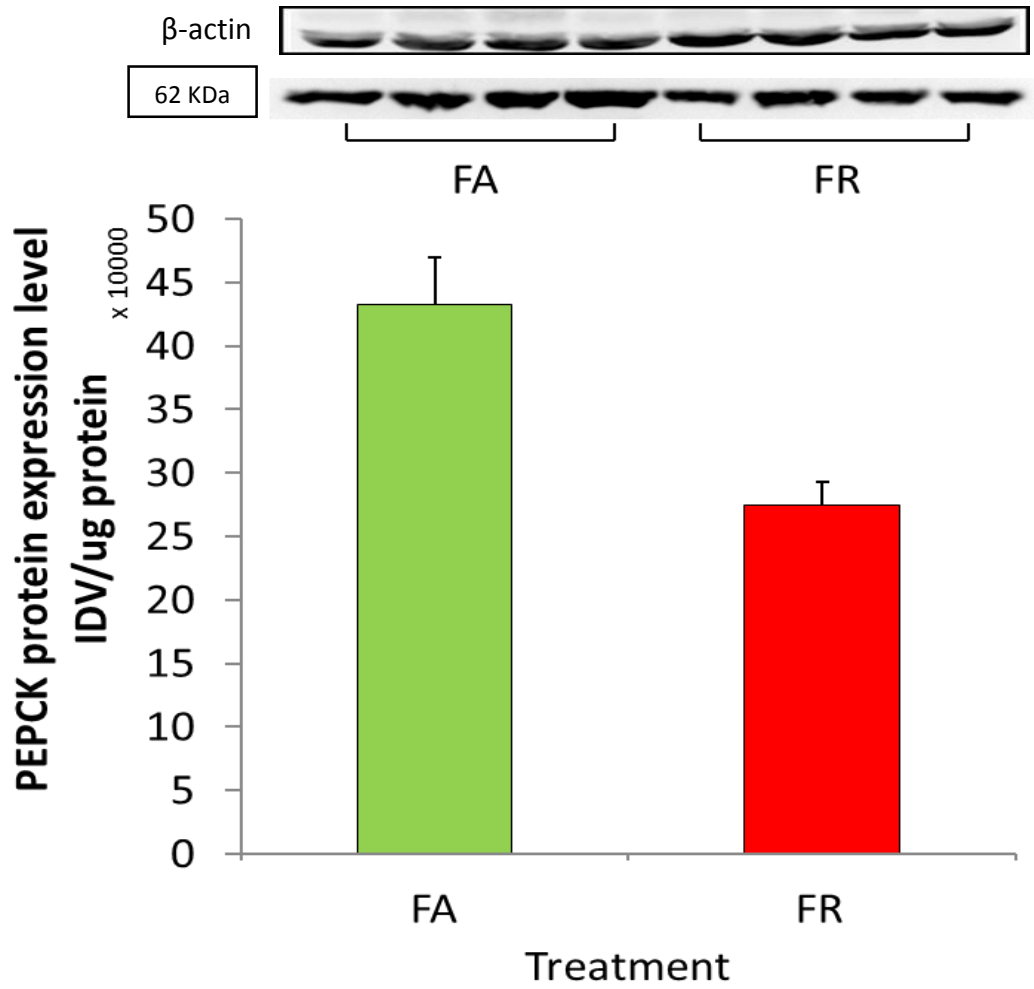
Figure 2.16: *Effect of folate restriction on 4EBP-1 phosphorylation.*

Figure 2.17: *Effect of folate restriction on S6K1 phosphorylation.* The level of S6K1 protein in 100 ug of liver whole cell extract from C57BL/6 mice fed FA or FR diets was determined by western blot analysis. The level of the phosphorylated form of S6K1 was determined first. The membrane was then stripped and tested for Total S6K1. The I.D.V (integrated density value) corresponding to the level of S6K1 protein (phosphorylated and total) was quantified by the BioRad Molecular Imager® System. Phosphorylated S6K1 was normalized to total and expressed as p-S6K1 protein expression ratio. Values represent an average [S.E.M] for data obtained from 4 mice in each group and are representative of separate identical experiments. * Significant differences at $P < 0.05$. FA: Folate adequate; FR: Folate Restricted.
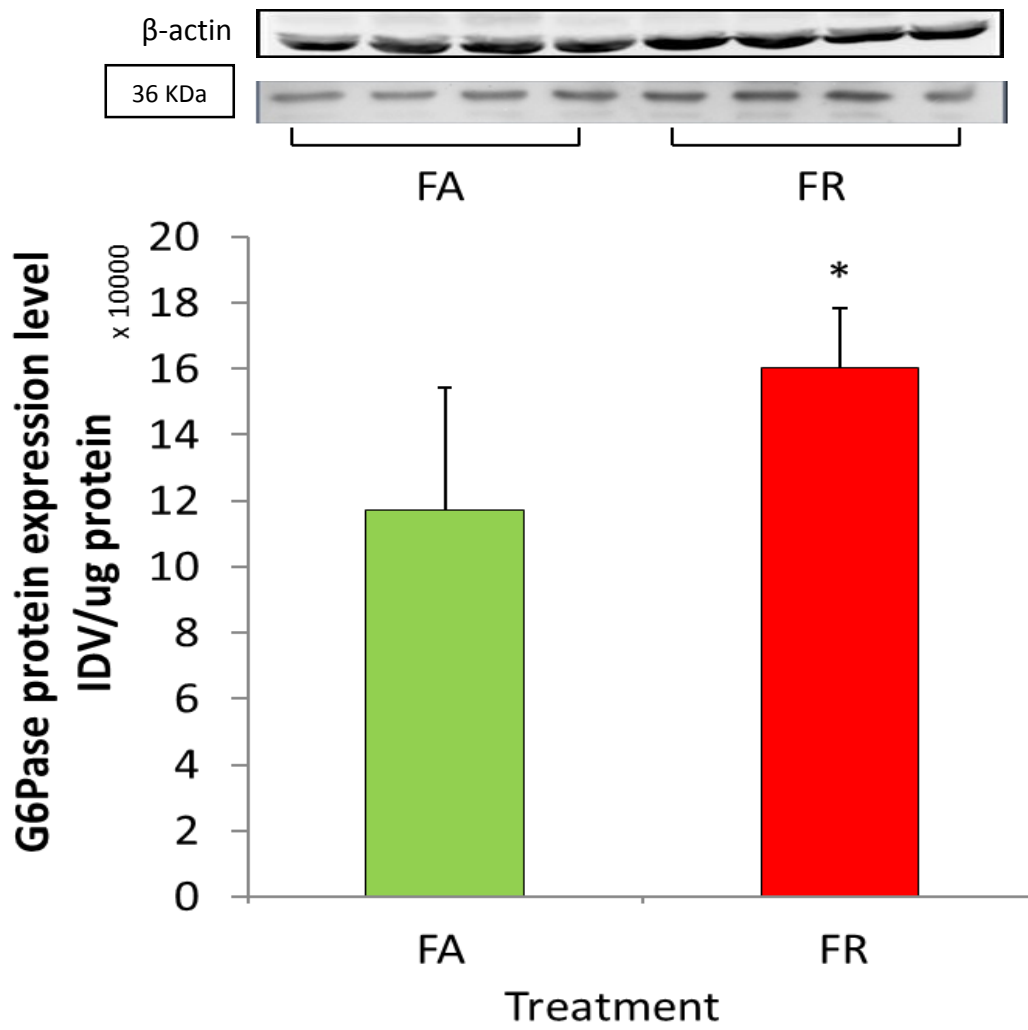
Figure 2.17: *Effect of folate restriction on S6K1 phosphorylation.*

Figure 2.18: *Effect of folate restriction on Gadd45g mRNA expression.* Gadd45g mRNA levels in the liver tissue of C57BL/6 mice fed FA or FR diets were quantified using real-time PCR. Normalized to RPLO. Values represent an average [S.E.M] for data obtained from 4 mice in each group and are representative of separate identical experiments. *Significant differences at $P < 0.05$. FA: Folate adequate; FR: Folate Restricted.

