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TIMING AND DURATION OF FOLATE RESTRICTION DIFFERENTIALLY IMPACTS COLON CARCINOGENESIS

by

ALI FARDOUS

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2020

MAJOR: NUTRITION AND FOOD SCIENCE

Approved By:

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Advisor Date

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DEDICATION

"Knowledge and understanding are life's faithful companions who will never prove untrue to you. For knowledge is your crown, and understanding your staff; and when they are with you, you can possess no greater treasures."

"Work is love made visible. And if you cannot work with love but only with distaste, it is better that you should leave your work and sit at the gate of the temple and take alms of those who work with joy."

-Gibran Khalil Gibran

I thank GOD first and foremost in his infinite mercy for all the blessings in my life.

This work is dedicated to my beautiful wife Safa, mother of our sons Yusuf and Yaseen, and best friend. Thank you!

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ACKNOWLEDGMENTS

A special thanks to my mentor Dr. Ahmad Heydari for his unwavering support and pushing me to be the best I can. You were there for me in my moments of weakness and did not hesitate to help me back on my feet whenever I faltered. For that you have my everlasting gratitude.

I thank my wife and partner for her love and unconditional support. I would also like to acknowledge my committee members: Dr. Diane Cress, Dr. David Pitts, Dr. Kequan Zhou for their time, support and feedback. I Thank the following who have assisted me in my research: Mike Saruna, John Sorge, Andrew James, Ali Beydoun, Hongzhi Ma, and Archana Unnikrishnan. A special remembrance to the late Dr. Lisa Lucente, her work became the inspiration and basis of my research. I thank the following lab members for their help and feedback: Sukayna Ismail, Rawia Khasawanah, Michelle Jones, Amanda Arrabi, and Tom Prychitko.

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CHAPTER 1: BACKGROUND AND SIGNIFICANCE

Folate

Folic acid, an essential water-soluble B vitamin also known as B9, was first discovered in the 1940's. The discovery came to light after the finding that a yeast extract corrected macrocytic anemia in pregnant women[1]. Most bacteria, yeast and plants can synthesize folates; however, animals require folate intake through the diet or through supplementation. Folate refers to compounds that include natural folates and folic acid, with folic acid (FA) referring to the fully oxidized form[1]. Folic acid, otherwise known as pteroylglutamic acid, was first extracted from spinach in 1941[2]. Chemical synthesis of folic acid first took place in 1943 by combining a pteridine ring, paraminobenzoic acid and glutamic acid[3]. Folate is essential for growth, homeostasis, and plays a critical role in cellular one carbon metabolism. Folate is involved in maintaining genomic integrity by regulating gene expression through methylation as well as purine and pyrimidine biosynthesis needed for repair and replication. Folate is also involved in amino acid metabolism, regeneration of the methyl donor S-adenosyl methionine, the antioxidant glutathione, as well as various critical cellular functions [4]. Folate deficiency is implicated in the pathogenesis of multiple diseases and conditions such as anemia, cardiovascular disease, neural tube defects, neurodegenerative diseases, cancer, and more [5]. Natural folates are found in adequate quantities in green leafy vegetables as well as legumes, fruits, yeast, and cereals. Animal food sources contain higher amounts of folates especially in liver and kidney [1].

Absorption

Absorption of folate from the diet occurs mainly in the proximal small intestine [1]. However, recent studies suggest that a significant amount of folate can be absorbed in the colon[6]. Absorption at the brush border occurs via a proton-coupled folate transporter (PCFT) and reduced

