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Effect Of Long Term Rapamycin Treatment On Mtor Signalling Network In Colon And Liver Of C57bl/6 Mice

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EFFECT OF LONG TERM RAPAMYCIN TREATMENT ON mTOR SIGNALLING NETWORK IN COLON AND LIVER OF YOUNG AND OLD C57BL/6 MICE

by

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_____

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DEDICATION

I would like to dedicate this thesis to my family.

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CHAPTER 1: INTRODUCTION

Nutritionists and molecular biologists have been interested in nutrigenomics to further understand the study between food and its encompassed nutrients within the genome. Cellular processes within the body can be analyzed based on diet composition, which can subsequently be targeted for alteration, leading to the expression of desired phenotypes. Dietary folate has is growing to be a topic of interest in nutrigenomics. It has been implied that folate intake has led to an increase in risk of various melanomas within liver, stomach, pancreas, colon, and the breast [1]. Although the initiation and progression of these tumors within these tissues due to folate status has not been fully defined [1], it has been implied that a diet deficient in folate may increase the likelihood of developing cancer in some tissues [2]. Worth noting is that folate deficiency may provide protection against cancer in mice with manipulated base excision repair (BER) mechanisms (β-polymerase haploinsufficiency), a pathway known to mask oxidative stress [3]. It is believed that folate deficiency may influence cancer development on the level of the mammalian target of rapamycin (mTOR) pathway. Rapamycin is a drug that targets mTOR, which is overly expressed in many cancers [4]. There is evidence indicating that rapamycin supplemented later in life may extend lifespan and provide protection against some cancers [5]. Although, there have not been any studies that have investigated the long-term effects in the supplementation of rapamycin in a cancer model. Similarly to folate deficiency providing a protective measure in mice, it is important to provide a protective measure against cancers that are prevalent in models without BER impairment. Hence, it is important to investigate the role of rapamycin and its long-term

supplementation prior to induction of cancer to see if it provides a protective/preventative mechanism in cancer models that do not have impaired BER pathways.

A. Folate

Although folate, or folic acid, is found in copious amounts within plant-derived foods, this water-soluble B-vitamin is commonly under-consumed in people that find themselves indulging in diets that neglect fruits and vegetables [6]. Folate deficiency (FD) during pregnancy is known to play a pivotal role in the development of birth defects and other chronic diseases, such as cardiovascular disease, Alzheimer's disease, and various cancers. FD has been known to increase the levels of single strand breaks [7,8,9,10,11], chromosomal aberrations [12,13], and mutational frequency [14,15]. Recently, studies imply that folic acid restriction may protect against some cancers [3]. Folic acid's roles extend to being an integral participant in the metabolism of amino acids and nucleic acids, where it acts as a coenzyme mediator, transferring methyl groups between substrates [16]. It is also a major contributor in the maintenance of the regulation of cellular division [16].

Folate that can be found in food is in the pterolypolyglutamate form. Within supplements, it is found in the monoglutamate folic acid form [16]. Folate has been fortified in most grains and cereal products within the United States and Canada.

Neural tube defects can occur in a developing fetus if the mother does not take in folate in sufficient amounts during pregnancy. A destruction of neural tissues occurs when the brain and spinal cord are left susceptible because of a failure in closure of the neural tubes [17].

In terms of cancer development, studies have shown that folate status has an impact on the development of cancer. It is believed that diets high in fruits and vegetables, which contain folate, will aid against the development of cancer [18]. Being that folate is involved in two majorly up-regulated processes, DNA synthesis and DNA methylation, it is believed that folate may exemplify its protective role through these pathways [19]. This involvement of folate is still being investigated. On the contrary, excess folic acid has also been linked to enhance the development of pre-existing tumors [19]. Folate deficiency in β-polymerase haploinsufficient mice compromised for the BER pathway has shown to stimulate the ability to reduce tumor progression and size [3].

Other than DNA repair and cellular division, one function that folate is involved in is the regulation of one-carbon metabolism [20]. The mechanism of one-carbon metabolism is displayed below:

Figure 1.1: Folate Metabolism [21]

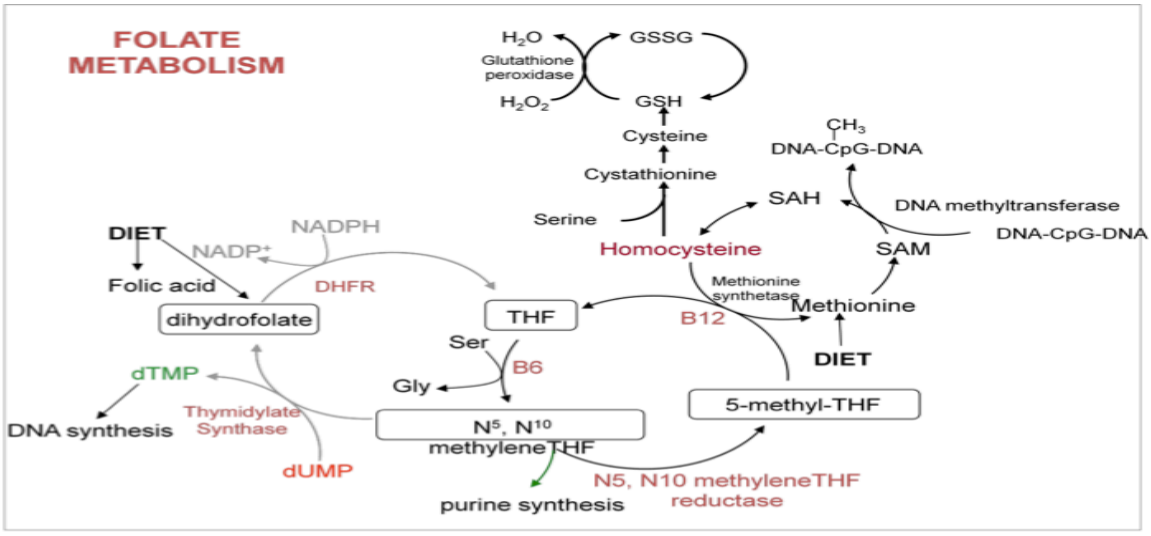


Figure 1.1 Folate Metabolism: The depicted figure is described in the below paragraph.

From the **Figure 1.1**, folate is shown as 5-methylenetetrahydrofolate (5-methylTHF). Methylenetetrahydrofolate reductase (MTHFR) is an enzyme whose function is to

convert 5,10-methylene THF to 5-methyl THF irreversibly. One can observe now how DNA repair and cellular division are processes that involve folate: DNA repair and cellular division is linked through the generation of purines and thymidylate through this process. When folate is deficient, this pathway is altered, compromising these processes [21].

B. Folate deficiency in DNA polymerase β haploinsufficient mice: Cancer

A study testing folate deficiency on haploinsufficient mice for DNA polymerase beta (β-pol$^{+/-}$) in the BER pathway showed that the progression of tumors in these mice was halted and the size was minimized [3]. Lucente et al. showed that in wild type (WT) mice treated with 1,2-dimethylhydrazine (DMH), a FD diet resulted in an accelerated generation of aberrant crypt formations (ACFs). In mice haploinsufficient for DNA polymerase β, an integral enzyme in the base excision repair pathway (BER), a FD diet resulted in reduced ACF onset and progression as well as prevented liver tumorigenesis. Amanda Arrabi's published data from Dr. Ahmad Heydari's laboratory at Wayne State University suggests that a FD diet in DNA polymerase β haploinsufficient mice further supports Lucente et al.'s work. Arrabi qualifies the proteins affected throughout the mTOR network. In her findings, β-pol$^{+/-}$ FA fed mice hit with DMH compared to their wild type controls developed significant colon carcinogenesis. She tested older mice as well. In these experiments, old β-pol$^{+/-}$ mice fed FA diets were also not able to sustain the entire DMH treatment process as most died before completion of the treatment. On the contrary, β-pol$^{+/-}$ mice fed FD diets and hit with DMH did survive the entire treatment, indicating the presence of a protective mechanism in the β-pol$^{+/-}$ mice from the development of cancer when folate is lacking in the diet. Arrabi's protein analysis shows

significant differences in pathways involving mTOR's activity, cellular proliferation and endurance [22], energy sensing [23], and amino acid level sensing [24], and the regulation of apoptosis [29].

Just recently, unpublished data involving FD diets supplemented long-term in wildtype mice has also mimicked the above conditions.

C. Folate Deficiency in DNA β Haploinsufficient Mice: Aging

To my understanding, there are no long term studies that have been completed that supplement rapamycin and investigates cancer simultaneously. This is the first. Although this is not a primary aging study, previous aging studies that experiment with folate deficiency and investigates cancer may bring some clarity to the results that will be observed with this study. One study in particular was performed by Lucente et al.. This study investigated the effect of FD on wild type (WT) and β-pol$^{+/-}$ mice. WT mice showed that when subjected to FD diets, ACFs were increased after treatment with DMH. It was also found that in β-pol$^{+/-}$ mice, FD diets showed protection and prevention of further advancement of ACFs and a halt in tumorigenesis. Since β-pol$^{+/-}$ mice are BER impaired and positively affected by this FD phenomenon, one could raise the question, "What treatment would yield similar results in WT mice treated with DMH with normal folate diets?" This study investigates this question.

D. mTOR Signaling pathway and general network

The mTOR signaling network can be seen below although the pathway is not completely understood [4]:

Figure 4.2: mTOR Signaling Pathway

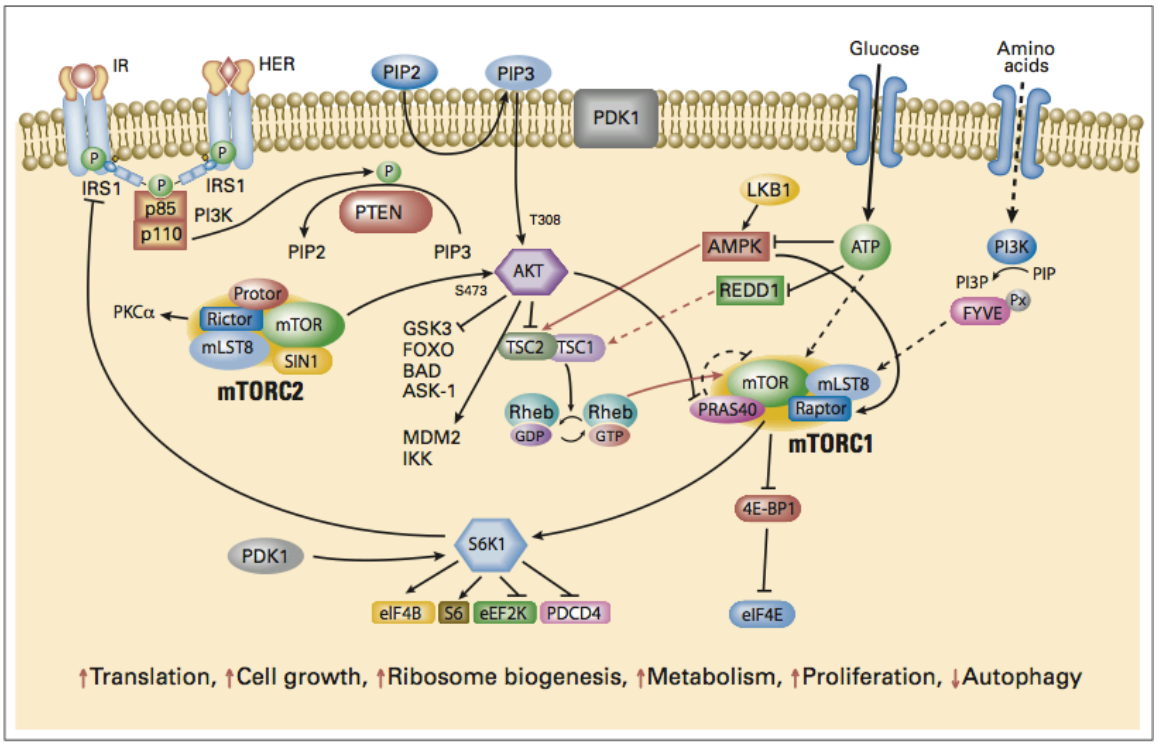


Figure 1.2 mTOR Signaling Pathway: The mTOR signaling pathway is described below.

The function of mTOR is to increase translation, cellular growth, proliferation and cellular metabolism. Apoptosis is a mechanism for which it downregulates [4]. mTOR consists of two multiprotein complex forms: mTORC1 and mTORC2. mTOR1 has been recognized as the rapamycin-sensitive form while mTORC2 has been recognized as the rapamycin-insensitive form, until recently where longer duration of rapamycin treatment has shown that it is actually effected as well [26]. mTORC1 is composed of mTOR, mammalian LST8 (mLST8), proline-rich Akt substrate 40 (PRAS40), and raptor [27]. The targets of mTORC1 are 4E-BP1 and S6K1. mTORC2 is composed of proteins mTOR, mLST8, mSIN1, PRR5, and rictor [28,29,30,31,32]. mTORC2 targets Akt at the Ser473 for phosphorylation and further regulates Akt activation indirectly by activating integrin-linked kinase, which acts on Akt. The full activation of Akt through mTORC2

induces Akt to inhibit FOXO (associated with longevity and apoptosis) and BAD (an apoptosis regulator), promoting cellular proliferation and tumor formation. Furthermore, Akt may also act on PKCα, which may promote tumor proliferation and generation.

The regulation of mTOR is influenced through growth factor signaling, nutrient signaling, and energy status [4]. Other protein signaling also regulates mTOR. PI3K/Akt and Ras/MAPK signaling promotes mTORC1's activity by inactivating TSC2 through phosphorylation, which subsequently would have acted to inhibit Rheb to decrease mTORC1's activity [33,34,35,36]. Cellular energy has an effect on the protein AMPK. When cellular energy is low, protein synthesis is inhibited. AMPK does this by phosphorylating TSC2, which subsequently leads to the inactivation of mTORC1. The way amino acids act to influence mTOR signaling is not entirely defined [37].

E. Rapamycin

Rapamycin, also known as Sirolimus, is a macrolide drug containing a macrolide ring that was disovered to be produced by the bacteria *Streptomyces hygroscopicus.* The original use for rapamycin was as an antifungal agent but this has long been attenuated as it was discovered that rapamycin prolonged the lifespan of mice and showed beneficial results in the prevention of some cancers [4,5].

Rapamycin targets the mechanistic target of rapamycin (mTOR). Specifically, it binds to mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2) (after longer exposures) [38]. Clinically, rapamycin typically acts as an immunosuppressant. It has been used in the treatment/prevention of transplant rejection. Additionally, it is used to coat coronary stents. Its anti-proliferative properties allow it to prevent the reoccurrence of narrowed coronary arteries [39].

There are a few adverse affects worth noting about rapamycin. It has been observed that rapamycin may cause lung toxicity. Particularly, patients with lung transplants have developed complications, such as interstitial pneumonitis, when treated with rapamycin as a prevention of lung transplant rejection [40]. The exact mechanism of which this occurs is not well defined. Another complication of rapamycin treatment is the possible induction of diabetes. When rapamycin targets mTORC2, diabetes like symptoms, such as insensitivity to insulin, occur [41].

F. Rapamycin and its Effects on Aging

Rapamycin has had differential effects on aging throughout literature. Harrison et al., 2009, Selman et al., 2009, Chen et al., 2009, and Anisimov et al., 2011 [5,42,43,44], show an extension in longevity in mice and knockout mice fed or injected with rapamycin. Alternatively, rapamycin treatments according to Houde et al., 2010, Chang et al., 2009, and Fraenkel et al., 2008 [45,46,47], show opposite results. These results include insulin resistance, hyperlipidemia, and glucose intolerance – all of which are associated with shortened longevity. These contrasting results signify a paradox. The question arose, "What could be the reason for these contrasting results?" The differences in these studies reside in the duration of rapamycin treatment that was performed.