Figure 2.19: *Effect of folate restriction on p53 nuclear localization.* The level p53 protein in 100 ug of liver nuclear extracts from C57BL/6 mice fed FA or FR diets was determined by western blot analysis. The level of p53 was normalized based on the amount of protein loaded. The I.D.V (integrated density value) corresponding to the level of nuclear p53 protein was quantified by the BioRad Molecular Imager® System, normalized to β-actin. Values represent an average [S.E.M] for data obtained from 3 mice in each group and are representative of separate identical experiments. * Significant differences at $P < 0.05$. FA: Folate adequate; FR: Folate Restricted.
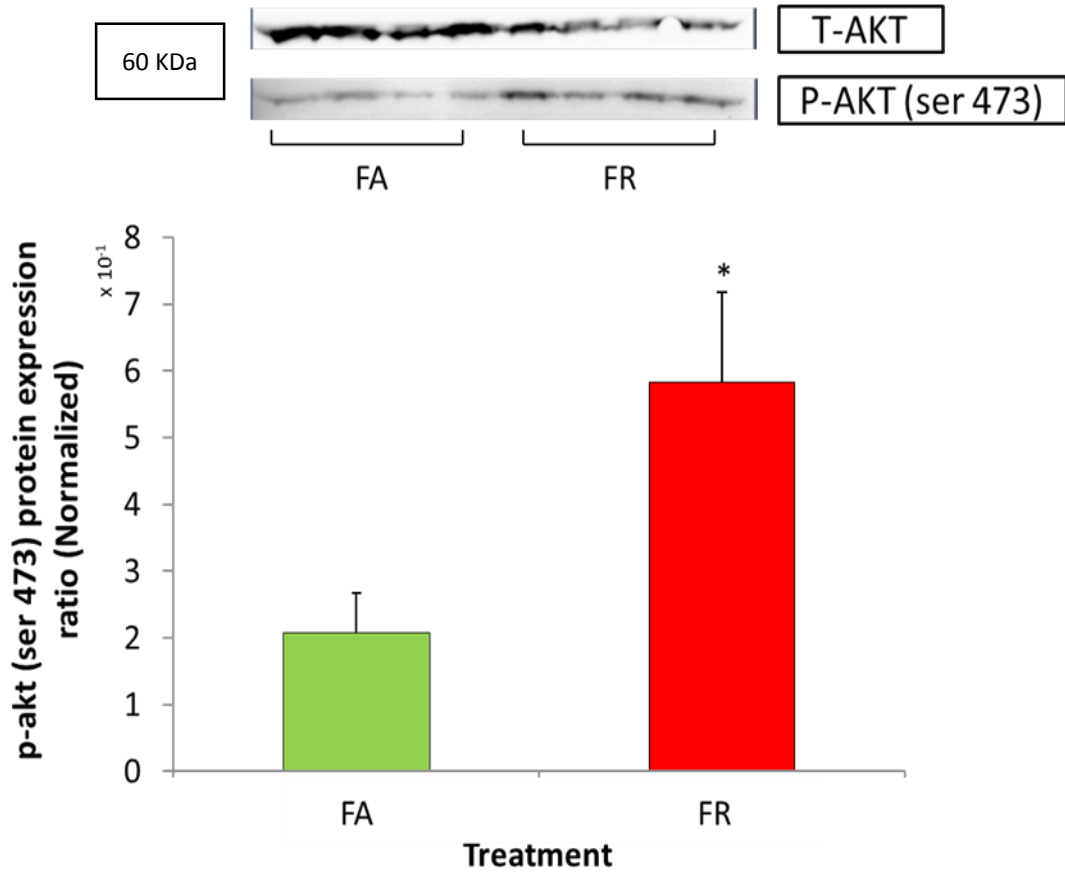
Figure 2.19: *Effect of folate restriction on p53 nuclear localization.*
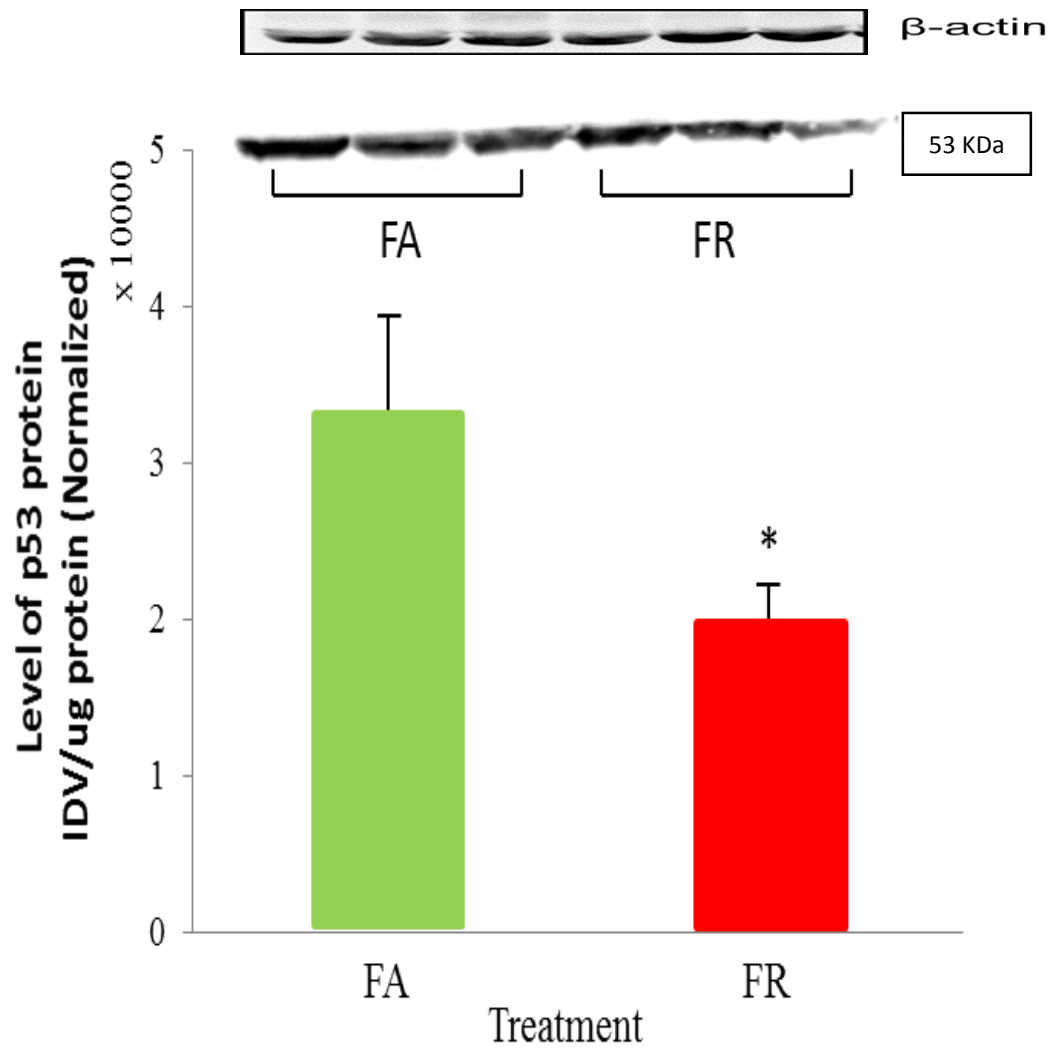
Figure 2.20: *Effect of folate restriction on Caspase-3 activity.* Cytosolic extracts were isolated using 250 mg liver. Extracts were incubated at room for 2 hours in a working solution containing synthetic caspase-3 substrate, Z-DEVD-AMC. Caspase mediated proteolytic cleavage of the substrate yields a bright blue-fluorescent product. An additional control assay was performed using reversible aldehyde inhibitor Ac-DEVD-CHO to confirm that the fluorescence observed in the sample assay was due to caspase activity. The fluorescence was measured using a fluorescence microplate reader (Genios plus, Tecan) at excitation: 342nm, emission: 441nm. The caspase activity was determined using an AMC (7-amino-4-methylcoumarin) standard curve (0-100mM), and reported as fluorescence per mg of protein. Values represent an average [S.E.M] for data obtained from 4 mice in each group and are representative of separate identical experiments. * Significant differences at $P < 0.05$. FA: Folate adequate; FR: Folate Restricted.
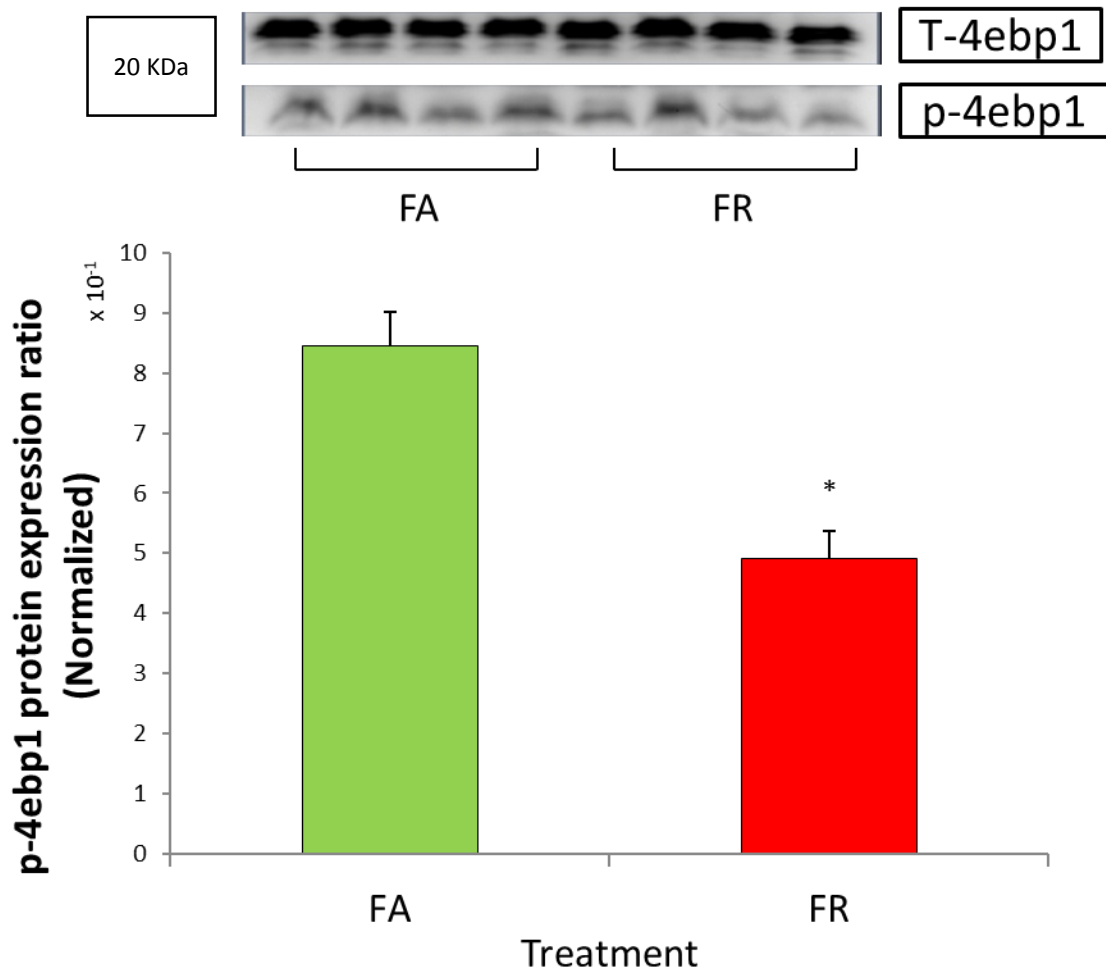
Figure 2.20: *Effect of folate restriction on Caspase-3 activity.*
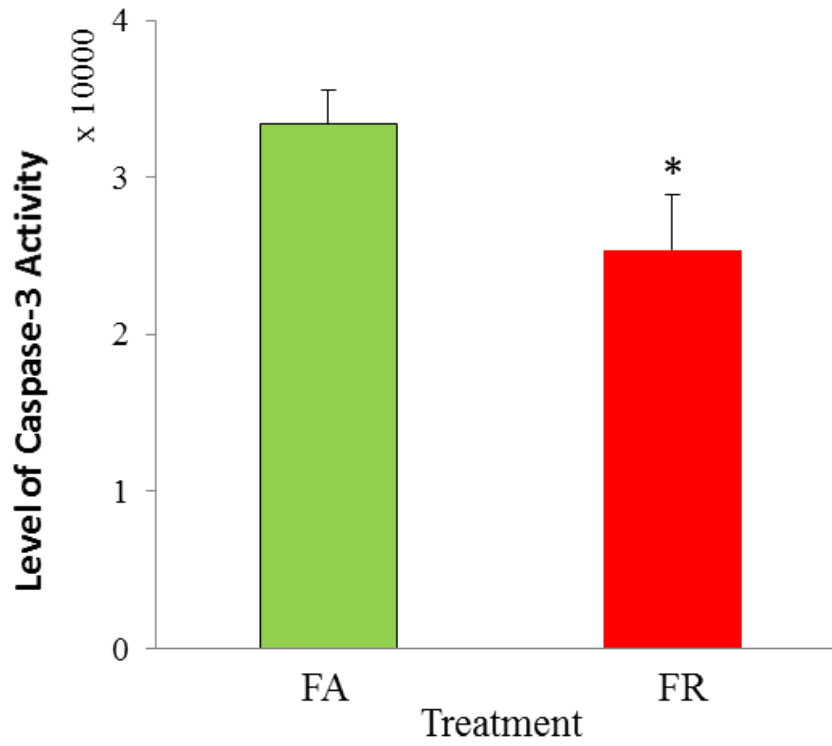
Figure 2.21: *Effect of folate restriction on PARP-1 cleavage.* The level of PARP-1 protein in 100 ug of liver whole cell extract from C57BL/6 mice fed FA or FR diets was determined by western blot analysis. The level of PARP-1 was normalized based on the amount of protein loaded. The I.D.V (integrated density value) corresponding to the cleavage of PARP-1 (cleaved/total) protein as quantified by the BioRad Molecular Imager® System, normalized to β-actin. Values represent an average [S.E.M] for data obtained from 3 mice in each group and are representative of separate identical experiments. * Significant differences at $P < 0.05$. FA: Folate adequate; FR: Folate Restricted.
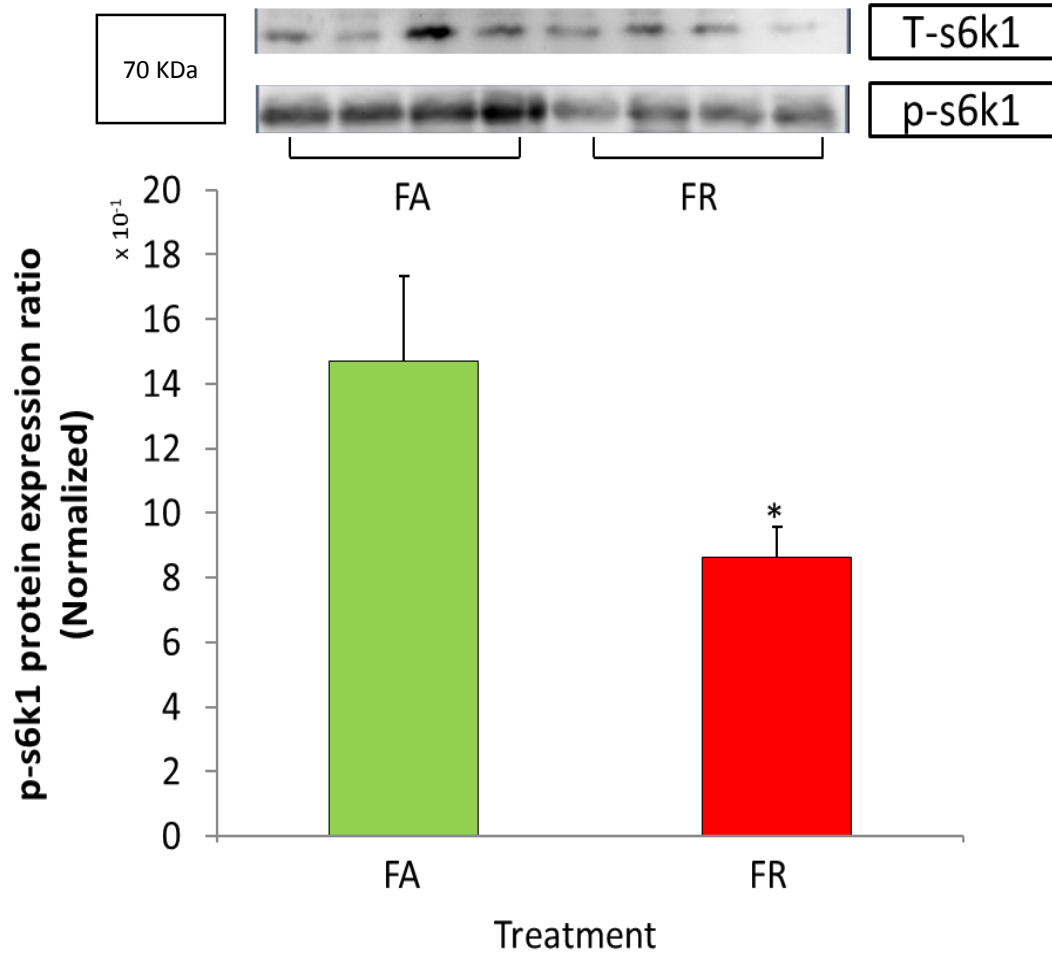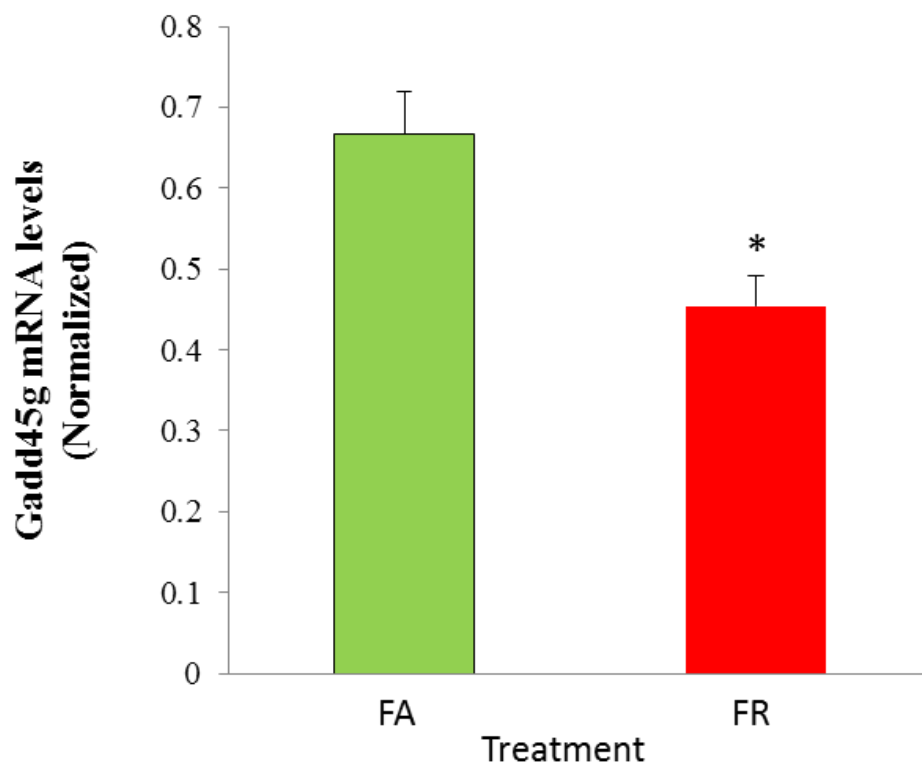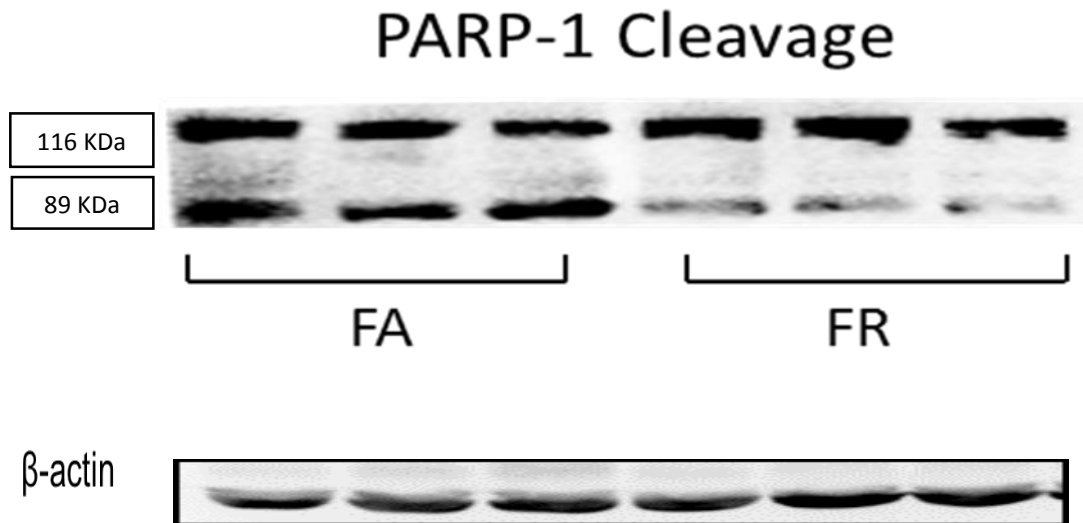
Figure 2.22: *Effect of folate restriction on survival in C57BL/6 mice*. The animals were fed either a folate adequate or a folate depleted AIN93G-purified isoenergetic diet (Dyets, Inc., Lehigh Valley, PA) as previously described [32]. The FA group received a folate adequate diet containing 2 mg of folic acid/kg diet. The FR group received a folate-deficient diet containing 0 mg of folic acid/kg diet. The animals remained on their respective diets and survival was periodically monitored until 33 months of age. FA: Folate adequate; FR: Folate Restricted.
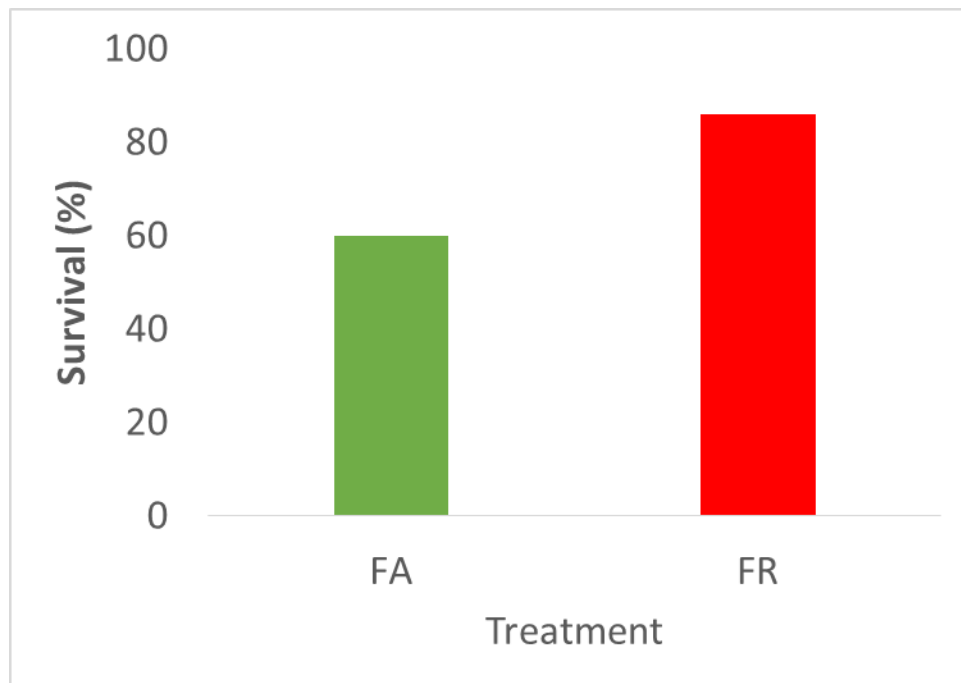
Table 2.1: *Effect of folate restriction on plasma amino acid.* Plasma was collected from C57BL/6 mice fed (FA) folate adequate diet (2 mg folic acid/kg diet) and (FR) folate restricted diet (0 mg folic acid/kg diet) upon sacrifice. Data obtained from 4 mice in each group. Samples were sent to MSU and UC-Davis for HPLC analysis. Amino aicd levels are expressed as ratio of FR to FA.