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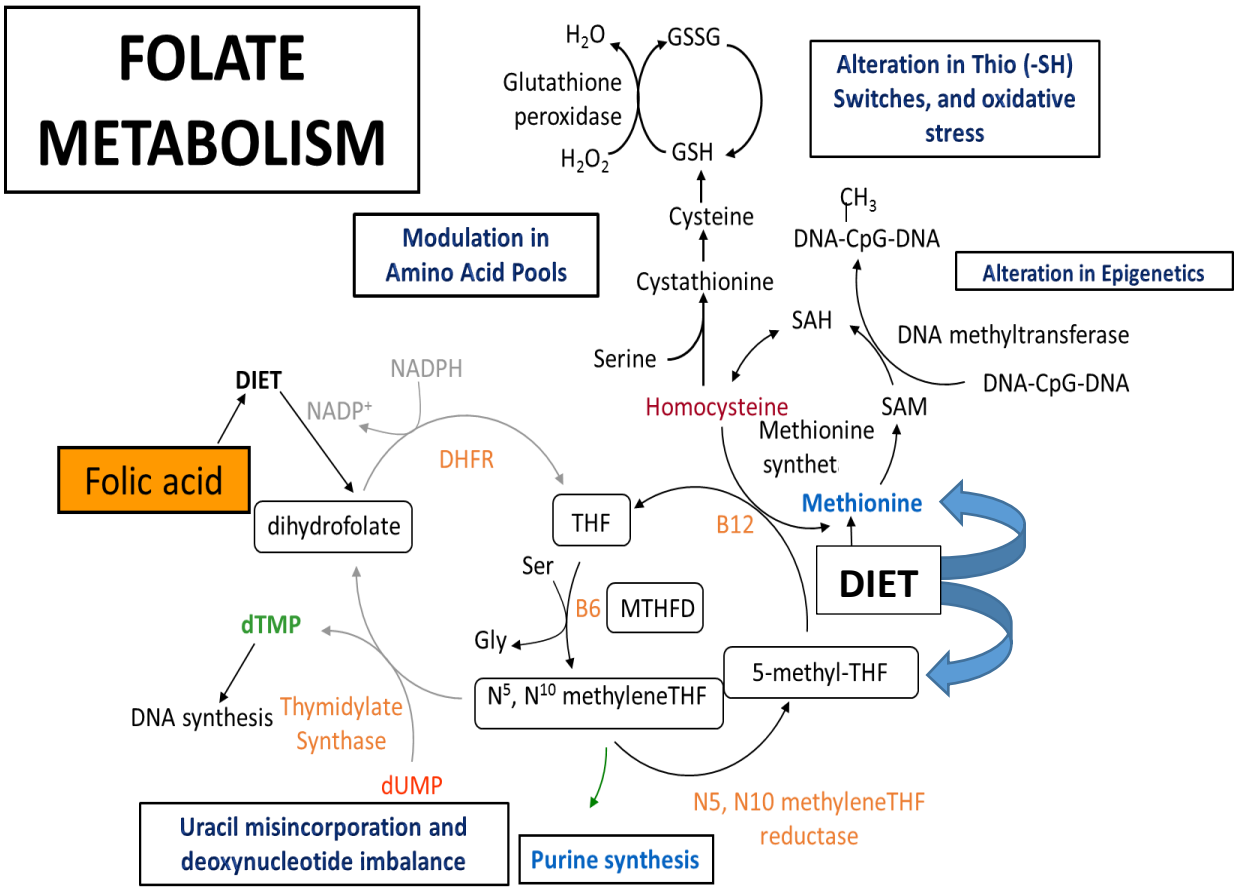
folate carriers (RFC) [1]. RFC is a membrane spanning transport protein that is necessary for the transport of folates. Some mutations of the gene encoding RFC can lead to alteration of folate and homocysteine levels [7]. The bioavailability of folates is dependent on multiple factors including dietary folate content, food sources and preparation methods. Various host factors such as mutations in the transporters or inflammatory conditions can limit absorption [1]. Folate absorption can be reduced through passage and destruction in the acidic environment of the stomach. Surviving folate must be reduced form the polyglutamate form to monoglutamate by folate conjugase to be absorbed. It is then converted back to the polyglutamate form and stored in the liver as well as secreted in the bile to be reabsorbed by enterohepatic recirculation [1].

Folate metabolism



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Folate refers to a diverse array of biological molecules that serve as cofactors in one carbon transfer and metabolism. Natural folates in the diet and in the body are found predominantly in the reduced form commonly as 5-methyl-THF [8]. Folic acid, a synthetic folate, must be reduced to dihydrofolate then further reduced to tetrahydrofolate (THF) by dihydrofolate reductase before entering the folate cycle. THF is the biologically active form of folate and serves as the starting point for one carbon transfer. THF picks up one carbon units from the conversion of serine to glycine catalyzed by the methylentetrahydrofolate dehydrogenase (MTHFD) with the vitamin B6 serving as a cofactor forming N5-N10-methylene-THF. As THF picks up the carbon units it then can be interconverted between various oxidative states leading to distinct metabolic outcomes. N5-N10-methylene THF provides carbon units to thymidylate synthase converting deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). Folate plays an essential role as a cofactor in the de novo biosynthesis of purines, thus maintaining DNA integrity during DNA synthesis, replication, and repair. N5-N10-methylene-THF is converted to10-formyl-THF, the most oxidized form of folate used in the de novo synthesis of purines, while 5-methyl-THF is used in the re-methylation of homocysteine to form methionine, the precursor of S-adenosylmethionine (SAM). SAM is involved in various biological methylations, most importantly the methylation of DNA and histones and epigenetic imprinting [9]. SAM is converted back to s-adenosylhomocysteine (SAH) and back to homocysteine. Due to the critical function of SAM, the SAM/SAH ratio is tightly controlled via numerous metabolic mechanisms and feedback loops. Dietary factors such as folate and methionine availability can affect SAM/SAH ratio leading to epigenetic alterations. Homocysteine, long considered to be a marker for various disease etiologies such as cardiovascular disease and cancer, is regulated by the transulfuration pathway.