G. Duration of Rapamycin Treatment

Fang et al., [5] showed that varying rapamycin treatment duration produced contrasting results. Their results showed that in mice, shorter duration treatments of rapamycin are detrimental to mouse quality of life while longer duration treatments enhance the mouse quality of life and extend longevity. Three rapamycin treatment periods were described in this experiment (2, 6, and 20 weeks respectively). Mice were

injected with rapamycin and subsequent analyses were performed. It was found that the mice subjected to the 2 and 6-week treatment periods, adiposity, body weight, and food consumptions were not altered (they did not improve). Alternatively, the 20-week treatment showed reduced levels in adiposity, body weight, and food consumption. Pancreas and liver size decreased and increased, respectively, in mice subjected to the 2-week treatment, which are symptoms of metabolic syndrome. Pancreas and liver size were restored (according to controls) in the 20-week treatment.

Insulin stimulates the uptake of glucose from the bloodstream. Subsequently, glucose can act as a building block in gluconeogenesis or lipogenesis. When individuals lose sensitivity to insulin, the gluconeogenesis process is impaired but the process of lipogenesis tends to remain unaffected [48]. It was found in this study that a 2-week rapamycin treatment increased insulin levels by 2.5fold compared to controls, which later lead to insulin-insensitivity. The 20-week rapamycin treatments lead to decreased levels of insulin with more sensitivity, thereby, having a restoring effect.

Lipid levels and metabolism was also correlated with duration of rapamycin treatment. High levels of insulin provide conditions stimulating lipogenesis and inhibit lipolysis. In the 2-week and 6-week rapamycin studies, there were high levels of insulin. Fang et al. found that this explained why there were lower levels of glycerol and fatty acids and higher levels of triglycerides within these experimental groups. With the 20-week experimental groups, there were lower levels of insulin associated with the prolonged rapamycin treatment. Accordingly, triglyceride levels dropped without an increased in non-esterified fatty acids, signifying a shift from lipogenesis to lipolysis and an improvement of metabolic activity [48].

Energy expenditure in the form of enhanced oxygen consumption (VO_2) was also shown in this study. This was done to show that fatty acid consumption took precedent over carbohydrate consumption at the 20-week mark of treatment. The 2-week rapamycin treatment mice showed to have lower VO_2 than their respective control, suggesting that they expend less energy than their controls. The 20-week rapamycin treatment showed to have higher VO_2 than their respective control, suggesting that they expend more energy than their controls. These findings were consistent with Cunningham et al. 2007 and Polak et al., 2008, who experimented with Raptor knockouts, indicating a shift from carbohydrate to fatty acid metabolism in the 20-week treatment [49,50]. To further verify that fatty acid breakdown was occurring, Fang et al. measured the formation of ketone bodies, as they are related to increased fatty acid breakdown. They found that there were higher concentrations of ketone bodies in the 20-week treated mice compared to those only treated with rapamycin for 2 and 6 weeks, respectively.

Hughes and Kennedy, suggested that mTORC1 and mTORC2 work together to balance the impact on insulin-sensitivity [51]. Their results are in accordance with Lamming et al.'s research in that hepatic mTORC2 mediated rapamycin-induced insulin resistance [52]. Later, in Lamming et al.'s study, a short rapamycin treatment of mice (2 to 4 weeks) that had hepatic deletion for Rictor (an important component of mTORC2) were found to exhibit large increases in insulin and glucose levels. Fang et al., show that the levels of phosphorylation of mTOR, S6K1, 4EBP1, and AKT actually increase with increasing duration of rapamycin treatment (20 weeks). On the contrary, Fang et al. show that blood levels of rapamycin are constant (with all durations of rapamycin treatments) and the phosphorylation of S6 does not seem to exhibit this reverse profile with

increasing the duration of treatment with rapamycin. Furthermore, it seems as though this reverse profile of rapamycin treatment is beneficial being that in improves insulin-sensitivity. Fang et al., has also shined some light on the direction of the response with increased duration of rapamycin treatment. mTORC2 may regulate the increase in expression of mTOR, S6K1, 4EBP1, and AKT while some other mechanism may repress the expression of S6 upon increased duration of exposure to rapamycin treatment.

H. Rapamycin and its Effects on Cancer

Many tumor types can develop through the over-activation of mTOR. A common mode of deregulation of mTOR is through the over expression of phospatidyl-inositol 3'-kinase (PI3K)/Akt signaling pathway. Over expression of growth factors, mutations of regulatory genes (such as PTEN – an inhibitor of the PI3K/Akt pathway), or amplification of genes can lead to the over expression of this pathway [53,54,55,56].

Rapamycin is known to administer its effectiveness against tumor progression in that it can cause cell cycle arrest. Additional to this halt in the cell cycle, it has also been recognized as an endothelial cell proliferation inhibitor as well as an inhibitor of hypoxia inducible factor 1, VEGF expression, angiogenesis, and vascular permeability [57,58]. mTORC1 is sensitive to rapamycin treatment while mTORC2 is known to be insensitive to rapamycin. Notable exceptions causing mTORC2 to be sensitive to rapamycin treatment (as said previously) are associated with the duration of exposure to rapamycin. Rapamycin exposure has also been identified to increase Akt activation in some models [59,60]. Insulin-like growth factor I (IGF-I) and insulin-dependent induction of the PI3K/Akt pathway is not able to be negatively regulated because rapamycin inhibits this

action through inhibition of mTOR. The mTOR/S6K-mediated phosphoylation of IRS-I provides this negative feedback loop [59,60].

Rapamycin's effectiveness to prevent Akt's activity may also depend on its dosage. Lower dosages seem to promote Akt's activity while higher dosages prevent it [58,61]. The higher dosages may be due to rapamycin's inhibitory effect on mTORC2. mTORC2 directly phosphorylates Akt at Ser473, activating it [62,63,64]. Higher dosages may inhibit mTORC2, preventing Akt from reaching its fully activated form, slowing down the Akt/mTOR pathway.

I. Clinical Significance and Adverse Effects of Rapamycin

Generally, the toleration of rapamycin and its respective rapalogs (including sirolimus, everolimus, and AP23573) has been well through various clinical trials. Toxicities of this drug has been associated with higher dosages and resulted in symptoms including asthenia, mucositis, nausea, cutaneous toxicity, diarrhea, hypertriglyceridemia, thrombocytopenia, hypercholesterolemia, elevated transaminases, hyperglycemia, and pneumonitis [65,66,67,68].

Rapamycin is also shown to be beneficial in treatment of diseases such as Alzheimer's disease, muscular dystrophy, and systemic lupus [4]. Neurofibromatosis, Cowden's Syndrome, and tuberous sclerosis are currently being investigated through clinical trials [4].

CHAPTER 2: SPECIFIC AIMS

The mTOR signaling pathway is commonly deregulated in many cancers. It is important to investigate its mechanisms of deregulation. Of more importance is to investigate how to restore functionality and regulation of the mTOR pathway. With this answer, many cancers can be prevented from occurring, prevented from spreading, hindered, and/or cured. We hypothesize that a long-term rapamycin diet will provide protection against the progression of cancer in mice. Additional to this, we hypothesize that there is a resemblance between the mechanisms in which FD acts to provide protective means against cancer in BER impaired mice and the way a long-term supplementation of rapamycin on normal mice acts to provide protection against cancer in wildtype mice.

Specific Aim 1:

To investigate the effect of long-term rapamycin supplementation on ACF in response to DMH in young and old mice

Specific Aim 2:

To determine the impact of long-term rapamycin on the mTOR signaling pathway in response to DMH in young and old mice.

CHAPTER 3: METHODS

A. Animals

Experiments were performed in young (9 months) and old (20 months) C57BL/6-specific pathogen-free male wildtype mice for 28 weeks. All practices performed on animals were in agreement with the National Institutes of Health guidelines for the care and use of laboratory animals. The Wayne State University Animal Investigation Committee approved the animal protocol. Mice were maintained on a 12-hour light/dark cycle and given water and food *ad libitum* [3].

B. Diets and Carcinogenic Treatment

After acclimation, the mice were put into 4 experimental groups: 1) young mice with control diets lacking rapamycin (YC), 2) young mice with diets incorporating rapamycin (YR), 3) old mice with control diets lacking rapamycin (OC), and 4) old mice with diets incorporating rapamycin (OR). Rapamycin diets were prepared according to Harrison et al. [26]. The Southwest Research Institute (San Antonio, Texas) microencapsulated rapamycin so that the agent can be protected against stomach digestion. The coating also provided the agent to be prevalent in the finished food product by three- to fourfold more. Encapsulated rapamycin was then incorporated into Purina 5LG6 mouse chow and distributed to our testing facility. Diets were stored at -20^{o}C.

Diets were given to the control and experimental groups for 4 months before injections started. At the four-month mark, all mice were injected with 1,2-dimethylhydroazine (DMH, 30mg/kg body weight) in 10mmol/liter of $NaHCO_3$ (Fisher Scientific) once per week for 6 weeks. Both food intake and body weights were checked

throughout the course of the study to monitor for signs of toxicity (i.e. weight loss). The diets continued up to 28 weeks [3].

C. Aberrant Colonic Crypt Formation (ACF) Analysis

Animals were anesthetized through CO_2 asphyxiation. Mice were sacrificed through cervical dislocation. Immediately, abdominal cavities were opened and the colon excised, risned with cold phosphate-buffered saline, cut longitudinally, and fixed flat overnight in 10% neutral buffered formalin. The colonic crypts were stained with 2g/liter and methylene blue in phosphate-buffered saline for 5 minutes. The number of ACF and aberrant crypts per foci were determined by light microscopy at x10 magnification in a blinded manner [3]. Livers were also excised and stored at -80^{o}C in liquid nitrogen until Western Blot experiments began.

D. Western Blot Analysis and Statistical Analysis

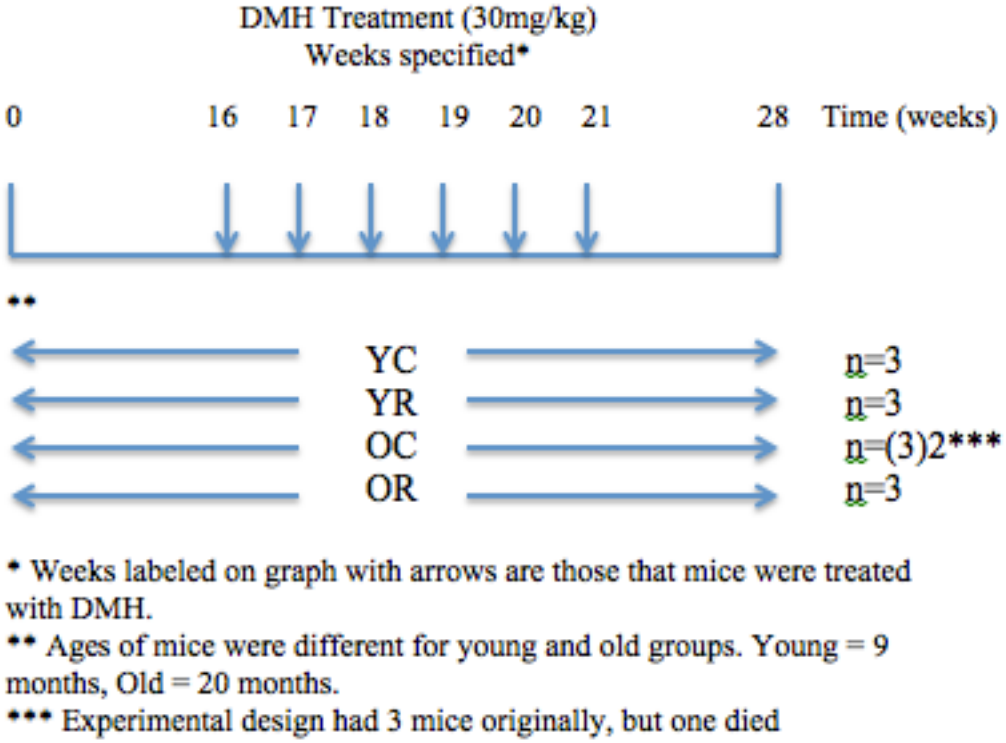
Western Blot analysis was performed on liver tissue normalized to 10μg of sample as previously described in Unnikrishnan et al. 2009 [74]. Samples were subjected to polyacrylamide gel electrophoresis. Staining of gels using GelCode blue stain reagent (Pierce Biotechnology) ensured consistent protein concentrations in all wells additional to the Bradford Assay performed through normalization. Western analysis was accomplished using affinity purified polyclonal antisera developed against mouse target proteins. As an internal control for protein loading, membranes were reprobed with anti-Lamin B antibody (Santa Cruz Biotechnology, santa Cruz, CA). The bands were visualized and quantified using a ChemiImager^{TM} System (AlphaInnotech San Leandro, CA) after incubation in SuperSignal$^{®}$ West Pico Cemiluminescent Substrate (Pierce

Biotechnology). Data are expressed as the integrated density value of the band per μg of protein loaded [3].

Statistical significance between means was determined using an "unpaired t-test." *P-values* less than 0.05 were considered statistically significant.

CHAPTER 4: FIGURES

Figure 4.1: Diet and DMH Treatment Schedule of Mice



• Weeks labeled on graph with arrows are those that mice were treated with DMH.

•• Ages of mice were different for young and old groups. Young = 9 months, Old = 20 months.

••• Experimental design had 3 mice originally, but one died

Figure 4.1 Diet and DMH Treatment Schedule of Mice: The above figure displays the number of weeks the respective diets took place (28 weeks). The arrows represent the respective weeks for which the mice were treated with 1,2-dimethylhydrazine (DMH). DMH was injected intraperitoneally in 30mg/kg body weight amounts. The control groups are labeled as Young Control diet (YC), Young Rapamycin diet (YR), Old Control diet (OC), and Old Rapamycin diet (OR). Young and old mice started diets at different ages, explained in the figure. Mice were sacrificed at week 28 via CO_2 asphyxiation. Protocol explained in the methods section.

Figure 4.2: Amount of Diet Eaten over the Course of Experiment

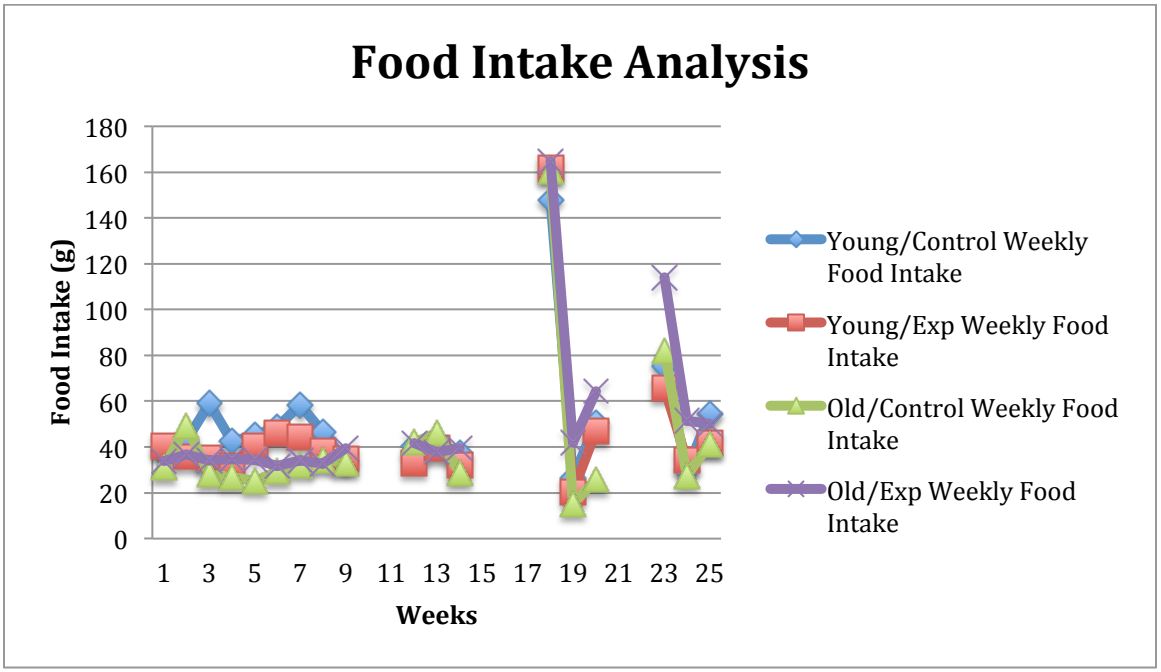


Figure 4.2 Amount eaten over the course of the experiment: Data for food intake analysis was recorded for 25 weeks although the diet persisted for 28 weeks. All mice showed the same eating habit trends. Gaps and spikes are due to lack of data being present in weeks prior to spikes. Purina 5LG6 mouse chow with rapamycin was used for mice on experimental diets. Purina 5LG6 mouse chow was used for mice on control diets. Diets are described in detail in the "Method" section.