Table 2.1: *Effect of folate restriction on plasma amino acid.*

Amino Acid	FR/FA
Aspartic acid	0.94
Threonine	0.60
Serine	0.82
Asparagine	0.78
Glutamic Acid	0.92
Glutamine	0.90
a-Aminoadipic acid	0.91
Proline	0.49
Glycine	0.89
Alanine	0.64
Citrulline	0.71
a-Amino-n-butyric acid	0.81
Valine	0.54
Methionine	0.64
Isoleucine	0.54
Leucine	0.57
Tyrosine	0.62
Phenylalanine	0.73
b-Alanine	1.4
Homocysteine	0.86
Ethanolamine	1.1
Ammonia	1.2
Ornithine	0.60
Tryptophan	0.79
Lysine	0.69
Histidine	0.72
3-Methylhistidine	1.69
Arginine	0.80
AE-Cys (int.std)	0.96

Table 2.2: *Effect of folate restriction on mTOR pathway genes.* Total RNA was isolated from the colon mucosa of folate adequate and folate restricted mice using the RNeasy Mini Kit (Qiagen, Valencia, CA) per manufacturer's protocol. RNA samples were quantified with NanoDrop ND-1000 (NanoDrop Technologies, Inc, Wilmington, DE) and 260/280 ratio in the range of 2.0-2.2 was defined as acceptable. A quality check of the total RNA was performed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Microarray expression profiling was conducted by Microarray & Bioinformatics Facility Core at Wayne State University (Institute of Environmental Health Sciences, Detroit, MI) according to the manufacturer's protocol. Data obtained from 4 mice in each group. Significant differences at $P < 0.01$. FR as compared to FA.

Accession No.	Gene Symbol	Gene Name	FR
NM_133781	Cab39	Calcium binding protein 39	-1.2
NM_011492	Stk11	Serine/threonine kinase 11	-1.2
BC043920	**Frap1**	**FK506 binding protein 12-rapamycin associated protein 1**	**-1.3**
AK029196	4921505C17Rik	RIKEN cDNA 4921505C17 gene	1.5
AK129326	4932417H02Rik	RIKEN cDNA 4932417H02 gene	ns
NM_007918	Eif4ebp1	Eukaryotic translation initiation factor 4E binding protein 1	ns

CHAPTER 3: RAPAMYCIN, CROWDED LITTER AND CALORIC RESTRICTION

Introduction

Aging and age related diseases have been a major focus for researchers in various fields. Aging is defined as "the decline and deterioration of functional properties at the cellular, tissue, and organ level" [4]. The loss of homeostasis and loss of functional capacity associated with aging increase susceptibility to developing many age related diseases [1]. Some of the associated diseases include: cancer, neurodegenerative disorders, autoimmune disease, cardiovascular disease and type II diabetes mellitus [5]. The high occurrence of these diseases in the geriatric population limits the benefit obtained from targeting them individually [34]. Researchers have been aiming to target the aging process as a whole in order to delay occurrence of age related diseases, and in turn delaying aging.

The first documented life extending strategy was over 60 years ago. McCay demonstrated that it was possible to extend lifespan of rodents by modifying their diet [6]. Since this discovery, various nutritional interventions, genetic manipulations, and drug treatments were also shown to delay aging [6, 9, 13, 19, 26-28, 35]. By delaying aging, these manipulations could potentially have a protective effect on human health as a whole, which far exceeds the effect seen on individual age related diseases. Since the published data on the beneficial effect of dietary interventions on age related diseases were sporadic, they were not considered reliable or reproducible by the scientific community [145]. This led the National Institute on Aging (NIA) to develop an interventions testing program (ITP) to evaluate candidate agents known to delay aging or age related diseases [145].

ITP approved different agents to be tested every year. Some of the compounds were tested because they showed promising results in worms, flies or small scale rodent studies [145]. Others

were chosen due to their impact on biochemical and physiological processes that are involved in regulating aging [145]. ITP used genetically heterogeneous mice that were produced by a standardized four way cross (UM-HET3) [146]. The use of heterogeneous mice would avoid missing a true positive effect of an agent that may fail to work in a specific genotype [145]. It would also reduce the chance of overemphasizing an agent that may work only in a specific genotype [145]. Other key features of the program include: replication at three test sites (the Jackson Laboratory, University of Michigan, and University of Texas), and a study design size with more than 80% power to detect a 10% increase or decrease in median lifespan [145].