Figure 4.3: Weight of young animals on control and experimental diets

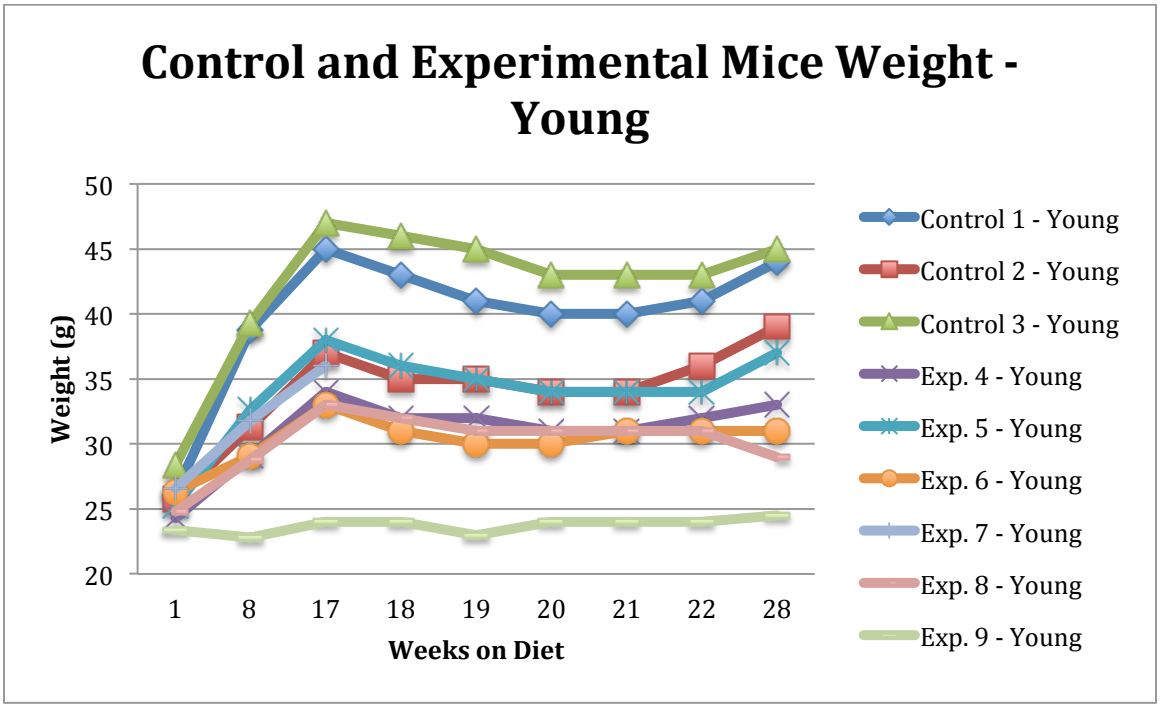


Figure 4.3 Weight of young animals on control and experimental diets: On average, the young control mice are of higher weight than the young experimental weight. At week 16 after being on the diet, the mice were hit with a carcinogen (DMH). The weights subsequently dropped – eventually to restore their weight at 28 weeks. Experimental mouse 9 is much lesser in weight than all other mice. Data in subsequent experiments did not incorporate mouse 9 due to its being an outlier.

Figure 4.4: Weight of old animals in control and experimental diets:

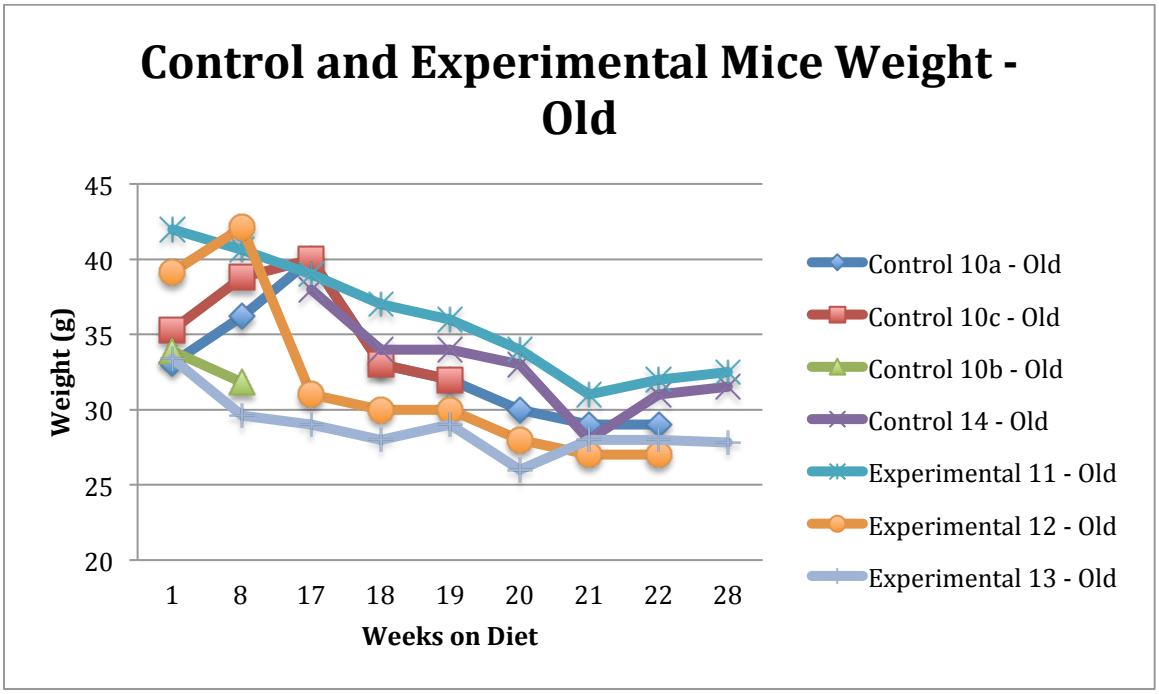


Figure 4.4 Weight of old animals on control and experimental diets: Mouse 10b died at 18 weeks. Weights for the old experimental mice were a bit less than control mice after injection with DMH (with the exception of experimental mouse 11). All weights of mice dropped after DMH injection. Weights were taken up to 28 weeks of being on the respective diets until animals were sacrificed and tissues were stored in liquid nitrogen, readied for subsequent analysis. Mouse control 10c is the same as mouse control 14. This mouse was moved to a different cage after control 10b died.

Figure 4.5: ACF analysis of control and experimental mice

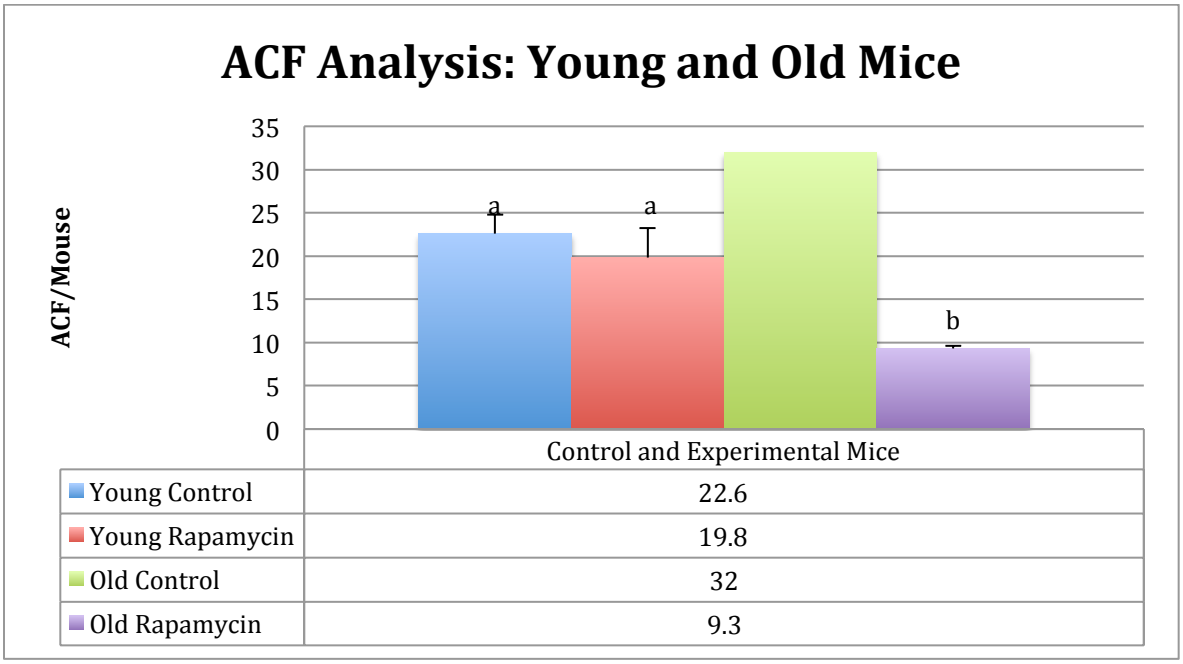


Figure 4.5 ACF analysis of control and experimental mice: This figure displays the impact of rapamycin on ACF formation in colon of mice injected with DMH. There is no significant difference between control mice and their respective experimental counterparts. Data in this figure for old control mice only incorporates two mice, thus significance cannot be accurately determined. Colons were processed and stored as described in "Methods." Colons were analyzed under light microscopy to visualize the number of ACF per mouse colon (ACF/Mouse). Statistical significance was deemed to be non-significant in the young mice. T-Test = 0.490464756.

Figure 4.6: Crypt multiplicity analysis of young and old mice

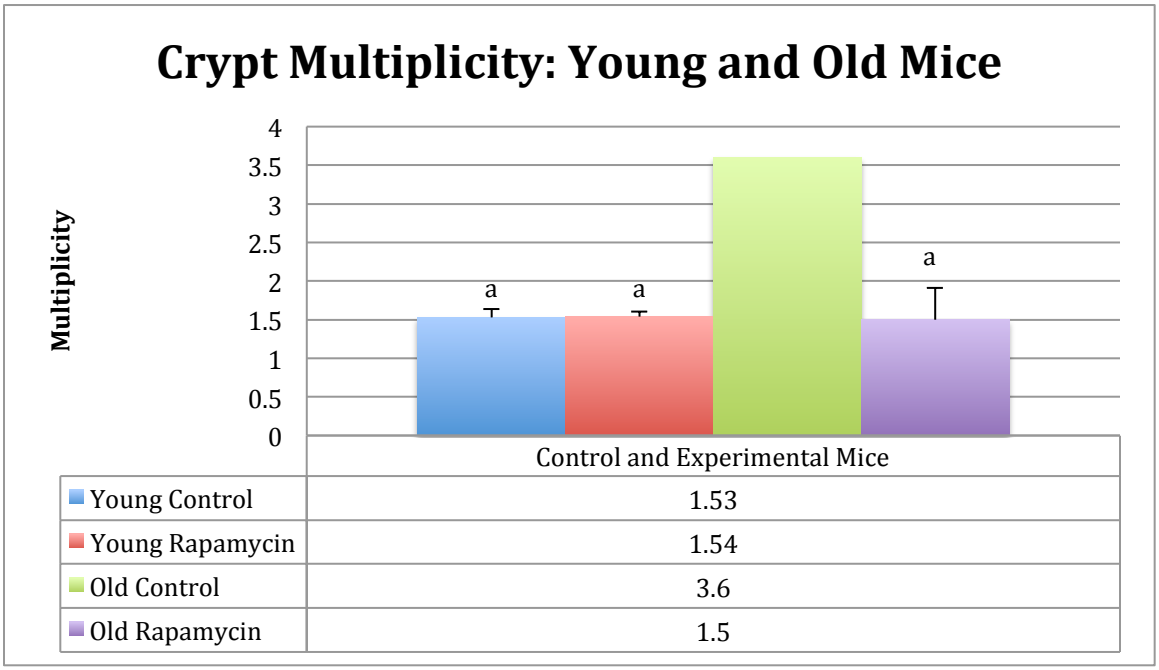


Figure 4.6 Crypt multiplicity analysis of young and old mice: The figure displays the development of ACFs in the respective control and experimental groups. Group Old Control only has data for two mice and can therefore not be considered for statistical analysis. Crypt multiplicity does not show any significant difference between young groups and old groups. Statistical significance was deemed to be non-significant in the young mice. T-Test = 0.938927271.

Figure 4.7: Effect of rapamycin diet and DMH treatment on protein levels of p-S6K in young mice

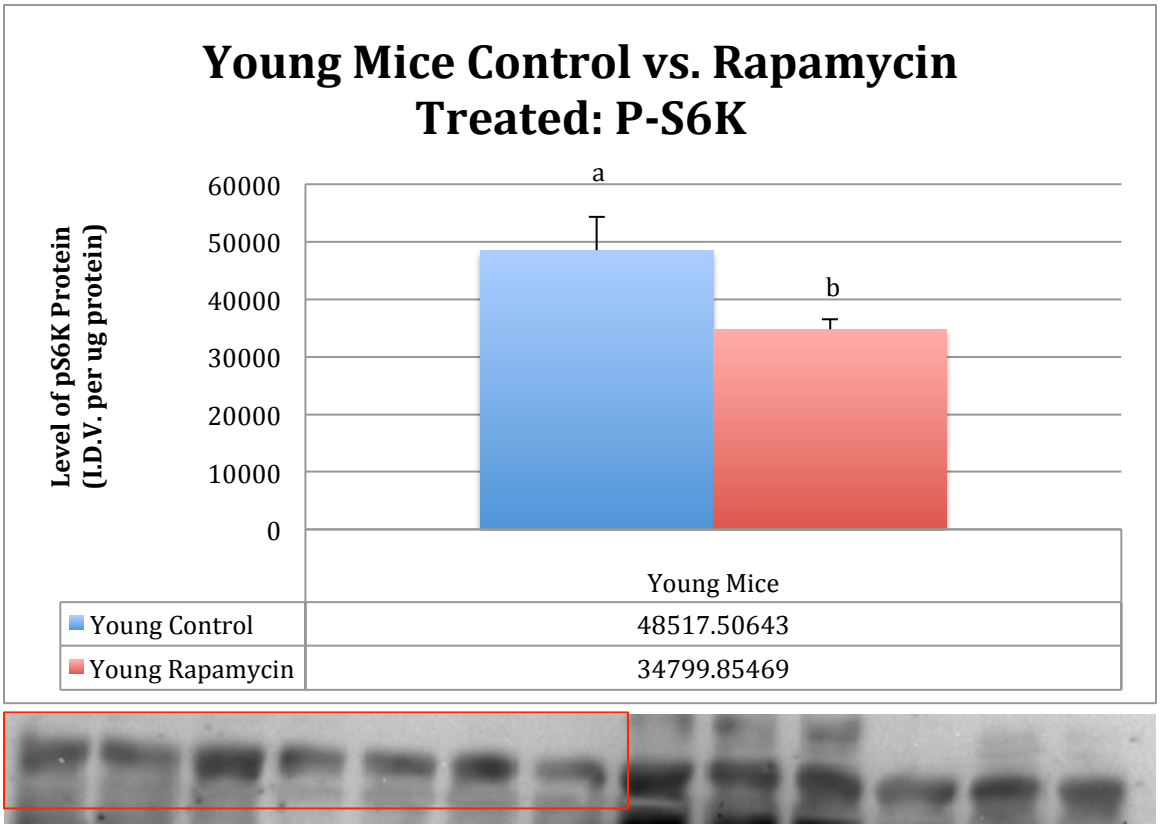


Figure 4.7 Effect of rapamycin diet and DMH treatment on protein levels of p-S6K in young mice: In the above figure, an analysis of p-S6K protein levels in liver of young wildtype mice is shown. Mice in both control and experimental groups were treated with intraperitoneal injections of DMH. Experimental mice were given rapamycin diets as described previously while control mice were given the same diets without rapamycin. Intraperitoneal treatment with DMH was done at week 16 for 6 weeks at 30mg/kg body weight, once per week. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 3 mice of group "Young Control" and 4 mice from group "Young Rapamycin," shown in sequence within the red box, and are representative of separate identical experiments. Values with different letter superscripts indicate significant differences ($p<0.05$). Levels of p-S6K show significant difference comparing control vs. experimental diets. T-test = 0.02447293.

Figure 4.8: Effect of rapamycin diet and DMH treatment on protein levels of p-S6K in old mice

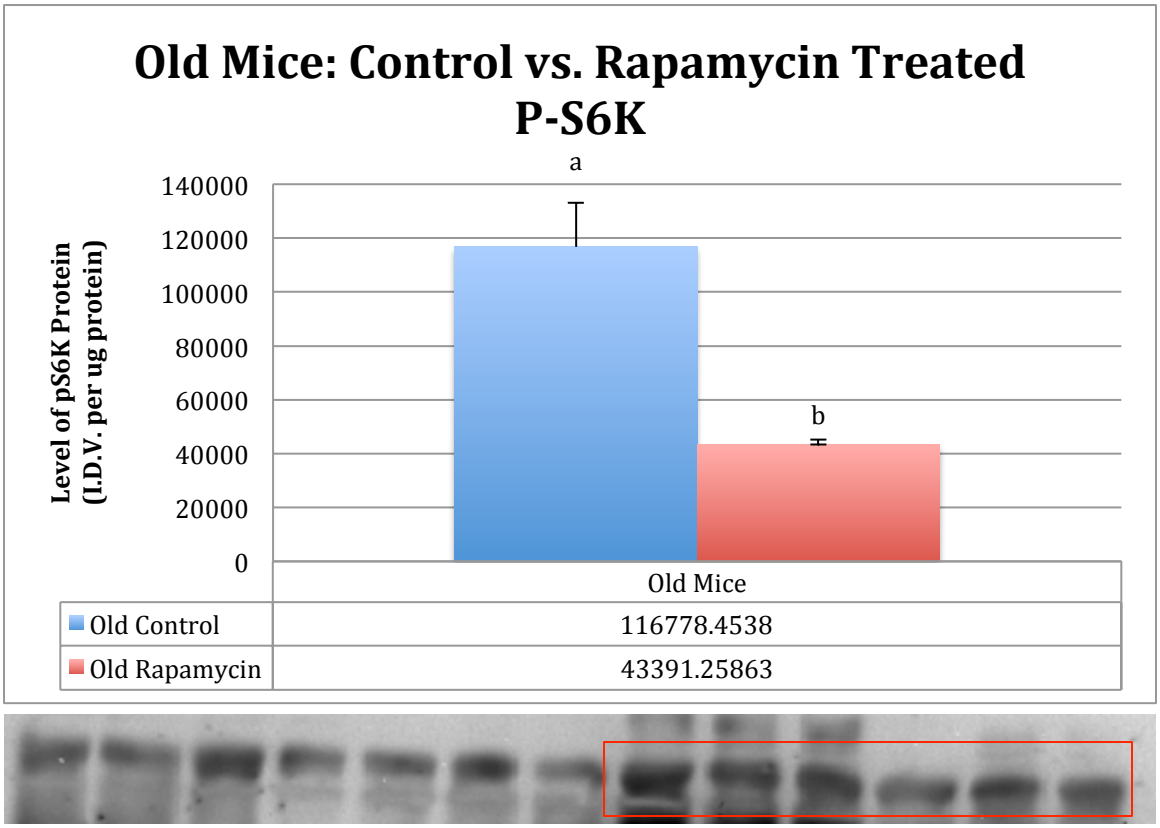


Figure 4.8 Effect of rapamycin diet and DMH treatment on protein levels of p-S6K in old mice: In the above figure, an analysis of p-S6K protein levels in liver of old wildtype mice is shown. Mice in both control and experimental groups were treated with intraperitoneal injections of DMH. Experimental mice were given rapamycin diets as described previously while control mice were given the same diets without rapamycin. Intraperitoneal treatment with DMH was done at week 16 for 6 weeks at 30mg/kg body weight, once per week. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 3 mice of each group, shown in sequence within the red box, and are representative of separate identical experiments. Group "Control Old" only has two mice due to an unexpected death of one of the mice. Values are represented in triplicate for this group, repeating one mouse. Values with different letter superscripts indicate significant differences ($p<0.05$). Levels of p-S6K show significant difference comparing control vs. experimental diets. T-test = 0.005383792.

Figure 4.9: Effect of rapamycin diet and DMH treatment on protein levels of p-4EBP1 in young mice

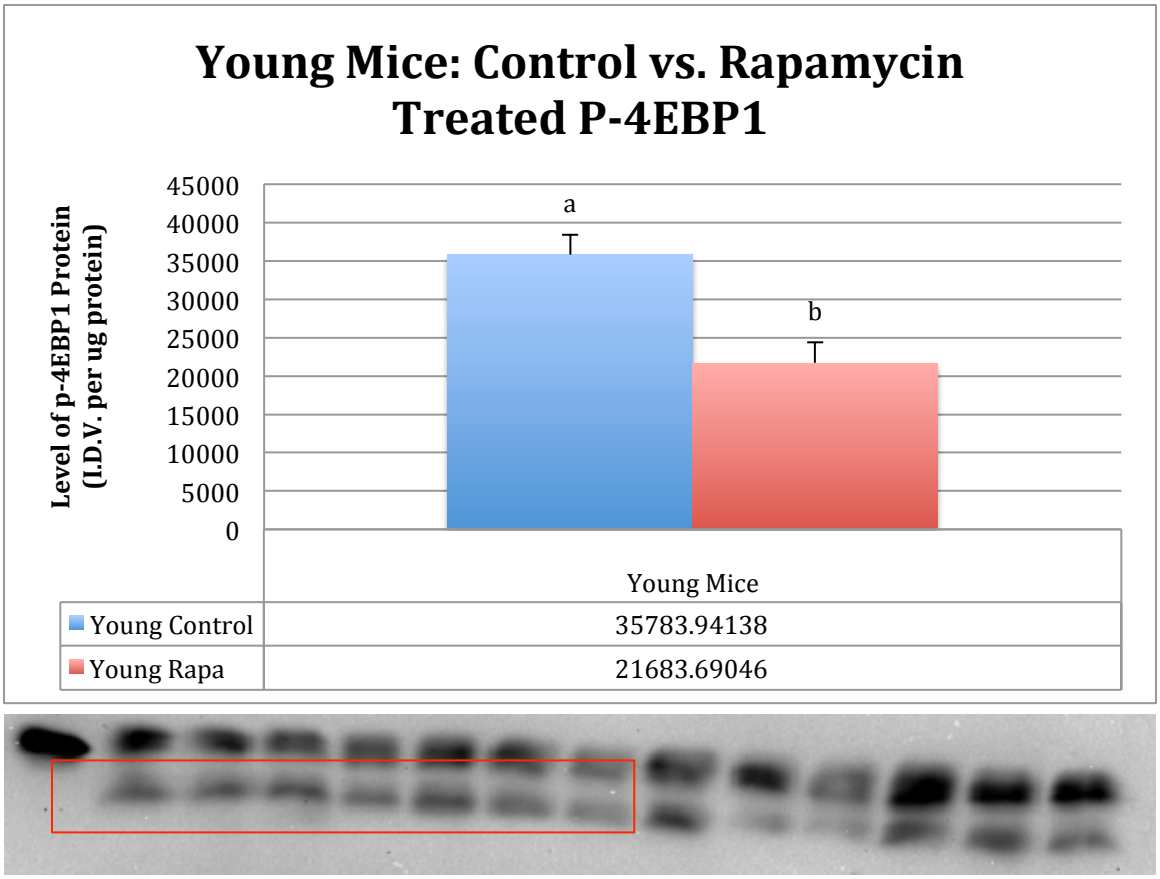


Figure 4.9 Effect of rapamycin diet and DMH treatment on protein levels of p-4EBP1 in young mice: In the above figure, an analysis of p-4EBP1 protein levels in liver of young wildtype mice is shown. Mice in both control and experimental groups were treated with intraperitoneal injections of DMH. Experimental mice were given rapamycin diets as described previously while control mice were given the same diets without rapamycin. Intraperitoneal treatment with DMH was done at week 16 for 6 weeks at 30mg/kg body weight, once per week. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 3 mice of group "Young Control" and 4 mice from group "Young Rapamycin," shown in sequence within the red box, and are representative of separate identical experiments. Values with different letter superscripts indicate significant differences ($p<0.05$). Levels of p-4EBP1 show significant difference comparing control vs. experimental diets. T-test = 0.010454087.

Figure 4.10 Effect of rapamycin diet and DMH treatment on protein levels of p-4EBP1 in old mice:

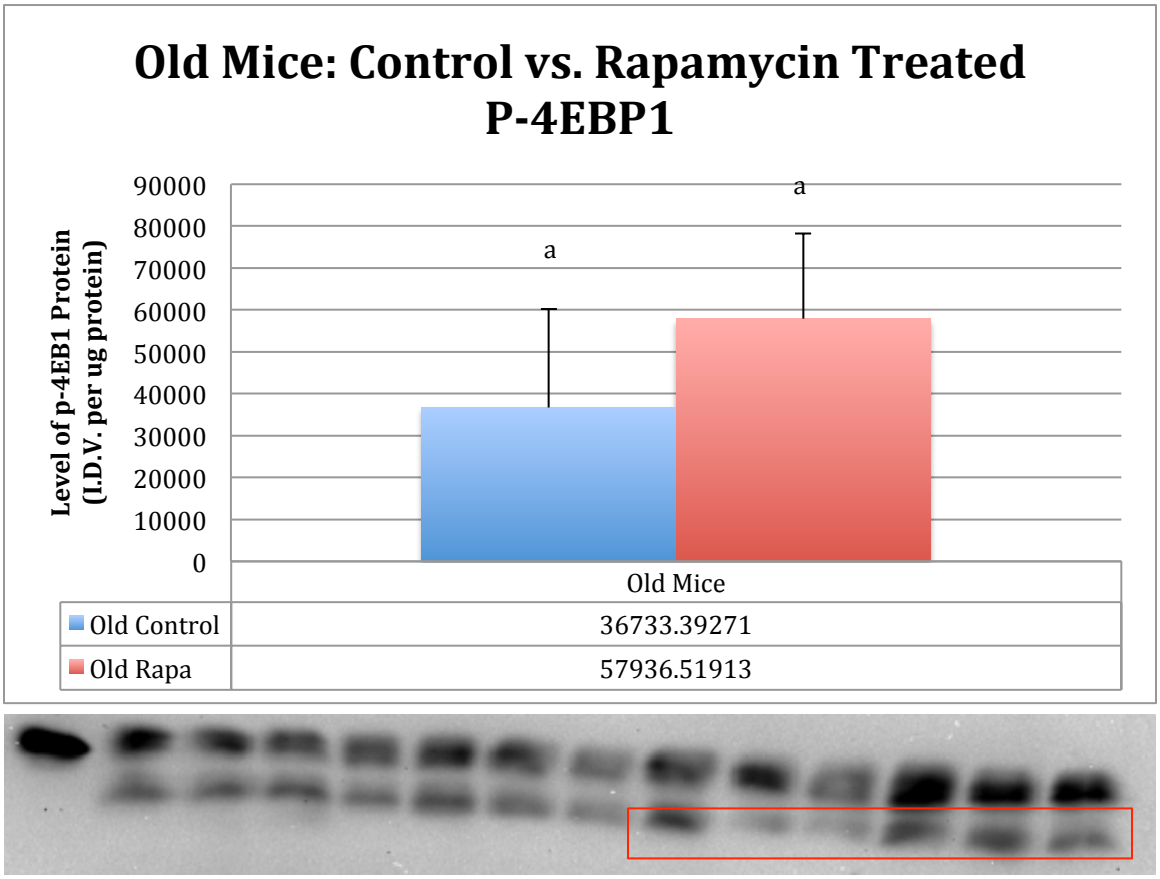


Figure 4.10 Effect of rapamycin diet and DMH treatment on protein levels of p-4EBP1 in old mice: In the above figure, an analysis of p-4EBP1 protein levels in liver of old wildtype mice is shown. Mice in both control and experimental groups were treated with intraperitoneal injections of DMH. Experimental mice were given rapamycin diets as described previously while control mice were given the same diets without rapamycin. Intraperitoneal treatment with DMH was done at week 16 for 6 weeks at 30mg/kg body weight, once per week. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 3 mice of each group, shown in sequence within the red box, and are representative of separate identical experiments. Group "Control Old" only has two mice due to an unexpected death of one of the mice. Values are represented in triplicate for this group, repeating one mouse. Values with different letter superscripts indicate significant differences ($p<0.05$). Levels of p-4EBP1 did not show significant difference comparing control vs. experimental diets. T-test = 0.449735204.