The emphasis of this research will be on a cohort study from the University of Michigan that included three life extending strategies: Rapamycin, Caloric Restriction and Crowded Litter. Rapamycin, also known as Sirolumus, is a macrolide immunosuppressant drug with anti-proliferative properties [14]. Rapamycin inhibits the TOR kinase of mTORC1 and extends lifespan in various animal models including: yeast, flies and mice [5]. Rapamycin extended lifespan of heterogeneous male and female mice even when started late in life [42]. Caloric restriction, a well-documented strategy in adults, also slows down aging and extends lifespan of rodents [6, 147-149]. However, detrimental effects were seen in the offspring when exposed to undernutrition during the gestation and/or lactation period [150, 151]. Recent evidence now suggests that pre-weaning food restriction increases lifespan in mice [152]. Crowded litter, a 50% increase in litter size and a transient food restriction period during the first 20 days of life, extended mean and maximal lifespan in mice [152]. The purpose of this study is to characterize the possible mechanism behind the extension of lifespan seen in these three life extending strategies.

Experimental Procedures

Animals- Genetically heterogeneous UM-HET3 mice were produced by crossing CByB6F1/J female mice with C3D2F1/J male mice at the University of Michigan (UM). Details of breeding have been explained in detail by Miller and coworkers [145, 153]. All experiments were performed in male pathogen–free young mice from this heterogeneous background in accordance with NIH guidelines for the use and care of laboratory animals.

Samples & Interventions- Liver samples from 32 UM-HET3 male mice were snap frozen in liquid nitrogen upon sacrifice at 9 months of age from the University of Michigan. Group 1: control mice were fed a chow diet (Purina 5LG6) from the time they were weaned until sacrifice (CON). Group 2: mice were fed encapsulated rapamycin (14 mg/kg food) incorporated into chow diet (Purina 5LG6) (RAPA) starting at 6 weeks of age. Group 3: mice from a crowded litter. The size of the litter was increased to 12 pups per nursing mother. After 20 days, pups were weaned onto a normal chow diet (Purina 5LG6) until sacrifice (CL). Group 4: mice from the caloric restricted group. Mice were fed 60% of food consumed by control mice from the time of weaning until sacrifice (CR). Mouse chow (Purina 5LG6) containing rapamycin, as well as control chow was prepared at one site (TestDiet, Richmond, IN) and shipped to each of the three test sites. Detailed description of diet preparation and interventions is described here [35, 42, 145, 154, 155].

Isolation of Whole cell extract- Whole cell extracts were isolated using hypotonic and hypertonic salt solutions from transfactor extraction kit (Clontech, Mountain View, CA). Briefly, 100mg of liver tissue was homogenized with the hypotonic salt solution to lyse the cell and further treated with the hypertonic salt solution to release the nuclear contents without any fractionations. The whole cell extract thus obtained was used for various assays.

Western Blot Analysis- Protein expression analysis was performed using western blot technique as previously described [76]. 100 μg nuclear protein was used to run the SDS-PAGE. Upon completion of SDS-PAGE, the region containing the proteins of interest was excised and prepared for western blot analysis while the remaining portion of the gel was stained with Gel Code Blue Stain Reagent (Pierce Biotechnology, Rockford, IL) to ensure equal protein loading. Manufacturer recommended dilutions of anti-sera developed against p-AMPK (Abcam), t-AMPK (Millipore), p-AKT (Cell Signaling), t-AKT (Cell Signaling), REDD1 (Abcam), IPMK (Abcam), p-S6K1 (Cell Signaling), t-S6K1 (Cell Signaling), p-4EBP1 (Cell Signaling), t-S6K1 (Cell Signaling), PEPCK (Santa Cruz) and G6PASE (Santa Cruz) were used to detect proteins of interest followed by incubation with HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The bands were visualized and quantified using a Molecular Imager System (Bio-Rad, Hercules, CA) after incubation in SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). Data are expressed as the integrated density value (I.D.V.) of the band normalized to β-actin (Sigma Aldrich) or ratio of phosphorylated form of protein to total normalized to β-actin.

NAD/NADH Assay: Total NAD (NAD and NADH) was extracted from 20 mg of liver using Abcam's NAD/NADH Assay kit. This is a calorimetric assay that detects intracellular nucleotides at OD 570 nm. Total NAD is extracted and detected. Then NAD^+ is decomposed to detect NADH. The values are used to calculate for NAD^+. NAD^+ levels are expressed as (uM). The ratio of NAD^+ to NADH is also calculated.

Statistical Analysis- Statistical significance between means was determined using t-test and analysis of variance followed by post tukey test wherever appropriate. P-values less than 0.05 were considered statistically significant.

Results

Analysis of the impact of rapamycin, crowded litter and caloric restriction on animal weight:

Genetically heterogeneous UM-HET3 mice were produced by crossing CByB6F1/J female mice with C3D2F1/J male mice at the University of Michigan [145, 153]. Animals were divided into 4 groups: Control, Rapamycin, Crowded Litter and Caloric Restriction. The mice in the control group were fed a chow diet after they were weaned (CON). The mice in the rapamycin group were fed a chow diet with encapsulated rapamycin (14 mg/kg food) starting at 6 weeks of age (RAPA). The mice in the crowded litter group received a transient food restriction during their first 20 days of life. Litter size was increased by 50% during this period, after which they were fed a regular chow diet. The mice in the caloric restricted group were fed 60% of food consumed by control after they were weaned. Animals were sacrificed at various time points for analysis, and the focus of this study will be on the first group sacrificed at 9 months.

It's been shown that Rapamycin, crowded litter and caloric restriction extend lifespan in animal models [5, 6, 42, 147-149, 152]. However, the mechanism behind the longevity seen is still elusive. We began by looking at the effect of these interventions on body weight at the time of sacrifice. As shown in Figure 3.1, there was no significant difference in body weight in the rapamycin or crowded litter group as compared to control. However, we saw a significant decrease in body weight in the caloric restricted group. Rapamycin, crowded litter and caloric restriction all increase lifespan in this heterogeneous population of mice, however their impact on the weight of the animals is not the same.

Analysis of the nutrient sensing pathway - mTOR in the liver of mice on rapamycin, crowded litter and caloric restriction:

We looked at the impact of these three interventions on the mTOR pathway. Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that regulates cell growth, autophagy and proliferation [23]. mTOR signaling is altered in many age related diseases including cancer [24]. Genetic downregulation of TOR signaling delays aging in evolutionary distant organisms from yeast to mammals [26]. mTOR exists in two multiprotein complexes: mTORC1 and mTORC2. mTORC1 pathway integrates input from various upstream intracellular and extracellular signals that include: growth factors (ILGF), stress, hypoxia, energy status (AMP/ATP, NAD^+/NADH), purine metabolism, and amino acid levels [101]. However, mTORC2 is not sensitive to nutrients but responds to growth factors by poorly defined mechanisms [23].

We collected liver samples from UM-HET3 male mice upon sacrifice at 9 months of age from the University of Michigan. We looked at the impact of rapamycin, crowded litter and caloric restriction on various upstream regulators of mTOR, starting with energy metabolism. NAD cycles between the oxidized (NAD+) and reduced (NADH) forms, partaking a central role in cellular metabolism and energy production [100]. We saw an increase in NAD^+ levels (Figure 3.2), and NAD^+/NADH ratio (Figure 3.3) in all three experimental groups as compared to control. We then looked at the impact of rapamycin, crowded litter and caloric restriction on IPMK. IPMK mediates the activation of mTORC1 in response to amino acids and glucose [106, 107]. There was no significant difference in the expression of IPMK in rapamycin and crowded litter Figure 3.4. However, we saw a significant increase in expression in the caloric restricted group; possibly a feedback mechanism due to downregulated mTOR. We looked at REDD1 levels, which increase following exposure to hypoxia or DNA damage, leading to inhibition of mTOR signaling. There was no significant difference in expression of REDD1 in the rapamycin group (Figure 3.5) which is what we expected. However, there was a significant increase in crowded litter and a significant

decrease in caloric restriction. This suggests that the transient food restriction early on in life in the crowded litter group is triggering alternate pathways that may be resulting in the extension of lifespan.

We looked at the phosphorylation of AMPK, a metabolic fuel gauge that detects changes in intracellular AMP/ATP ratio [108]. AMPK is activated in response to nutrient depletion and works to maintain energy stores [108]. AMPK directly inhibits mTORC1, and neutralizes PI3K activation of mTORC1. We saw a significant increase in AMPK phosphorylation in all three group, with the highest increase in the caloric restricted group (Figure 3.6). Activation of AMPK in response to low energy inhibits hepatic gluconeogenesis in an insulin independent manner [111]. However, there was no significant difference on the effect of these interventions on PEPCK expression (rate limiting enzyme in gluconeogenesis) (Figure 3.7). On the contrary, we did see a significant decrease in G6PASE expression in response to rapamycin only (Figure 3.8). When we analyzed the effect of rapamycin, crowded litter and caloric restriction on phosphorylation of AKT S473, which is mTORC2 dependent, we saw a significant increase in expression in the caloric restricted group only (Figure 3.9).

The final part that we analyzed was the impact of rapamycin, crowded litter and caloric restriction on protein synthesis. Protein synthesis is the best characterized process downstream of mTORC1 through phosphorylation of two downstream target proteins: S6K1 and 4E-BP1[23]. There was a significant decrease in phosphorylation of 4EBP1 in the caloric restricted group only (Figure 3.10). However, we saw a significant decrease in phosphorylation of S6K1 in both rapamycin and caloric restricted groups (Figure 3.11). This differential effect of rapamycin on protein synthesis was also shown by Choo et al. [156].

Rapamycin, crowded litter and caloric restriction have been shown to extend lifespan in animal models. However, the mechanisms by which they do so are not fully understood. Here we show that although these three interventions extend lifespan, they have a differential effect on the mTOR signaling pathway. The mTOR signaling pathway may be one of the mechanisms by which rapamycin and caloric restriction are working to extend lifespan. However, we speculate that alternate mechanisms are being activated in the crowded litter group. The transient food restriction during their first 20 days of life may be resulting in the extension of lifespan irrespective of mTOR.

Discussion

The National Institute on Aging (NIA) developed an Interventions Testing Program (ITP) to evaluate possible agents that may delay the rates of aging. Researchers speculate that delaying aging in general could greatly benefit human health as a whole. The beneficial effect could potentially be greater than any effect seen by targeting individual forms of age related diseases [145]. They evaluated various candidates every year using genetically heterogeneous mice. Studies were replicated at three test sites (the Jackson Laboratory (TJL), University of Michigan (UM), and University of Texas (UT)) and included sufficient statistical power to detect a 10% change in lifespan [145].

The focus of this research was on a cohort study from UM evaluating the effects of rapamycin, crowded litter and caloric restriction on aging. It's been shown that Rapamycin, crowded litter and caloric restriction extend lifespan in animal models [5, 6, 42, 147-149, 152]. Rapamycin is an immunosuppressant drug that directly inhibits the TOR kinase of mTORC1. Caloric restriction has also been shown to inhibit mTOR [36]. However data is lacking on the effect of crowded litter on mTOR, and whether these interventions affect the upstream regulators of mTOR similarly.

Animals were divided into 4 groups: Control (Chow diet), Rapamycin (14 mg/kg diet encapsulated rapamycin), Crowded Litter (transient food restriction during first 20 days of life) and Caloric Restriction (60% of food consumed by control). At 9 months of age, animals were sacrificed for analysis. Upon examining their body weights, we did not see any significant difference in the rapamycin or crowded litter groups as compared to control. However, there was a significant decrease in body weight in the caloric restricted group as compared to the rest.

Since the focus of our lab is mTOR, we characterized the impact of these life extending strategies on the mTOR signaling pathway. mTOR is a major nutrient sensing pathway that impacts aging, and many age-related diseases. Inhibition of mTOR extends life-span in many laboratory models including yeast, worms, flies and rodents [35, 119, 143, 144]. mTOR is impacted by changes in NAD^+ levels and $NAD^+/NADH$ ratio. We showed that there was an increase in NAD^+ levels and $NAD^+/NADH$ ratio in all three groups. Rapamycin, crowded litter and caloric restriction also increased phosphorylation of AMPK, a metabolic fuel gauge that senses changes in AMP/ATP ratio, and modulates mTORC1. Rapamycin and caloric restriction downregulate mTORC1 as observed by the decreased activation of two downstream targets: S6K1 and/or 4E-BP. However, we did not see any significant difference on the impact of crowded litter on mTOR, suggesting that crowded litter may be activating alternate pathways that may be resulting in the beneficial longevity effect.