Figure 4.11: Effect of rapamycin diet and DMH treatment on protein levels of IPMK in young mice

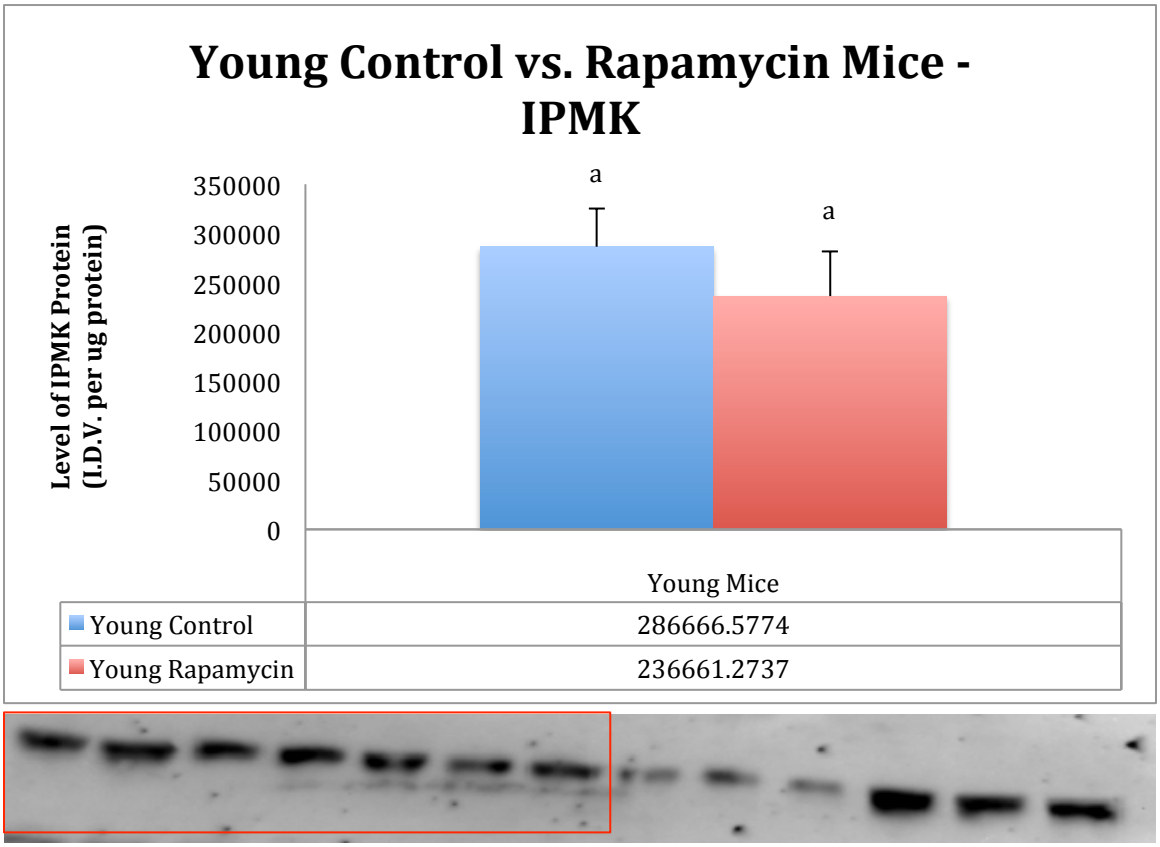


Figure 4.11 Effect of rapamycin diet and DMH treatment on protein levels of IPMK in young mice: In the above figure, an analysis of IPMK protein levels in liver of young wildtype mice is shown. Mice in both control and experimental groups were treated with intraperitoneal injections of DMH. Experimental mice were given rapamycin diets as described previously while control mice were given the same diets without rapamycin. Intraperitoneal treatment with DMH was done at week 16 for 6 weeks at 30mg/kg body weight, once per week. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 3 mice of group "Young Control" and 4 mice from group "Young Rapamycin," shown in sequence within the red box, and are representative of separate identical experiments. Values with different letter superscripts indicate significant differences ($p<0.05$). Levels of IPMK do not show significant difference comparing control vs. experimental diets. T-test = 0.393128514.

Figure 4.12: Effect of rapamycin diet and DMH treatment on protein levels of IPMK in old mice

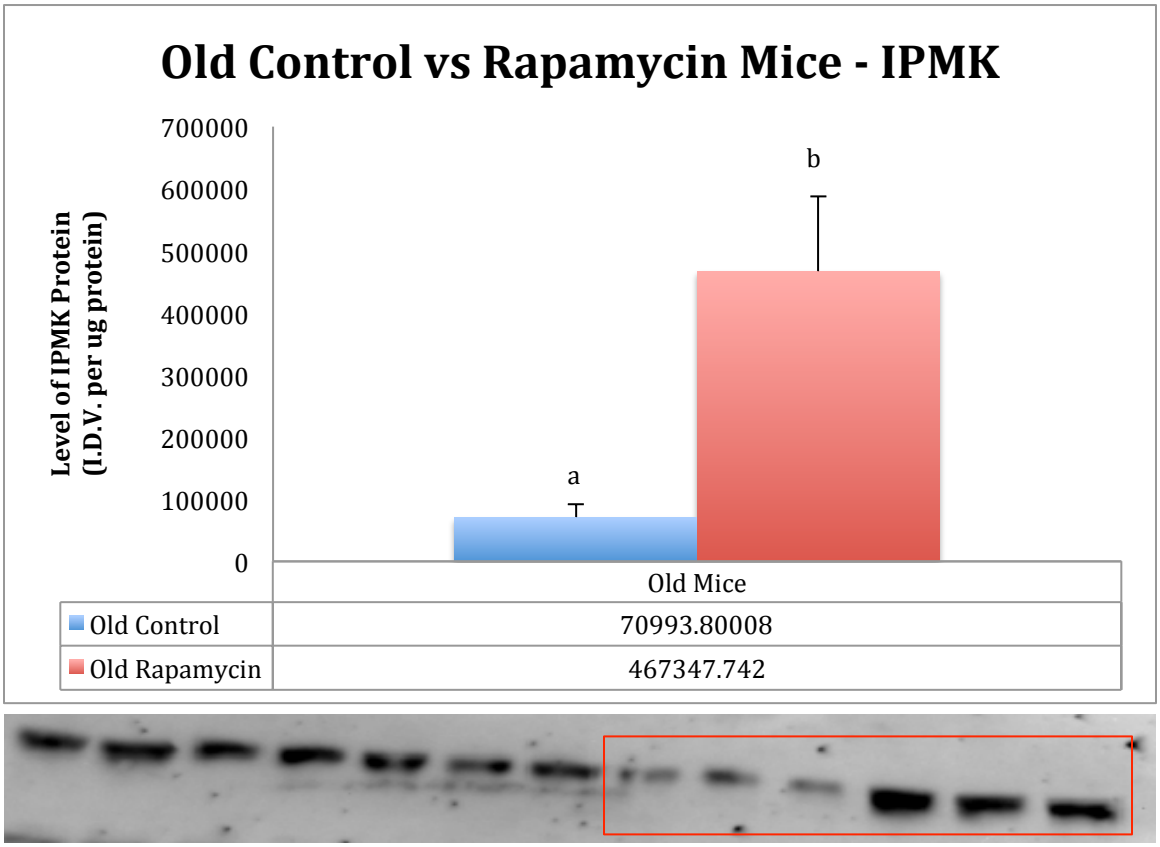


Figure 4.12 Effect of rapamycin diet and DMH treatment on protein levels of IPMK in old mice: In the above figure, an analysis of IPMK protein levels in liver of old wildtype mice is shown. Mice in both control and experimental groups were treated with intraperitoneal injections of DMH. Experimental mice were given rapamycin diets as described previously while control mice were given the same diets without rapamycin. Intraperitoneal treatment with DMH was done at week 16 for 6 weeks at 30mg/kg body weight, once per week. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 3 mice of each group, shown in sequence within the red box, and are representative of separate identical experiments. Group "Control Old" only has two mice due to an unexpected death of one of the mice. Values are represented in triplicate for this group, repeating one mouse. Values with different letter superscripts indicate significant differences ($p<0.05$). Levels of IPMK show significant difference comparing control vs. experimental diets. T-test = 0.016413721.

Figure 4.13: Effect of rapamycin diet and DMH treatment on protein levels of pAMPK in young mice

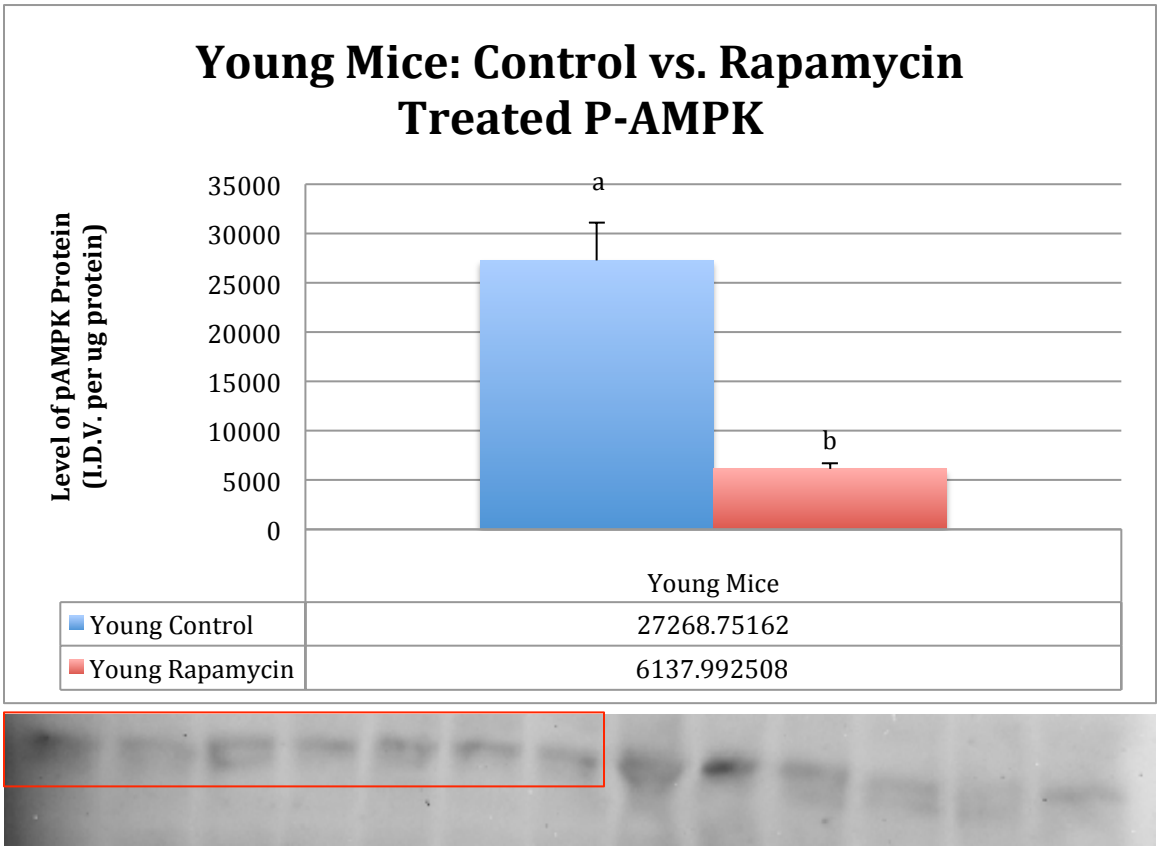


Figure 4.13 Effect of rapamycin diet and DMH treatment on protein levels of pAMPK in young mice: In the above figure, an analysis of pAMPK protein levels in liver of young wildtype mice is shown. Mice in both control and experimental groups were treated with intraperitoneal injections of DMH. Experimental mice were given rapamycin diets as described previously while control mice were given the same diets without rapamycin. Intraperitoneal treatment with DMH was done at week 16 for 6 weeks at 30mg/kg body weight, once per week. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 3 mice of group "Young Control" and 4 mice from group "Young Rapamycin," shown in sequence within the red box, and are representative of separate identical experiments. Values with different letter superscripts indicate significant differences ($p<0.05$). Levels of pAMPK show significant difference comparing control vs. experimental diets. T-test = 0.002687152.

Figure 4.14: Effect of rapamycin diet and DMH treatment on protein levels of AMPK in old mice

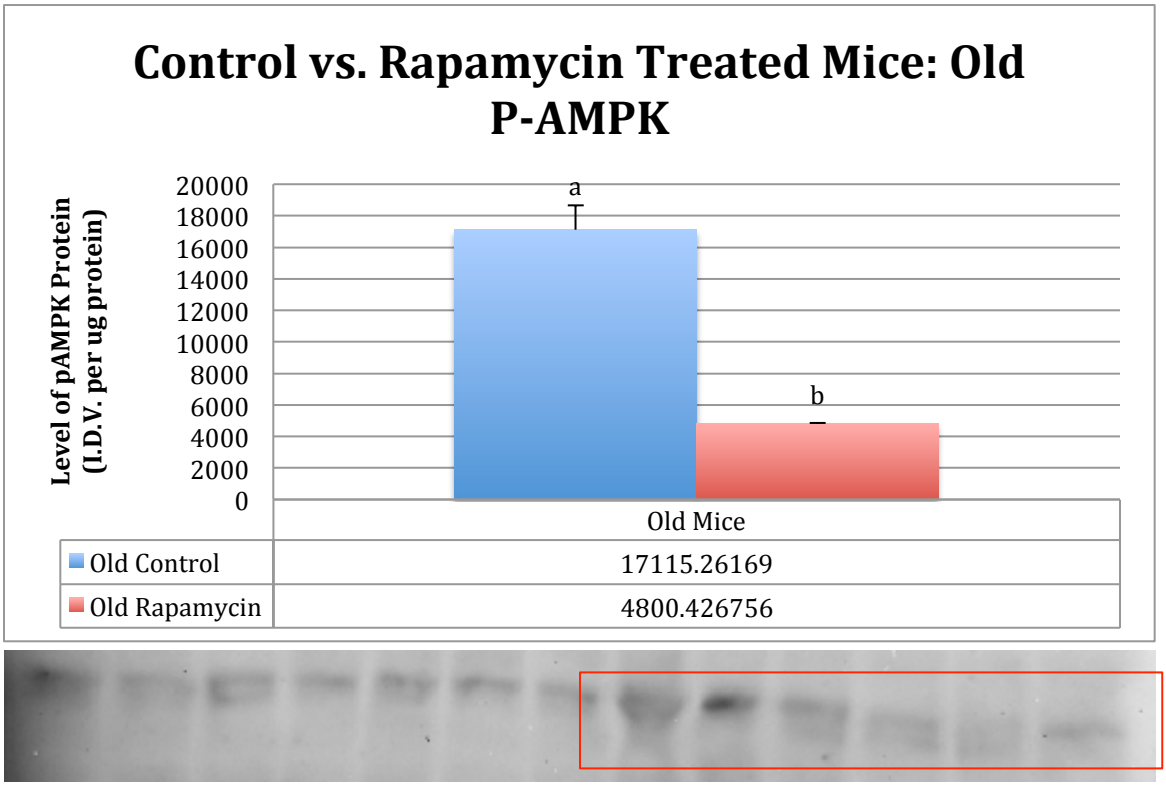


Figure 4.14 Effect of rapamycin diet and DMH treatment on protein levels of p-AMPK in old mice: In the above figure, an analysis of p-AMPK protein levels in liver of old wildtype mice is shown. Mice in both control and experimental groups were treated with intraperitoneal injections of DMH. Experimental mice were given rapamycin diets as described previously while control mice were given the same diets without rapamycin. Intraperitoneal treatment with DMH was done at week 16 for 6 weeks at 30mg/kg body weight, once per week. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 3 mice of each group, shown in sequence within the red box, and are representative of separate identical experiments. Group "Control Old" only has two mice due to an unexpected death of one of the mice. Values are represented in triplicate for this group, repeating one mouse. Values with different letter superscripts indicate significant differences ($p<0.05$). Levels of p-AMPK show significant difference comparing control vs. experimental diets. T-test = 0.000634124.

Figure 4.15: Effect of rapamycin diet and DMH treatment on protein levels of REDD1 in young mice

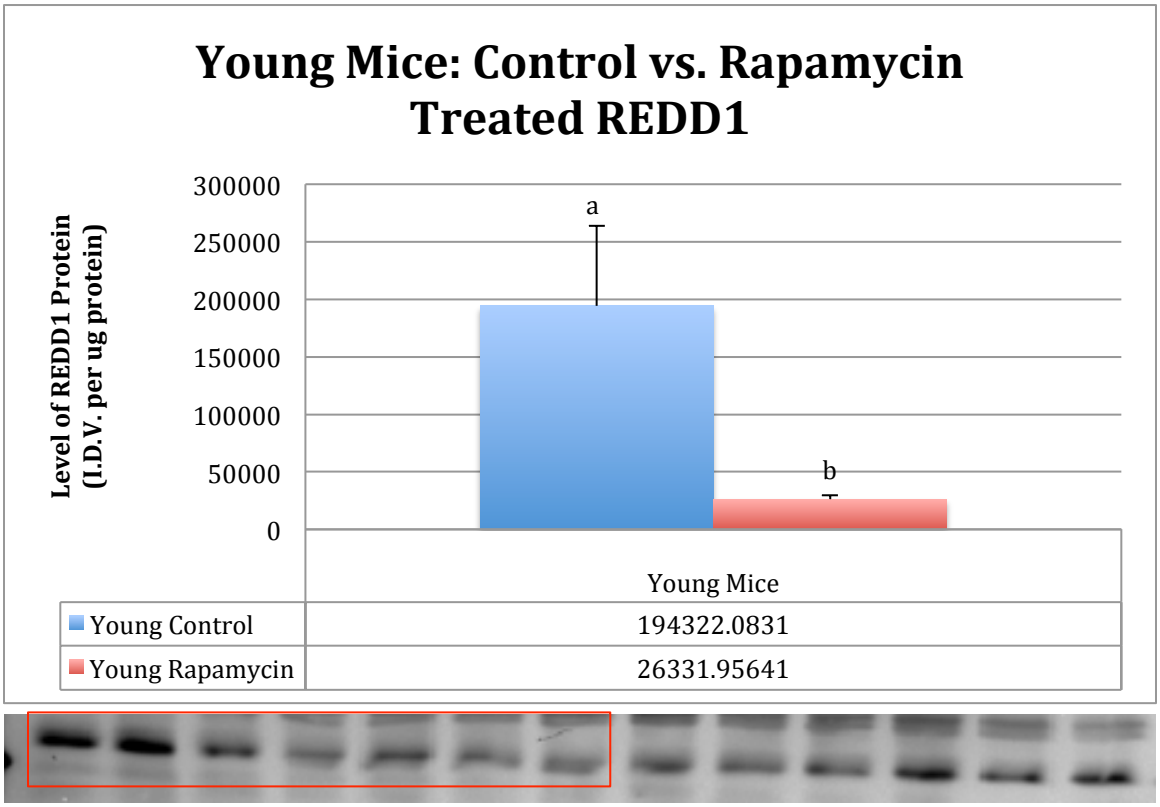


Figure 4.15 Effect of rapamycin diet and DMH treatment on protein levels of REDD1 in young mice: In the above figure, an analysis of REDD1 protein levels in liver of young wildtype mice is shown. Mice in both control and experimental groups were treated with intraperitoneal injections of DMH. Experimental mice were given rapamycin diets as described previously while control mice were given the same diets without rapamycin. Intraperitoneal treatment with DMH was done at week 16 for 6 weeks at 30mg/kg body weight, once per week. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 3 mice of group "Young Control" and 4 mice from group "Young Rapamycin," shown in sequence within the red box, and are representative of separate identical experiments. Values with different letter superscripts indicate significant differences ($p<0.05$). Levels of REDD1 show significant difference comparing control vs. experimental diets. T-test = 0.041440458.

Figure 4.16: Effect of rapamycin diet and DMH treatment on protein levels of REDD1 in old mice

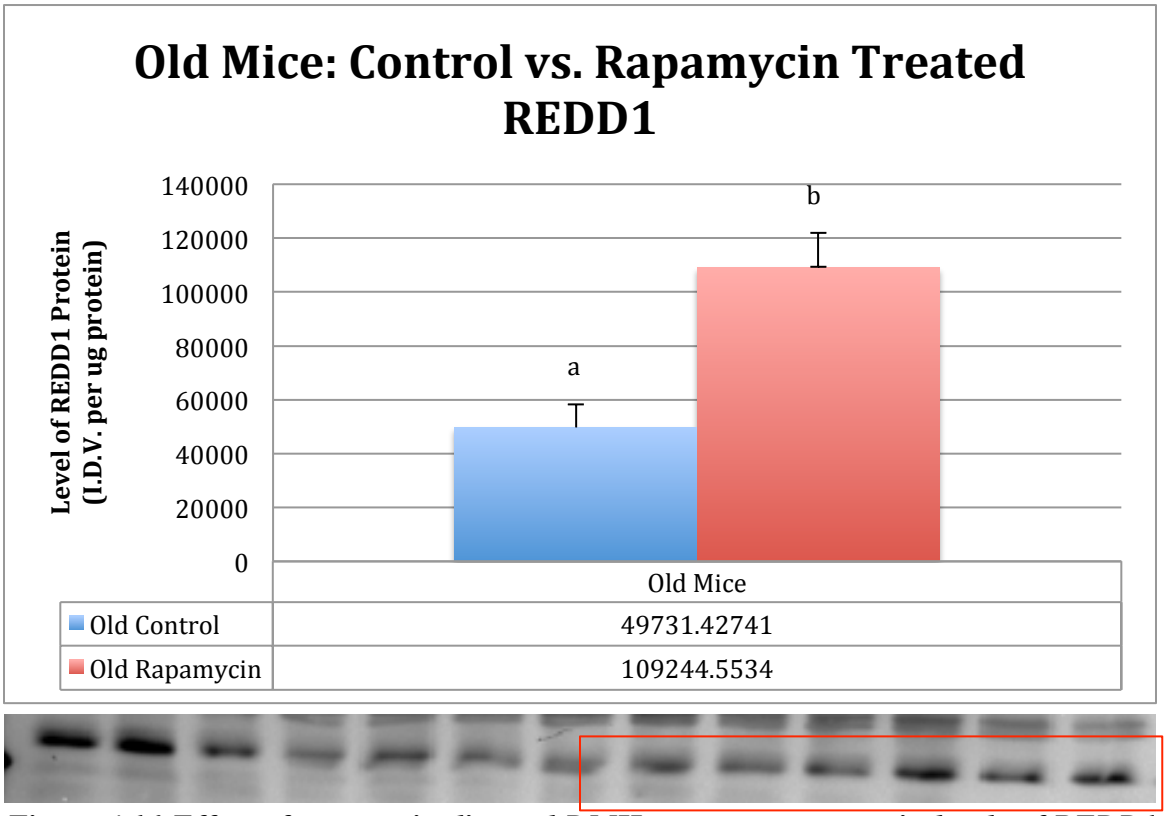


Figure 4.16 Effect of rapamycin diet and DMH treatment on protein levels of REDD1 in old mice: In the above figure, an analysis of REDD1 protein levels in liver of old wildtype mice is shown. Mice in both control and experimental groups were treated with intraperitoneal injections of DMH. Experimental mice were given rapamycin diets as described previously while control mice were given the same diets without rapamycin. Intraperitoneal treatment with DMH was done at week 16 for 6 weeks at 30mg/kg body weight, once per week. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 3 mice of each group, shown in sequence within the red box, and are representative of separate identical experiments. Group "Control Old" only has two mice due to an unexpected death of one of the mice. Values are represented in triplicate for this group, repeating one mouse. Values with different letter superscripts indicate significant differences ($p<0.05$). Levels of REDD1 show significant difference comparing control vs. experimental diets. T-test = 0.008856092.

Figure 4.17: Effect of rapamycin diet and DMH treatment on protein levels of LC3 (cleavage ratio) in young mice

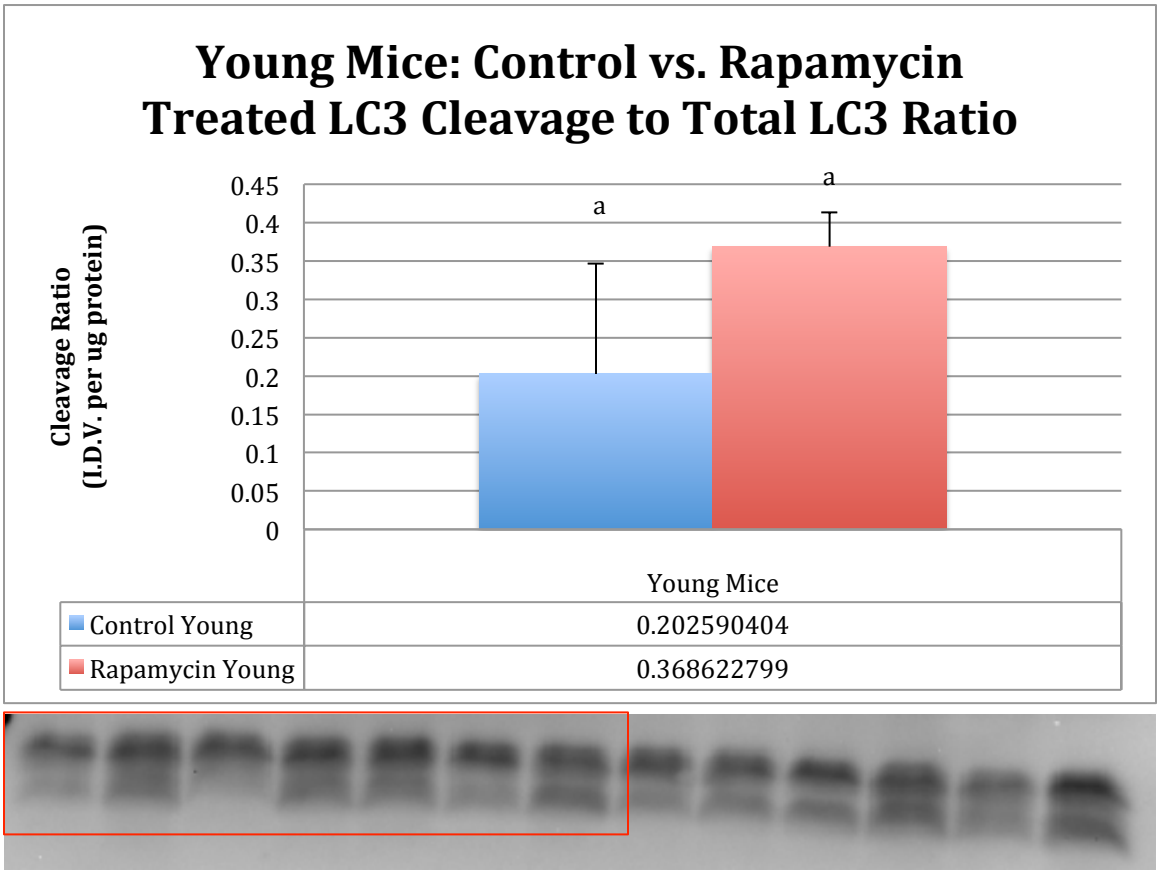
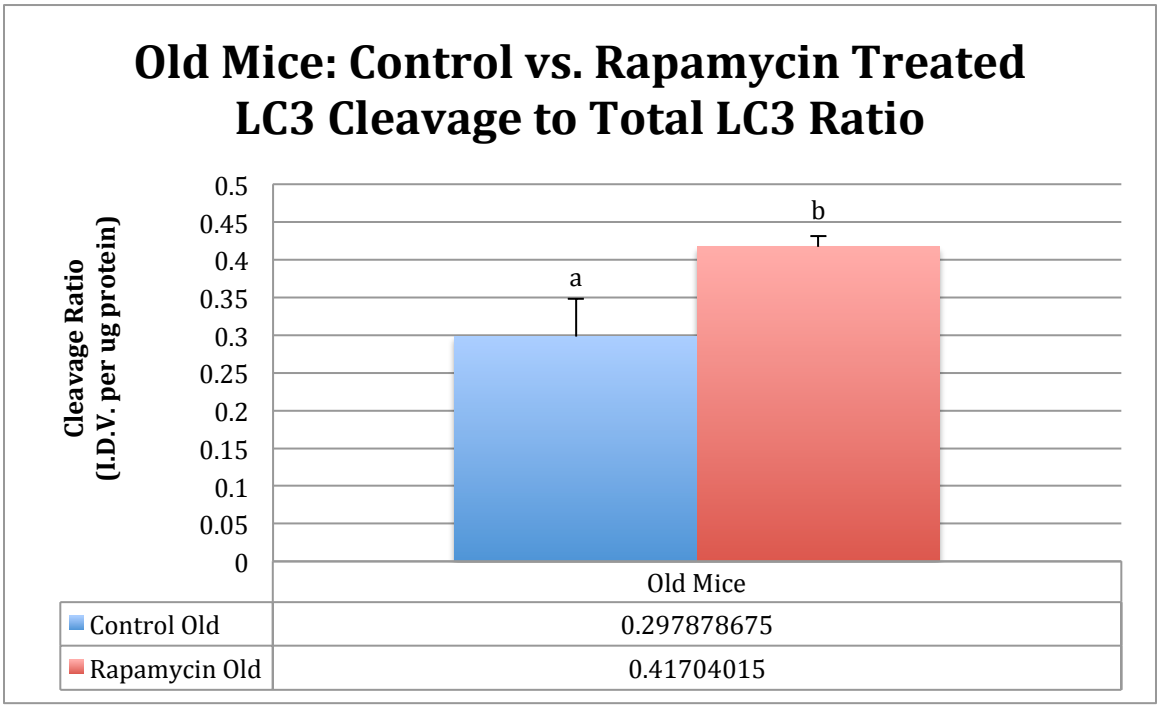


Figure 4.17 Effect of rapamycin diet and DMH treatment on protein levels (cleavage ratio) of LC3 in young mice: In the above figure, an analysis of LC3 protein levels in liver of young wildtype mice is shown. Mice in both control and experimental groups were treated with intraperitoneal injections of DMH. Experimental mice were given rapamycin diets as described previously while control mice were given the same diets without rapamycin. Intraperitoneal treatment with DMH was done at week 16 for 6 weeks at 30mg/kg body weight, once per week. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 3 mice of group "Young Control" and 4 mice from group "Young Rapamycin," shown in sequence within the red box, and are representative of separate identical experiments. Values with different letter superscripts indicate significant differences ($p<0.05$). Levels of LC3 do not show significant difference comparing control vs. experimental diets. T-test = 0.143829826.