Furthermore, caloric restriction significantly increased AKT S473 phosphorylation. This site is phosphorylated by mTORC2. Caloric restriction increased phosphorylation of AKT at the S473 residue possibly as a compensatory mechanism to maintain insulin sensitivity [5, 140]. However, we did not see any significant difference on phosphorylation of AKT S473 in the rapamycin or crowded litter group. Interestingly, there was no significant difference in the

expression of the gluconeogenesis enzyme PEPCK. However, G6PASE expression was significantly less in the rapamycin group.

When we analyzed the impact on REDD1 and IMPK we did not see any significant difference in the rapamycin group. Crowded litter only impacted REDD1 expression, while caloric restriction significantly impacted both. These data indicate that Rapamycin and caloric restriction are impacting and downregulating the mTOR signaling pathway, as shown by the decrease in downstream protein synthesis. The longevity effect may be regulated in part by downregulation of mTOR, however, they impact upstream regulators of mTOR differently. On the other hand, crowded litter does not impact the mTOR pathway. We speculate that crowded litter is impacting alternate pathways early on in life that are resulting in this beneficial longevity effect, irrespective of mTOR.

Figure 3.1: *Effect of Rapamycin, crowded litter and caloric restriction on weight of heterogeneous UM-HET3 male mice.* Genetically heterogeneous UM-HET3 mice were produced by crossing CByB6F1/J female mice with C3D2F1/J male mice at three test sites: the Jackson laboratory (TJL), the University of Michigan (UM), or the University of Texas Health Center at San Antonio (UT) [145, 153]. Animals were divided into 4 different groups at the University of Michigan. Group 1: control mice were fed a chow diet (Purina 5LG6) from the time they were weaned until sacrifice (CON). Group 2: mice were fed encapsulated rapamycin (14 mg/kg food) incorporated into chow diet (Purina 5LG6) (RAPA) starting at 6 weeks of age. Group 3: mice from a crowded litter. The size of the litter was increased to 12 pups per nursing mother. After 20 days, pups were weaned onto a normal chow diet (Purina 5LG6) until sacrifice (CL). Group 4: mice from the caloric restricted group. Mice were fed 60% of food consumed by control mice from the time of weaning until sacrifice (CR). The weights of animals were taken upon sacrifice at 9 months of age. Values represent an average [S.E.M] for data obtained from 8 mice in each group. Different letters significantly different at $P < 0.05$. CON: Control Group; RAPA: Rapamycin Group; CL: Crowded Litter Group; CR: Caloric Restriction Group.

Figure 3.1: *Effect of Rapamycin, crowded litter and caloric restriction on weight of heterogeneous UM-HET3 male mice.*

Figure 3.2: *Effect of Rapamycin, crowded litter and caloric restriction on NAD$^+$ levels.* Total NAD (NAD$^+$ and NADH) was extracted from the liver of heterogeneous UM-HET3 male mice from all 4 experimental groups (CON, RAPA, CL, and CR), using Abcam's NAD/NADH Assay kit. Intracellular nucleotides were detected using a plate reader at OD 570 nm. Total NAD is extracted and detected. Then NAD$^+$ is decomposed to detect NADH. The values are then used to calculate for NAD$^+$. NAD$^+$ levels are expressed as (uM). Values represent an average [S.E.M] for data obtained from 4 mice in each group and are representative of separate identical experiments. CON: Control Group; RAPA: Rapamycin Group; CL: Crowded Litter Group; CR: Caloric Restriction Group.

Figure 3.2: *Effect of Rapamycin, crowded litter and caloric restriction on NAD$^+$ levels.*

Figure 3.3: *Effect of Rapamycin, crowded litter and caloric restriction on $NAD^+/NADH$ ratio.*
Total NAD (NAD^+ and NADH) was extracted from the liver of heterogeneous UM-HET3 male
mice from all 4 experimental groups (CON, RAPA, CL, and CR), using Abcam's NAD/NADH
Assay kit. Intracellular nucleotides were detected using a plate reader at OD 570 nm. First, total
NAD is extracted and detected. Then NAD^+ is decomposed to detect NADH. The values are then
used to calculate for NAD^+. $NAD^+/NADH$ ratio calculated. Values represent an average [S.E.M]
for data obtained from 4 mice in each group and are representative of separate identical
experiments. CON: Control Group; RAPA: Rapamycin Group; CL: Crowded Litter Group; CR:
Caloric Restriction Group.

Figure 3.3: *Effect of Rapamycin, crowded litter and caloric restriction on NAD$^+$/NADH ratio.*

Figure 3.4: *Effect of Rapamycin, crowded litter and caloric restriction on expression of IPMK protein.* The level of IPMK protein in 100 ug of liver whole cell extract from heterogeneous UM-HET3 male mice from all 4 experimental groups (CON, RAPA, CL, and CR) was determined by western blot analysis. The level of IPMK was normalized based on the amount of protein loaded. The I.D.V (integrated density value) corresponding to the level of IPMK protein as quantified by the BioRad Molecular Imager® System, normalized to β-actin. Values represent an average [S.E.M] for data obtained from 3 mice in each group and are representative of separate identical experiments. Different letters significantly different at $P < 0.05$. CON: Control Group; RAPA: Rapamycin Group; CL: Crowded Litter Group; CR: Caloric Restriction Group.

Figure 3.4: *Effect of Rapamycin, crowded litter and caloric restriction on expression of IPMK protein.*

Figure 3.5: *Effect of Rapamycin, crowded litter and caloric restriction on expression of REDD1 protein.* The level of REDD1 protein in 100 ug of liver whole cell extract from heterogeneous UM-HET3 male mice from all 4 experimental groups (CON, RAPA, CL, and CR) was determined by western blot analysis. The level of REDD1 was normalized based on the amount of protein loaded. The I.D.V (integrated density value) corresponding to the level of REDD1 protein as quantified by the BioRad Molecular Imager® System, normalized to β-actin. Values represent an average [S.E.M] for data obtained from 3 mice in each group and are representative of separate identical experiments. Different letters significantly different at $P < 0.05$. CON: Control Group; RAPA: Rapamycin Group; CL: Crowded Litter Group; CR: Caloric Restriction Group.

Figure 3.5: *Effect of Rapamycin, crowded litter and caloric restriction on expression of REDD1 protein.*
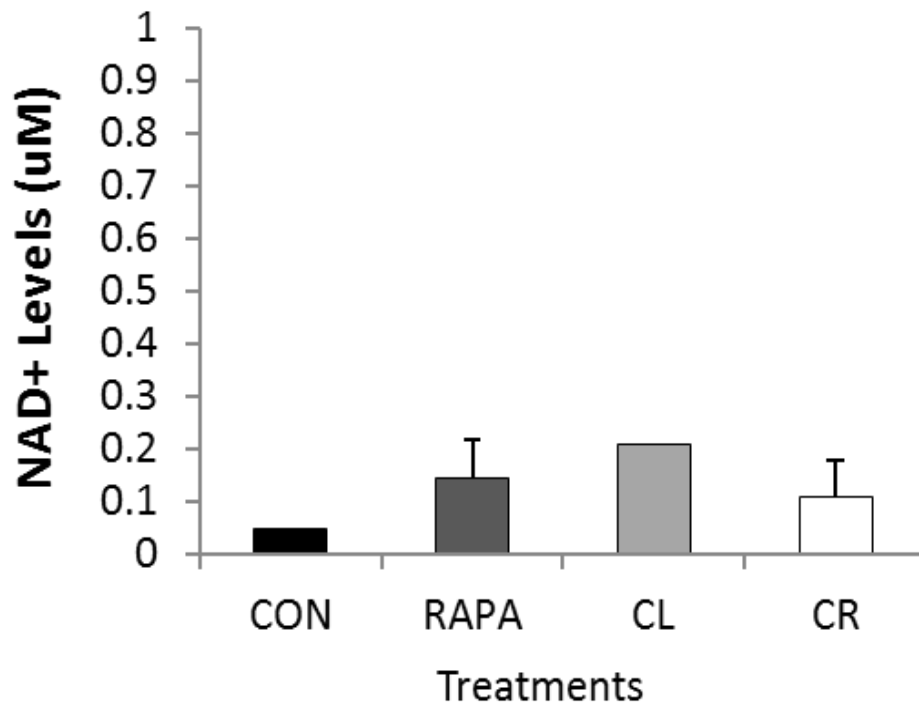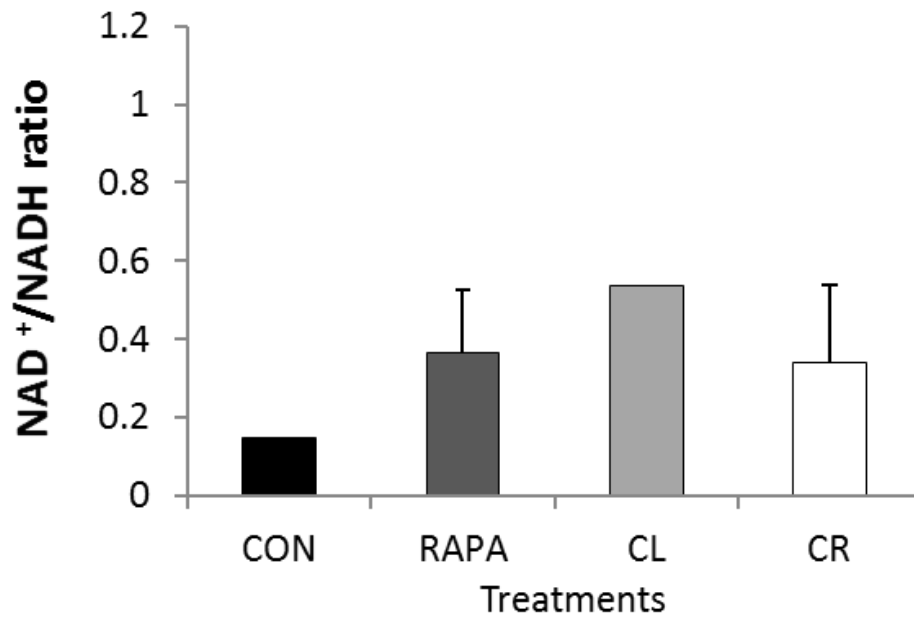
Figure 3.6: *Effect of Rapamycin, crowded litter and caloric restriction on AMPK phosphorylation.* The level of AMPK protein in 100 ug of liver whole cell extract from heterogeneous UM-HET3 male mice from all 4 experimental groups (CON, RAPA, CL, and CR) was determined by western blot analysis. The level of the phosphorylated form of AMPK was determined first. The membrane was then stripped and tested for Total AMPK. The I.D.V (integrated density value) corresponding to the level of AMPK protein (phosphorylated and total) was quantified by the BioRad Molecular Imager® System. Phosphorylated AMPK was normalized to total and expressed as p-AMPK protein expression ratio. Values represent an average [S.E.M] for data obtained from 3 mice in each group and are representative of separate identical experiments. Different letters significantly different at $P < 0.05$. CON: Control Group; RAPA: Rapamycin Group; CL: Crowded Litter Group; CR: Caloric Restriction Group.

Figure 3.6: *Effect of Rapamycin, crowded litter and caloric restriction on AMPK phosphorylation.*

Figure 3.7: *Effect of Rapamycin, crowded litter and caloric restriction on gluconeogenesis enzyme PEPCK.* The levels of PEPCK protein in 100 ug of liver whole cell extract from heterogeneous UM-HET3 male mice from all 4 experimental groups (CON, RAPA, CL, and CR) were determined by western blot analysis. The level of PEPCK was normalized based on the amount of protein loaded. The I.D.V (integrated density value) corresponding to the level of PEPCK protein as quantified by the BioRad Molecular Imager® System, normalized to β-actin. Values represent an average [S.E.M] for data obtained from 3 mice in each group and are representative of separate identical experiments. Different letters significantly different at $P < 0.05$. CON: Control Group; RAPA: Rapamycin Group; CL: Crowded Litter Group; CR: Caloric Restriction Group.

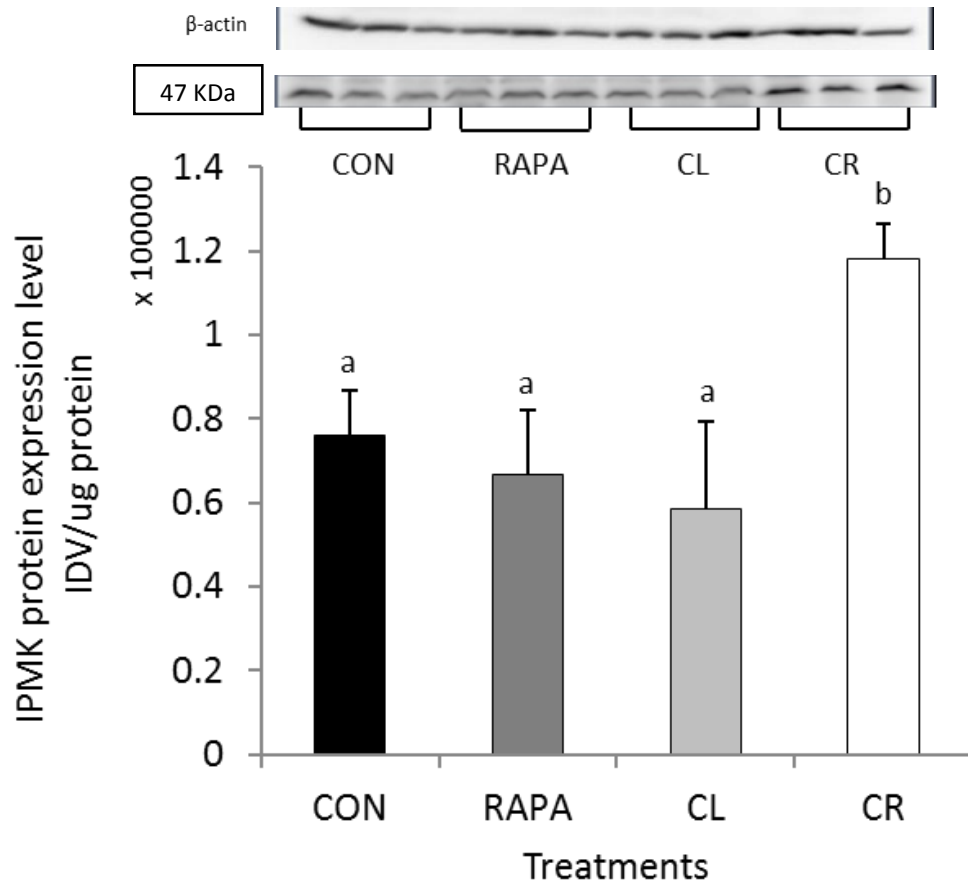Figure 3.7: *Effect of Rapamycin, crowded litter and caloric restriction on gluconeogenesis enzyme PEPCK.*

Figure 3.8: *Effect of Rapamycin, crowded litter and caloric restriction on gluconeogenesis enzyme G6PASE.* The levels of G6PASE protein in 100 ug of liver whole cell extract from heterogeneous UM-HET3 male mice from all 4 experimental groups (CON, RAPA, CL, and CR) were determined by western blot analysis. The level of G6PASE was normalized based on the amount of protein loaded. The I.D.V (integrated density value) corresponding to the level of G6PASE protein as quantified by the BioRad Molecular Imager® System, normalized to β-actin. Values represent an average [S.E.M] for data obtained from 3 mice in each group and are representative of separate identical experiments. Different letters significantly different at $P < 0.05$. CON: Control Group; RAPA: Rapamycin Group; CL: Crowded Litter Group; CR: Caloric Restriction Group.

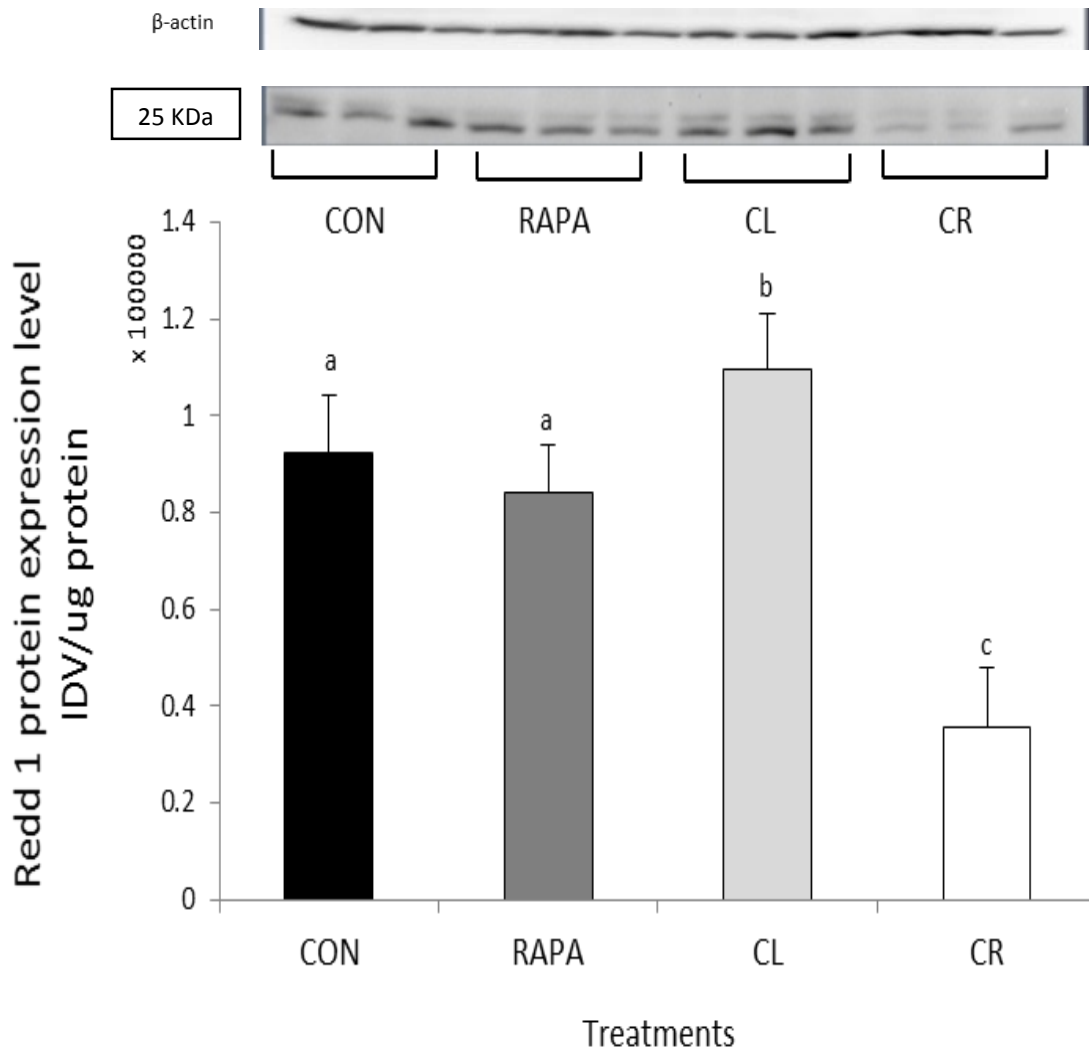Figure 3.8: *Effect of Rapamycin, crowded litter and caloric restriction on gluconeogenesis enzyme G6PASE.*

Figure 3.9: *Effect of Rapamycin, crowded litter and caloric restriction on AKT SER473 phosphorylation.* The level of AKT protein in 100 ug of liver whole cell extract from heterogeneous UM-HET3 male mice from all 4 experimental groups (CON, RAPA, CL, and CR) was determined by western blot analysis. The level of the phosphorylated form of AKT at SER473 residue was determined first. The membrane was then stripped and tested for Total AKT. The I.D.V (integrated density value) corresponding to the level of AKT protein (phosphorylated and total) was quantified by the BioRad Molecular Imager® System. P-AKT (ser473) was normalized to total AKT and expressed as p-AKT ser473 protein expression ratio. Values represent an average [S.E.M] for data obtained from 3 mice in each group and are representative of separate identical experiments. Different letters significantly different at P < 0.05. CON: Control Group; RAPA: Rapamycin Group; CL: Crowded Litter Group; CR: Caloric Restriction Group.

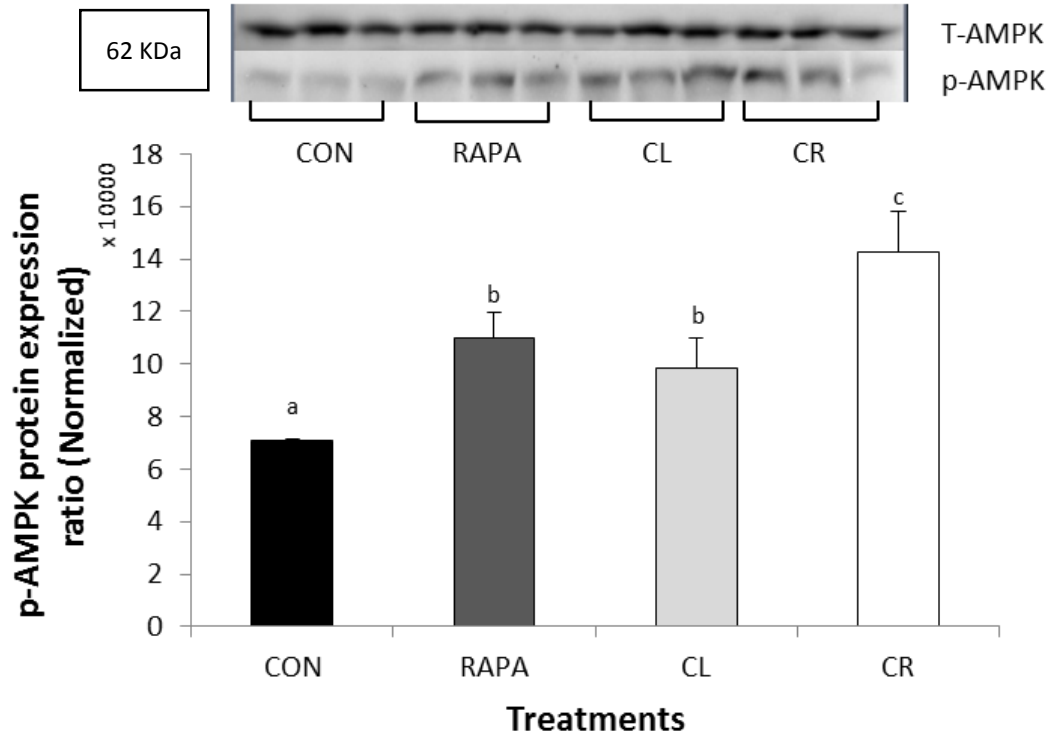Figure 3.9: *Effect of Rapamycin, crowded litter and caloric restriction on AKT SER473 phosphorylation.*

Figure 3.10: *Effect of Rapamycin, crowded litter and caloric restriction on 4EBP-1 phosphorylation.* The level of 4EBP-1 protein in 100 ug of liver whole cell extract from heterogeneous UM-HET3 male mice from all 4 experimental groups (CON, RAPA, CL, and CR) was determined by western blot analysis. The level of the phosphorylated form of 4EBP-1 was determined first. The membrane was then stripped and tested for Total 4EBP-1. The I.D.V (integrated density value) corresponding to the level of 4EBP-1 protein (phosphorylated and total) was quantified by the BioRad Molecular Imager® System. Phosphorylated 4EBP-1 was normalized to total and expressed as p-4EBP-1 protein expression ratio. Values represent an average [S.E.M] for data obtained from 3 mice in each group and are representative of separate identical experiments. Different letters significantly different at P < 0.05. CON: Control Group; RAPA: Rapamycin Group; CL: Crowded Litter Group; CR: Caloric Restriction Group.