Figure 4.18: Effect of rapamycin diet and DMH treatment on protein levels (cleavage ratio) of LC3 in old mice



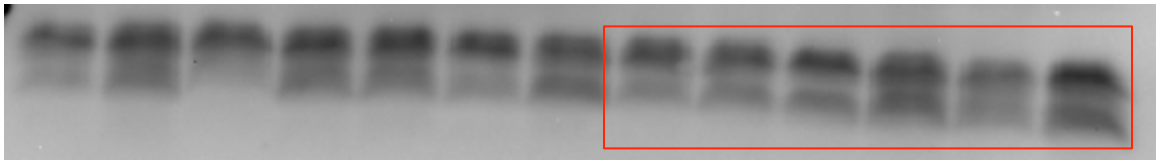
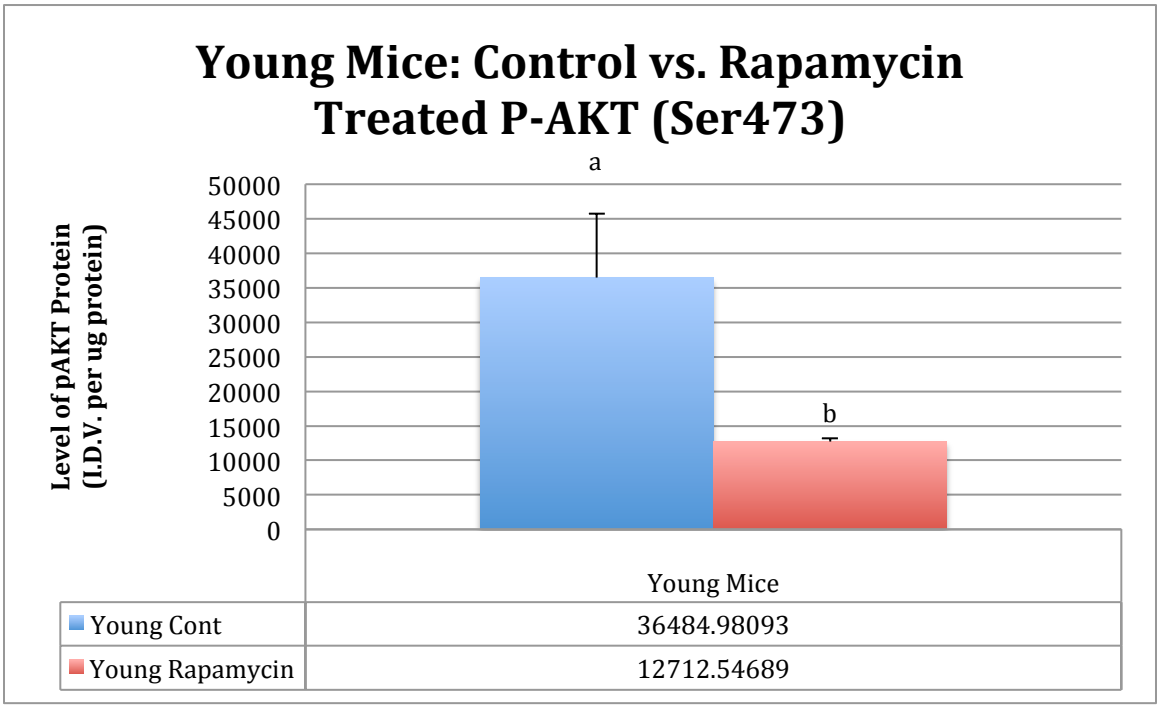


Figure 4.18 Effect of rapamycin diet and DMH treatment on protein levels (cleavage ratio) of LC3 in old mice: In the above figure, an analysis of LC3 protein levels in liver of old wildtype mice is shown. Mice in both control and experimental groups were treated with intraperitoneal injections of DMH. Experimental mice were given rapamycin diets as described previously while control mice were given the same diets without rapamycin. Intraperitoneal treatment with DMH was done at week 16 for 6 weeks at 30mg/kg body weight, once per week. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 3 mice of each group, shown in sequence within the red box, and are representative of separate identical experiments. Group "Control Old" only has two mice due to an unexpected death of one of the mice. Values are represented in triplicate for this group, repeating one mouse. Values with different letter superscripts indicate significant differences ($p<0.05$). Levels of LC3 show significant difference comparing control vs. experimental diets. T-test = 0.049844425.

Figure 4.19: Effect of rapamycin diet and DMH treatment on protein levels of p-Akt in young mice



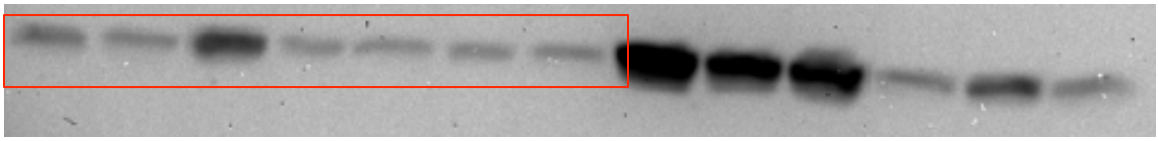


Figure 4.19 Effect of rapamycin diet and DMH treatment on protein levels) of p-Akt in young mice: In the above figure, an analysis of p-Akt protein levels in liver of young wildtype mice is shown. Mice in both control and experimental groups were treated with intraperitoneal injections of DMH. Experimental mice were given rapamycin diets as described previously while control mice were given the same diets without rapamycin. Intraperitoneal treatment with DMH was done at week 16 for 6 weeks at 30mg/kg body weight, once per week. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 3 mice of group "Young Control" and 4 mice from group "Young Rapamycin," shown in sequence within the red box, and are representative of separate identical experiments. Values with different letter superscripts indicate significant differences ($p<0.05$). Levels of p-Akt show significant difference comparing control vs. experimental diets. T-test = 0.013169954.

Figure 4.20: Effect of rapamycin diet and DMH treatment on protein levels of p-AKT in old mice

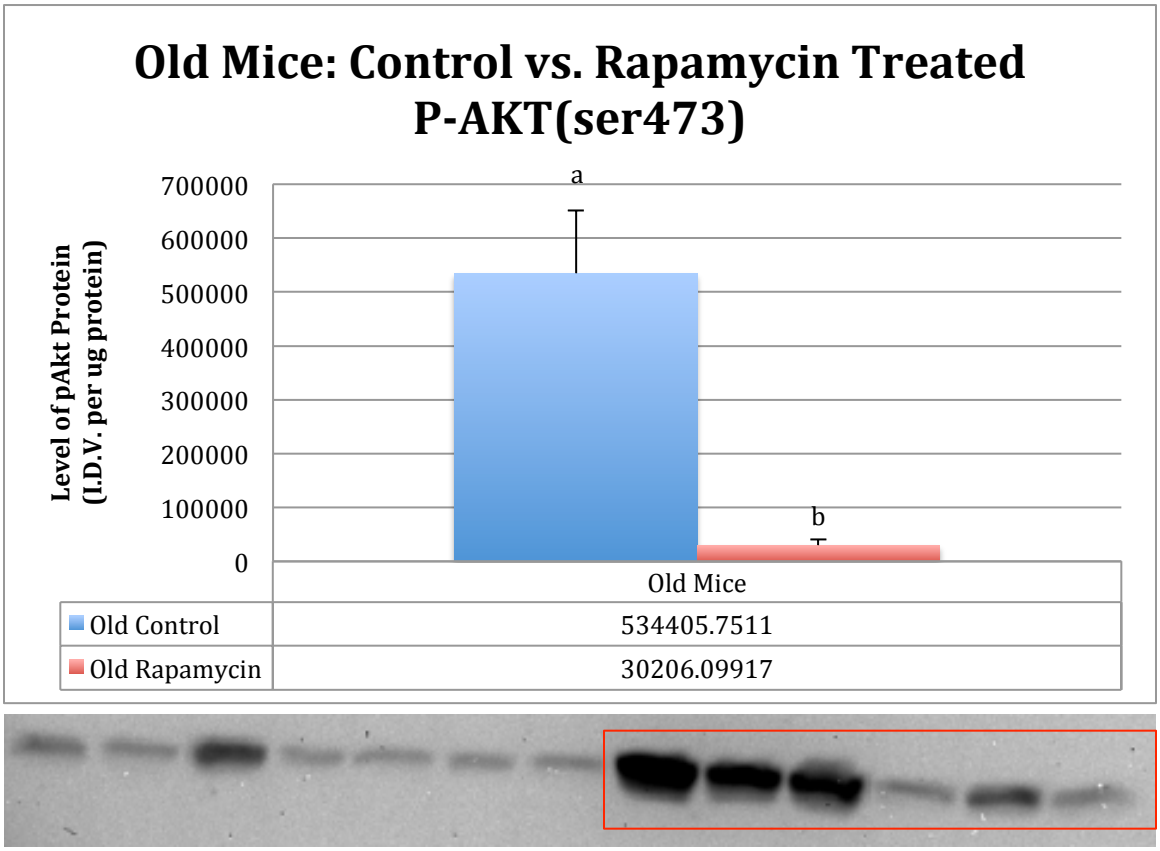


Figure 4.20 Effect of rapamycin diet and DMH treatment on protein levels of p-Akt in old mice: In the above figure, an analysis of p-Akt protein levels in liver of old wildtype mice is shown. Mice in both control and experimental groups were treated with intraperitoneal injections of DMH. Experimental mice were given rapamycin diets as described previously while control mice were given the same diets without rapamycin. Intraperitoneal treatment with DMH was done at week 16 for 6 weeks at 30mg/kg body weight, once per week. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 3 mice of each group, shown in sequence within the red box, and are representative of separate identical experiments. Group "Control Old" only has two mice due to an unexpected death of one of the mice. Values are represented in triplicate for this group, repeating one mouse. Values with different letter superscripts indicate significant differences ($p<0.05$). Levels of p-Akt show significant difference comparing control vs. experimental diets. T-test = 0.006157672.

Figure 4.21: Measurement of total-4E-BP1, total-AMPK, and total-AKT

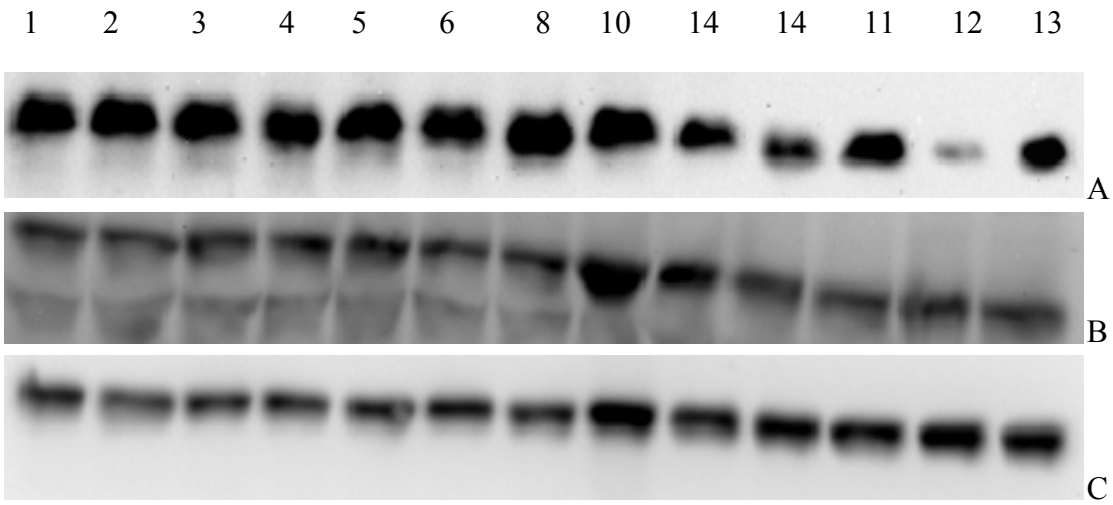


Figure 4.21 Measurement of total-4E-BP1, total-AMPK, and total-Akt: Measurements of total-antibody was done to establish a standard. Protein concentrations should be consistent in all wells. Figure 4.19A, 4.19B, and 4.19C represent total protein levels of 4EBP1, AMPK, and Akt, respectively. Sample 10 in well 8 generally exhibits more of some protein due to the nature of the mouse. Gel staining confirmed that the concentrations in each well are the same. Figure 4.19A is in the process of being repeated to confirm the results shown, despite the gel having equal staining patterns per well. Figure 4.19B and 4.19C show equal staining patterns in each of the wells, with the exception of sample 10 in lane 8. Lane 9 and 10 represent sample 14, duplicated. Young mice represent lanes 1 through 7. Old mice represent lanes 8 through 13. Sample numbers are labeled at the top of the diagram to signify which sample is duplicated.

Figure 4.22: Measurement of B-actin

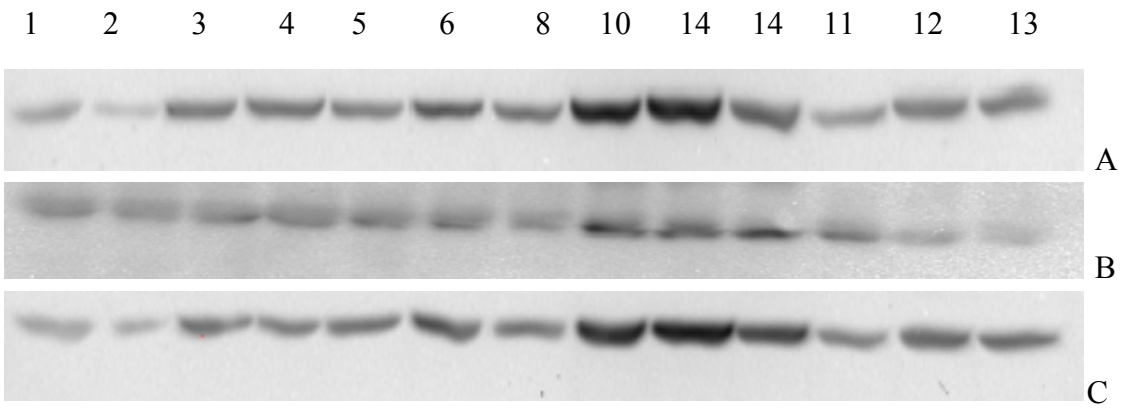


Figure 4.22 Measurement of B-actin: Figures 4.20A, 4.20B, and 4.20C are three separate Western Blot trials for B-actin. Each trial shows a similar pattern of expression. Gel staining showed that each of the respective runs exhibited equal concentrations. Young mice represent lanes 1 through 7. Old mice represent lanes 8 through 13. Sample numbers are labeled at the top of the diagram to signify which sample is duplicated.

CHAPTER 5: RESULTS AND DISCUSSION

There have been no previous studies that investigate the effects of long-term supplementation of rapamycin on a cancer model. There have been studies that have shown the effect of rapamycin in aging models through various durations of treatment, including 2-week, 6-week, and 20-week treatments of rapamycin on mice. Being that one of rapamycin's primary purposes is in the attenuation of mTORC1, which subsequently impacts the flux of the PI3K/Akt pathway, hindering the progression of cancer cells, it is important to investigate the effects of long-term supplementation of rapamycin in a cancer model. An experimental diet designed and developed by Harrison et al., was practiced in our laboratory for this study. This diet was tested to see its effects on the development and or hindrance of cancer progression in C57BL/6-specific pathogen-free male wildtype mice. Mice were given food *ad libitum* for 28 weeks and were treated with 1,2-dimethylhydrizine (DMH) one time per week for 6 weeks (30mg/kg body weight starting at week 16 of diet implementation) and were sacrificed at the end of the rapamycin treatment for analysis (**figure 4.1**). There were two types of groups, young (9 months of age) and old (20 months of age).

Figure 4.2 displays the food intake analysis throughout the 28-week diet. All mice showed the same trend in eating habits. Diets did not affect the outcome of the study. Gaps and subsequent spikes are due to data not being recorded for weeks missed. Purina 5LG6 mouse chow with encapsulated rapamycin was used for mice on experimental diets. Purina 5LG6 mouse chow was used for mice on control diets. Diets are described in detail in the "Method" section.

Figure 4.3 and 4.4 are representative of the weights of the animals subjected to experimental analysis. **Figure 4.3** represents the weight of young mice on the control and experimental diets with the subsequent induction of DMH. On average, the young control mice are of higher weight than the young experimental weight. At week 16 after being on the diet, the mice were hit with a carcinogen (DMH). The weights subsequently dropped, eventually to restore their weight at 28 weeks of age. This restoration may have been due to the general growth of the animal. Experimental mouse 9 is much lesser in weight than all other mice. It was not considered in the experimental analysis. **Figure 4.4** represents the weight of old mice on the control and experimental diets with subsequent induction of DMH. Unfortunately, control mouse 10b had died unexpectedly and tissues were not salvaged for experimental analysis at the time. Regardless, the unexpected death would not have provided valid results for the purposes of this experiment. Mice control 10a and control 10c were then separated (10c was placed into cage 14 and then renamed as mouse control 14). Weights for the old experimental mice were a bit less than control mice (with the exception of experimental mouse 11). All weights of mice dropped after DMH injection but roughly plateau at around week 21 (the last DMH injection date).