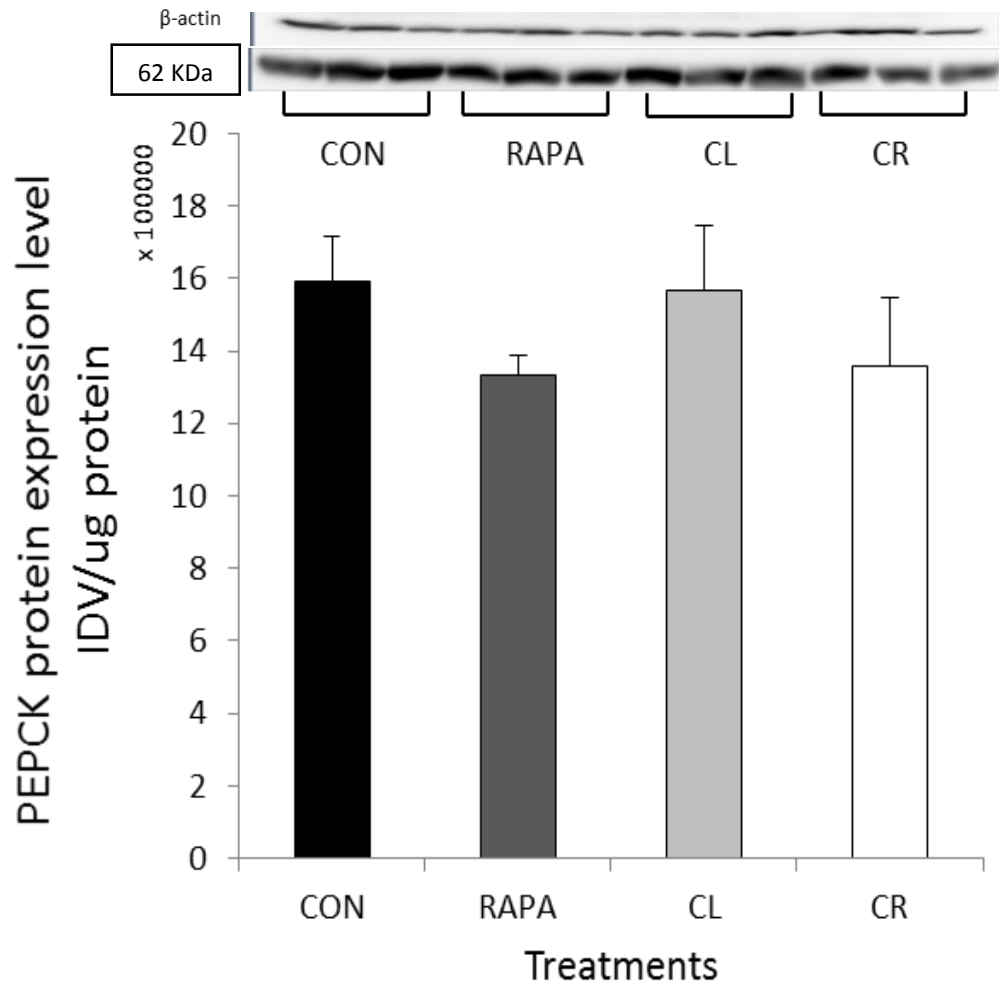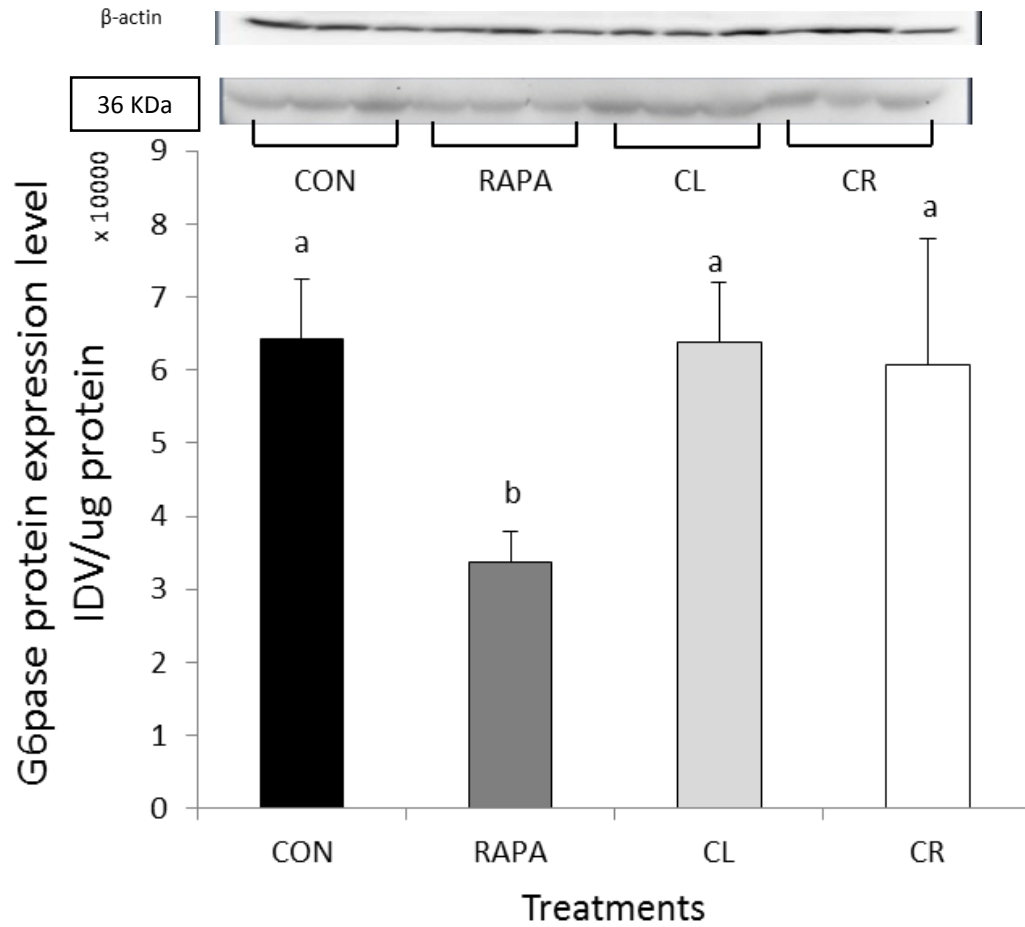Figure 3.10: *Effect of Rapamycin, crowded litter and caloric restriction on 4EBP-1 phosphorylation.*

Figure 3.11: *Effect of Rapamycin, crowded litter and caloric restriction on S6K1 phosphorylation.* The level of S6K1 protein in 100 ug of liver whole cell extract from heterogeneous UM-HET3 male mice from all 4 experimental groups (CON, RAPA, CL, and CR) was determined by western blot analysis. The level of the phosphorylated form of S6K1 was determined first. The membrane was then stripped and tested for Total S6K1. The I.D.V (integrated density value) corresponding to the level of S6K1 protein (phosphorylated and total) was quantified by the BioRad Molecular Imager® System. Phosphorylated S6K1 was normalized to total and expressed as p-S6K1 protein expression ratio. Values represent an average [S.E.M] for data obtained from 3 mice in each group and are representative of separate identical experiments. Different letters significantly different at P < 0.05. CON: Control Group; RAPA: Rapamycin Group; CL: Crowded Litter Group; CR: Caloric Restriction Group.

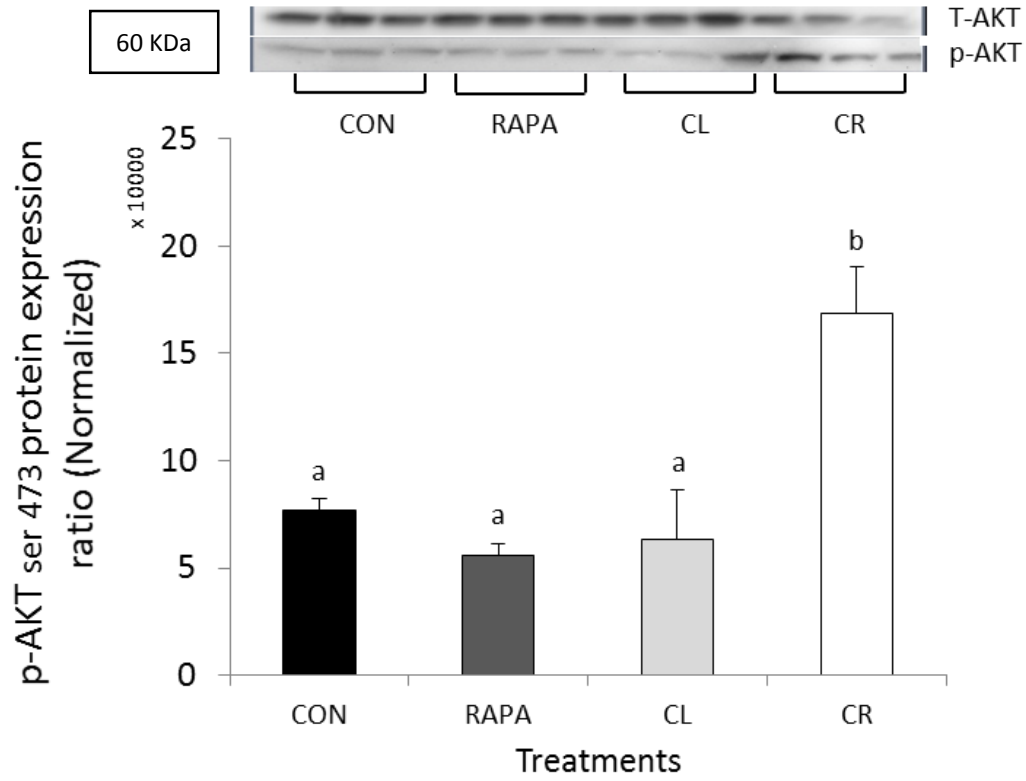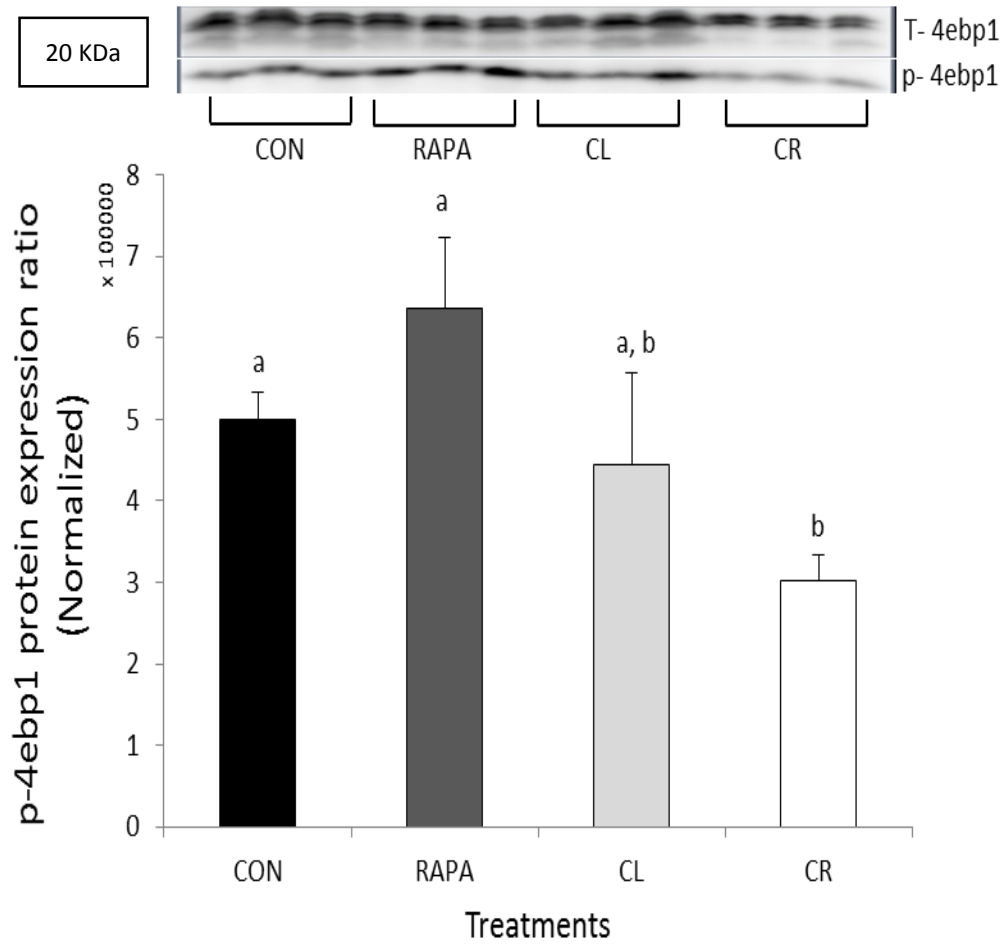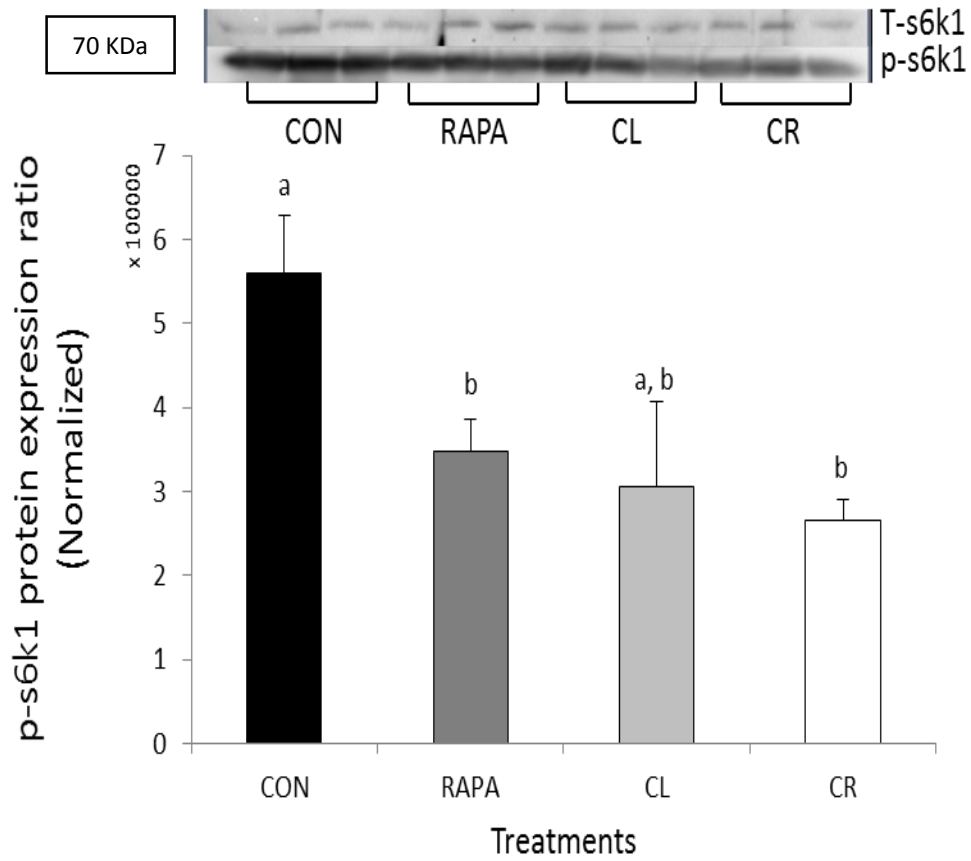Figure 3.11: *Effect of Rapamycin, crowded litter and caloric restriction on S6K1 phosphorylation.*