Mice were sacrificed, as described in the methods section. Subsequent tissue harvesting was conducted until analysis was ready to begin, also described in the methods section. **Figure 4.5** and **Figure 4.6** describe the aberrant crypt formation (ACF) analysis and crypt multiplicity analysis, respectively, in colon of the mice. Young control mice and young experimental mice had n=3 and 5, respectively. ACF averages were taken and presented in **figure 4.5**. Similarly to young mice, ACF averages for old mice are also displayed in **figure 4.5**. Old control and old experimental mice had n=2 and 3,

respectively. Thus, significant differences could not be adequately determined for ACF formation within colons of old groups. Instead, experiments continued for observational purposes. From **figure 4.5,** there seems to be a decrease in number of ACF formations in young mice treated with rapamycin compared to the control mice, although this difference is not statistically significant. In old mice, there seems to be more of a significant difference, although certainty cannot be guaranteed because n=2 for old control mice in this figure. What is interesting to note is that a trend exists showing that ACF formation actually decreases with age as rapamycin is supplemented. Crypt multiplicity is shown in **figure 4.6**. In young mice, there does not seem to be any difference in crypt multiplicity of ACF formations in the colon of the mice. However, in the old mice, there seems to be a small reduction in crypt multiplicity as mice are treated with rapamycin. This may be due to the effects of rapamycin, but we cannot be certain because only one control mouse is present for analysis in this figure.

Western blot analysis was constructed on liver tissues to investigate the effects of the diet and carcinogenic treatment, as colon cancer metastasizes to the liver [69]. **Figure 4.20** represents three separate trials for control protein, β-actin. Three separate trials, 4.20a, 4.20b, and 4.20c, respectively, were performed. From the figure, β-actin is clearly not expressed in a consistent expression pattern throughout all samples and cannot be used as a control protein. However, β-actin has shown to have consistent expression amongst the three separate trials, implying that the results shown are valid. β-actin must be manipulated by the experimental conditions. An untransferred gel was stained and analyzed for equal protein concentrations. The gel showed equal density of the staining

reagent throughout the lanes, implying equal protein concentrations per well (data not shown).

Rapamycin acts on mTORC1, which regulates cell growth, proliferation, and cell survival. Unregulation of the mTOR pathway can result in tumorigenesis, adipogenesis, and insulin resistance. The information provided in the results section has indicated an alteration in the mTOR pathway.

Phosphorylated-S6K is a protein that is acted upon directly by mTORC1 and responsible for increasing protein synthesis, cellular proliferation, and increase the activity of glycogen synthase kinase, which increases glucose concentrations. **Figure 4.7** displays the results observed in young mice throughout the course of this experiment. There is a significant decrease in level of p-S6K in mice that have been given the experimental diet, suggesting primarily that rapamycin is working in its known fashion – inhibiting the action of mTORC1. These results may also indicate that, in young mice, levels of cellular proliferation may be hindered under the conditions of this experiment, providing a method of protection against cancer. Similar results are seen in old mice (**figure 4.8**), although n=2 in all liver western blot old control groups. Trials were completed in triplicate, repeating sample "old control 14" in these experiments. Significant differences were based on these experimental stringencies.

Protein 4-EBP1 plays a role in the promotion of translation, increasing in the presence of mTORC1 activation, insulin resistance, and UV light. Young mice show a significant difference in the level of 4-EBP1 when exposed to the experimental conditions (**figure 4.9**). Interestingly, although not significantly different, old mice show

a reverse profile. This may be due to the possible development of insulin resistance, or a change in the cellular environment, due to its age nature (**figure 4.10**).

Protein inositol-phosphate multikinase (IPMK) works as a cofactor of mTOR and stimulates its expression through response of amino acid levels [24]. It senses the level of branched-chain amino acids present in the cell and acts accordingly to activate mTORC1 for protein translation to occur. Many different transducing steps have been observed recently, outlining the pathway of signal transduction from the amino acid through to mTORC1 [70]. **Figure 4.11** shows that in young mice, there is no significant difference in the level of expression of IPMK within control and experimental groups. However, within old mice, there is a significant difference that has been observed. Old mice treated with rapamycin have higher levels of IPMK expression than do control mice (**figure 4.12**). This may be due to rapamycin's inhibition and prevention of protein synthesis. An accumulation of amino acids may be inducing this increased expression of IPMK. One proposal for this behavior may be that old mice may not have the ability to compensate for the accumulation of amino acids as their young counterparts do. Some validity of this proposal may come from the level the total protein synthesized in these liver tissues. Normalization values were higher in old mice tissues that were treated with rapamycin diets, suggesting that this proposal may have some truth.

Protein p-AMPK works as a master regulator of several cellular processes such as cellular uptake of glucose, β-oxidation of fatty acids, and biogenesis of glucose transporter 4 (GLUT4) [23]. Additional to this, p-AMPK works to inhibit the mTOR cascade with its energy sensing capabilities. High AMP:ATP ratios, indicating low energy of the cell, are associated with high levels of activated AMPK (p-AMPK).

Subsequently, the activation of AMPK inhibits the mTORC1 cascade in two ways: 1) directly interacting with raptor and, 2) activating TSC proteins to inhibit Rheb and subsequently decreasing the level of mTORC1. **Figure 4.13** shows that mice treated with rapamycin and exposed to the experimental conditions have less activation of p-AMPK. Based on this observation, control mice should express higher AMP:ATP energy ratios (possibly due to DMH since mice are fed diets *ad libitum*), thereby making it necessary to decrease mTOR expression through increased p-AMPK levels. In young mice supplemented with rapamycin, p-AMPK is significantly lower. Rapamycin may be somehow acting to replenish the energy available in the cell. Alternatively, this may also be due to the fact that mTORC1 is already knocked down in expression with rapamycin supplementation. AMP:ATP energy ratios in rapamycin treated mice would still be higher for this to be possible. Similar results are seen in **figure 4.14** with the old groups. Either energy balance is improving in rapamycin treated tissues or tissues may be acting in a conservation scheme by allocating their energy elsewhere (i.e. toward β-oxidation and glycogen breakdown), where rapamycin does not deeply influence. To further investigate the mechanism around this protein, energy levels would be wise to look into.

mTORC1-dependent phosphorylation of S6K1 and 4-EBP1 has been shown to be effectively inhibited in hypoxic conditions. It was later discovered that REDD1 expression was necessary for this to occur [71]. **Figure 4.15** shows that in experimental mice, there is significantly less expression of REDD1 when compared to its respective control. REDD1 is expressed during hypoxic environments, energy stress, and conditions promoting reactive oxygen species (ROS). Hypoxia is a well-known condition that develops in cancer cells because of their rapid use of oxygen, eventually exhausting

energy stores. The induction of DMH may have induced a low energy level environment (as implied through increased levels of p-AMPK in **figure 4.13** and **figure 4.14**). ROS are most likely present being that these tissues exhibit cancer and metabolize rapidly. In young control mice, **figure 4.15** shows that they exhibit increased expression of REDD1 in comparison to their young rapamycin treated counterparts. The increase in activity of REDD1 may be due to compensating the increased flux of the mTORC1 pathway due to hypoxia, energy state, and ROS. Lower levels are observed within the experimental group mostly likely because rapamycin inhibits the increased flux of the mTOR pathway, rendering REDD1's expression to diminish by preventing its stimulants. These findings may support that REDD1 is needed to control the excessive activation of mTOR. Rapamycin fed mice have less REDD1 expression, implying a non-stimulatory environment (of REDD1) is present in these tissues. The effects of rapamycin may conserve the tissue environment while simultaneously preventing the increased flux of the mTOR-signaling pathway under cancer induced conditions. With this being said, it is possible that the treatment may have prevented the onset of hypoxic conditions while simultaneously negating the requirement of REDD1 to shut down mTORC1 due to rapamycin's direct target mechanism of mTOR inhibition. This would make sense since rapamycin is an inhibitor of hypoxia inducible factor 1 (as stated in the introduction). Interestingly, **figure 4.16** shows the inverse of this. REDD1 expression has significantly increased in mice fed rapamycin diets in comparison to the controls. This may also lead to possible insight as to why there were increased levels of 4-EBP1 in old mice fed rapamycin diets as well. Since REDD1 degredation is necessary to restore mTORC1 signalling [71], this is an interesting contradicting observation. This finding may be a

critical understanding point for this experiment and furthermore, provide an insight to how rapamycin may manipulate the cellular environment. More investigation must be done to satisfy this contradiction.

LC3 is a protein that is used as a marker for autophagy as it is the only protein associated with the inner and outer membrane of the autophagosome [72]. Autophagy as a dual role in that it can act as a tumor suppressor by preventing the accumulation of proteins that damage the cell and stimulate proliferation, and also as a mechanism of survival for tumor cells, metabolizing and renewing resources to promote growth [73]. In western blotting, LC3 is detected as two bands (signifying cleavage). The ratio of the two bands is determined in order to analyze the autophagy process. **Figure 4.17** displays the ratio of LC3 cleavage (bottom band:Total LC3) in young mice. Higher ratios signify that autophagy is occurring. Young control mice compared to young experimental mice do not exhibit a significant difference in expression patterns. **Figure 4.18** shows that old control mice and experimental mice differ in that there is significantly more autophagy occurring in experimental mice than in controls. This implies that the rapamycin diet may influence the autophagy process. Whether or not rapamycin influences the autophagy in a tumor demoting or promoting fashion is still to be determined.

Possibly the most important implication of this study resides in the significance drawn from analysis of p-Akt levels within these liver tissues. Akt is involved in the PI3K/Akt/mTOR pathway responsible for roles involving apoptosis and cellular proliferation. In many cancers this pathway is overactive, promoting cellular proliferation and hindering apoptosis. It has the ability to cleave BAD (a pro-apoptotic protein) and has been shown to escape cell cycle arrest. This can be detrimental, especially if

mutations in the cells have accumulated up to this point. In **figure 4.19** and **figure 4.20**, both young and old experimental mice show a significant decrease in levels of p-Akt compared to their respective controls. Some studies have suggested that Akt can become activated again through mTORC2, further promoting the cascade (as stated in the introduction). However, this experiment suggests that this cascade is hindered significantly in mice that have participated in the experimental diet. This ultimately suggests that a long-term supplementation of rapamycin is protective against some cancers.

Figure 4.19a, b, and c shows total protein levels for 4-EBP1, AMPK, and AKT, respectfully. AMPK and AKT show equal expression level throughout the samples, where 4-EBP1 does not. 4-EBP1 may have been manipulated under experimental conditions.

What is interesting to note is that the results from this study are slightly similar to results seen in another study constructed by our lab. Arrabi et al., experimented with haploinsufficient mice for DNA polymerase β (β-pol$^{+/-}$) and assessed how a folate deficient diet would impact the base excision repair (BER) pathway in this model compared to a wildtype model. In particular, β-pol$^{+/-}$ mice share similar results for proteins p-AMPK and p-AKT. A folate deficient diet in β-pol$^{+/-}$ mice shares the same expression pattern for these proteins as a folate adequate diet for wildtype mice long-term supplemented with rapamycin. Also, unpublished data involving long-term folate deficient diets in wildtype mice have shown similar results to those seen in β-pol$^{+/-}$ mice. This implies that a folate-deficient diet may lead to answers that question long-term supplementation experiments with rapamycin in a cancer model.

CHAPTER 6: CONCLUSION

Several studies have investigated the impact of rapamycin on aging, including short and long-term studies. But, there have not been any long-term rapamycin that have investigated the effects of long-term rapamycin supplementation on a cancer model. Being that rapamycin has successfully treated some cancers, it was important to investigate its effects through long-term diet means. ACF analyses determined that in colon, long-term supplementation did not show any significant differences in ACF formation within young or old mice when compared to the controls. Analysis of p-S6K and 4-EBP1, direct targets of mTOR phosphorylation, show significant differences in that rapamycin is working properly, downregulating the activity of mTORC1. A notable exception is seen in old mice for 4-EBP1 where the expression of 4-EBP1 in old mice treated with rapamycin diets actually exhibits increased expression levels of 4-EBP1. This may be due to the inherent nature of the mouse as it ages, or it may be due to an environmental influence that rapamycin has on the cell. The findings with protein REDD1 support this claim in old mice. An unexpected reverse profile with REDD1 expression is found to be similar to that shown of 4-EBP1. More investigation is needed to explain this finding. In young mice, REDD1 seems to have been expressed through its normal stimulation factors (hypoxia, cellular energy state, and reactive oxygen species). Young control mice exhibit higher levels of REDD1 whereas young experimental mice have lower levels of REDD1. This is possibly due to a partial relief of responsibility of REDD1 through the actions of rapamycin in young mice. Amino acid level sensitivity was investigated through analysis of IPMK during this experiment. Young mice did not show any significant difference between control and experimental models. However, in

old mice, there was a significant increase in expression of IPMK in experimental mice fed with rapamycin. One proposal for this behavior is that rapamycin prevents protein synthesis by inhibiting mTORC1, which could subsequently lead to a buildup of amino acids. This could then lead to over-expression of IPMK without stimulating mTORC1. Interesting to note is that normalization values for samples are higher for old mice fed diets with rapamycin. This finding provides support for this proposal. Phosphorylated AMPK acts as an energy sensor in the cell, inhibiting mTOR when energy is low. DMH may have hindered the energy production mechanisms in the cell (more investigation would be necessary to confirm this) which p-AMPK analysis could provide insight. There is significantly less expression of p-AMPK in both young and old mice when comparing control and experimental groups. Reason for this could be because rapamycin is claiming responsibility for p-AMPK's natural mechanism to inhibit mTOR. P-AMPK may be able to focus on alternative responsibilities that it has due to rapamycin's relieving properties. Protein LC3 is a marker used to sense autophagy. Whether or not autophagy acts in a protective manner in this experiment is unknown, but results expressed in this experiment do not show a significant difference in young mice. LC3 cleavage ratio levels are significantly different in old mice favoring autophagy in older mice supplemented with rapamycin when compared to controls. Possibly the most interesting part of this experiment is shown through protein p-AKT. P-Akt is typically overactive in many cancers. In both young and old mice, long-term supplementation of rapamycin has acted to prevent Akt from becoming activated. This means that the long-term supplementation of rapamycin may prevent the onset of cancer. Interestingly, some of these results mimic another study that was done in this lab by Arrabi et al. Folate

restricted diets in β-pol$^{+/-}$ mice show similar results to long-term rapamycin diets in wildtype mice. ACF analysis show that the studies are not entirely the same, but a more in-depth ACF analysis of old mice may provide some insight to the mechanism for which folate deficiency acts to protect against cancer in β-pol$^{+/-}$ mice. However, the levels of p-Akt and p-AMPK show similar expression patters. This implies that questions involving energy balance and cancer may be answered with information from a folate-restricted diet in models that have impaired BER pathways and/or through wildtype animals exhibiting long-term supplementation of rapamycin.

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ABSTRACT

EFFECT OF LONG TERM RAPAMYCIN TREATMENT ON mTOR SIGNALLING NETWORK IN COLON AND LIVER OF C57BL/6 MICE

by

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Many studies have investigated the effects of rapamycin on aging and cancer. However, the effects of long-term rapamycin supplementation on a cancer model have not been performed. This is the first study that investigates the effects of long-term supplementation of rapamycin in a cancer model. ACF analysis of colon tissues in mice showed no significant difference between controls and those supplemented with rapamycin. Factors such as energy balance, cellular environment, PI3K/Akt/mTOR pathway, and more have been assessed in this study. The duration of rapamycin supplementation seems to play an important role in the protection against cancer. Ultimately, this study suggests that the long-term supplementation of rapamycin in C57BL/6 mice provides a protective mechanism and influences many cellular processes involved in cancer. Interestingly, a correlation between this study and folate deficiency's influence and protection of β-pol$^{+/-}$ haploinsufficient mice has also been noticed.

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