CHAPTER 4: SUMMARY AND FUTURE DIRECTIONS

Aging is a multifactorial process that is implicated with alterations in several physiological functions, such as loss of homeostasis and loss of functional capacity [1]. It is associated with many diseases including cardiovascular disease, cancer and type II diabetes mellitus [5]. Since these diseases are highly prevalent in the aging population, targeting the aging process as a whole may delay the occurrence of these age related disease, in turn delaying aging. Various interventions have been shown to delay aging including: caloric restriction [6], methionine restriction [9], genetic alterations such as Ames dwarf mice [13], rapamycin [35], and metformin [19]. These interventions inhibit a major nutrient sensing pathway, mammalian target of rapamycin (mTOR), a pathway shown to be altered in many cancers [26, 35-39]. Down-regulation of this pathway also delays aging in a number of organisms, ranging from yeast to mammals [27, 28, 38, 40-42]. Alterations in one carbon metabolism, a major pathway upstream of mTOR, also extends lifespan in animal models [70, 71]. Metformin, an emerging gerosuppresant, impairs one carbon metabolism in a manner similar to anti-folate drugs [21]. We have previously shown that folate restriction decreased markers of proliferation such as mTOR in our β-pol haploinsufficient background [31]. In this study, we present folate restriction as a possible nutritional intervention to extend lifespan and improve health span in our animal model, modulating the one carbon metabolism and the mTOR signaling pathway.

We established an animal model to study the impact of folate status in the diet on plasma folate levels. We showed that by feeding the animals a folate depleted diet (0 mg folic acid per kg of diet), their plasma folate levels dropped by the third day on the diet, and plateaued at a 90% decrease for the duration of the study. Since we did not observe any further decrease in plasma folate, and the animals did not show any signs of anemia, we termed this condition folate restriction

(FR) rather than folate deficiency. Low folate status has been associated with the development and progression of various types of cancer, however, some studies show that low folate may be protective against cancer [52-54, 77-79]. Inconsistencies in these studies may be due to differences in experimental design, dosages, timing and duration of folate deficiency. We showed that folate restriction did not result in the development of spontaneous preneoplastic lesions. However, when exposed to a carcinogen, the timing and duration of folate restriction had a differential effect on the development of ACF. When we placed the animals on one week of folate restriction before exposing them to a carcinogen, we saw a significant increase in preneoplastic lesions. However, when the animals were given time to adapt to folate restriction before we exposed them to a carcinogen, we saw significantly less ACF. We showed that folate restriction by itself is a low penetrance event and does not independently give rise to spontaneous preneoplastic legions. We also showed that the timing and duration of folate restriction before exposure to a carcinogen resulted in a differential effect on ACF formation. Future studies will focus on understanding the mechanism behind the differential effect seen due to timing and duration of folate restriction.

Folate restriction altered the one carbon metabolism and the mTOR signaling pathway, shedding light on the anti-proliferative and anti-cancer effects observed. We showed that folate restriction resulted in a decrease in branched chain amino acids that have regulatory effects on cellular proliferation and growth. Folate restriction also decreased ATP levels directly impacting energy metabolism, and activating AMPK. AMPK, a direct inhibitor of mTORC1, enhanced SIRT1 activity by increasing cellular levels of NAD^+, decreasing proliferation. Folate restriction downregulated the mTOR signaling pathway decreasing protein synthesis, as shown by the decrease in p-S6K1 and p-4ebp1. Furthermore, folate restriction reduced apoptotic activity, possibly slowing down the aging process as a result of modulation between reduced damage and

reduced proliferation. Folate restriction also increased median lifespan in our aging colony. In this study, we have identified a direct link between the folate cycle and the nutrient sensing mTOR pathway that converge on target genes, mainly activation of AMPK, impacting aging and cancer.

Insulin, Growth factors

PIP3

↓Redd 1

↓ Amino Acids   ↓ ATP   Glucose

↓ PTEN —| AKT↑

ADP

IPMK ↓

AMP ↑

TSC1 TSC2–Ⓟ ← ↑AMPK ← AICAR

Rheb GTP → Rheb GDP

↑NAD⁺

Metformin

Rapamycin ==|| ↓mTOR Raptor–Ⓟ ↓   ↑SIRT 1

↓4E-BP1   ↓S6K1 —| Mdm2 ↓ ←→ Mdm2 ↑

eIF4E   BAD ↓   p53 ↓

Anti-Cancer

Reduced Translation
Reduced cell Growth and Metabolism
Reduced survival
Anti-Cancer and Anti-Aging

cell-cycle arrest/apoptosis
Anti-Aging (resting phenotype)
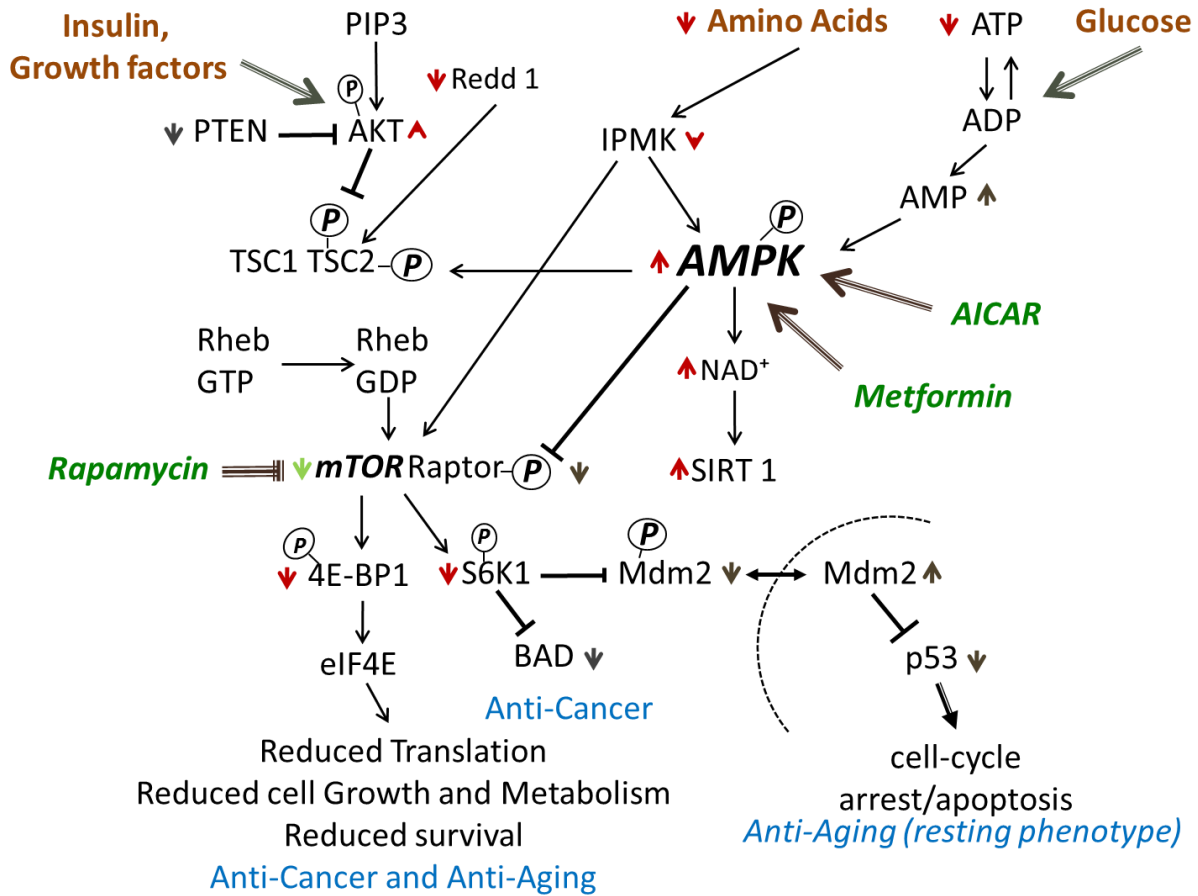
As a comparative analysis, we studied the impact of three known life extending strategies on the mTOR signaling pathway. We were given access to samples from a longevity study done at the University of Michigan as part of an Interventions testing program. The interventions included Rapamycin, crowded litter and caloric restriction which have all been shown to extend lifespan [5, 6, 42, 147-149, 152]. We showed that at 9 months of age, rapamycin and caloric restriction decreased expression of downstream targets of mTOR similar to folate restriction. However, when we analyzed the upstream regulators of mTOR, we saw a differential impact.

Although rapamycin, caloric restriction and folate restriction downregulated mTOR, the means by which they did differed. Crowded litter on the other hand did not impact the mTOR signaling pathway. We speculate that the transient food restriction early on in life triggered alternate pathways that provided this beneficial long lasting anti-aging effect irrespective of mTOR.

Future studies will include investigating the protective mechanism behind the longevity effect seen in the crowded litter group. We propose that the period of stress imposed by the transient food restriction early on in life may result in epigenetic changes that render these animals less susceptible to aging. It may also converge on the concept of mitohormesis, a response to mild mitochondrial stress that renders the cell less susceptible to subsequent perturbations, impacting susceptibility to disease and aging.

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ABSTRACT

THE IMPACT OF FOLATE RESTRICTION ON CANCER AND AGING: A MECHANISTIC ANALYSIS

by

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Aging is a multifactorial process associated with alterations in several physiological functions. It increases susceptibility to disease and ultimately results in mortality. Since the associated diseases of aging are highly prevalent in the geriatric population, targeting the aging process as a whole may provide a better way to delay these age related diseases, in turn delaying aging. Various interventions have been shown to delay aging and age related diseases. They impact a major nutrient sensing pathway, mTOR. mTOR signaling is altered in many cancers and its downregulation was shown to delay aging. Other interventions extend lifespan by altering the one carbon metabolism. In this study, we present folate restriction as a possible nutritional intervention to extend lifespan and improve health span impacting one carbon metabolism and the mTOR signaling pathway.

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