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Homocysteine undergoes irreversible degradation to cysteine and ultimately forming the antioxidant glutathione, an important regulator of cellular redox homeostasis[9].

Folate and neural tube defects

In 1998 the FDA mandated the addition of folic acid (FA) to all enriched cereal grain foods in order to reduce the incidence of neural tube defects in the offspring of women of childbearing age [10]. Folate rose to prominence after evidence for interventional trials and observational studies revealed that periconceptual folate supplementation protected against neural tube defects (NTD) [5]. This revelation led policymakers, the US health service, and the institute of medicine to recommend a daily intake of 400 ug folic acid to all women of childbearing age [5]. Soon after, the FDA mandated the fortification of all flour and uncooked cereal-grain products in the United States with 140 ug/100g of folic acid by 1998 [11], [12]. Similar recommendations were passed in Canada, Chile and Western Australia [5].

Argument for fortification

Folate supplementation was seen as safe and beneficial for disease prevention [13]. This argument was based on abundance of evidence from various studies linking folate deficiency to various diseases including, anemia, cardiovascular disease, neurological disease, cancer, neural tube defects (NTD), and low birth weight [13], [14]. Prior to FA fortification, targeted supplementation with FA in women at risk and of childbearing age failed to yield encouraging results due to failure to abide to the vitamin regimen. Despite the presence of massive education and awareness programs, the results did not translate into behavioral changes in the participants [5]. The risk of non-compliance with the recommendations was augmented by the fact that the neural tube closes during the fourth week of gestation, a time most women are unaware they are pregnant. These findings, along with the assumption that folate could provide additional health

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benefits in the general population encouraged health policy advocates to push for generalized folic acid fortification [5].

Effectiveness of fortification

The goal of the fortification program initiated in 1998 by the FDA was to increase daily intake of folates by about 100 ug/d while maintaining total intake under 1000 ug/d (safe upper limit of daily intake) [10]. However, recent studies revealed the predicted increase in folic acid from fortified food to be much higher (215-240 ug/d) [10]. Fortification lead to an equivalent decrease of homocysteine [13]. The effect of the fortification on NTD was significant as studies from the US and Canada showed a reduction of 15-50% in the incidence of NTD [13]. The dramatic decrease in NTD, however, cannot be solely attributed to the increase in folate levels due to fortification. Other factors such as improved nutrition and prenatal care had been playing a role in the steady decrease of NTD even before fortification began[5].

Controversies

For every neural tube defect prevention, several hundred thousand people are exposed to increased levels and intake of folic acid [15]. Animal studies show that a diet high in folate can affect DNA and histone methylation leading to epigenetic changes that can be inherited, affecting successive future generations[15]. Folic acid supplementation can lead to elevated levels of naturally occurring folates as well as unmetabolized folic acid in the blood [15]. There is currently a raging controversy among researchers on the risk vs benefit of supplementing the whole population with high levels of synthetic folates to reduce the risk of comparatively small number of neural tube defects[15]. In the United kingdom, it is estimated that for every neural tube defect prevention, there are up to three-quarter million people exposed to extra folic acid with similar ratios in the united states and Canada [15]. Furthermore, FA interventional studies did not support

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that folate supplementation reduces cardiovascular and stroke risk [13]. The controversies surrounding FA has led many countries into adopting a more cautious approach to supplementation[16].

Is Folate harmful?

Folate was deemed generally safe with almost no toxicities [5], however, its use in food fortification is controversial. High folate intake can mask vitamin B-12 deficiency, a condition with relatively high prevalence among the geriatric population [17]. Some argue that even moderate intake is capable of producing a masking effect, prompting several countries to delay or reject adopting folate fortification mandates [5]. FA intake raises concerns of negative adverse effects in various subpopulation groups that do not stand to benefit from supplementation [5]. Safety Data for long term exposure to high dose synthetic folates do not exist and is not well understood [5]. There is mounting evidence that FA may promote the growth and progression of existing preneoplastic or neoplastic lesions [18]. An increasing percentage of the population is exposed to folates in levels that exceeds the upper safety limit caused by fortification and additional supplementation [19]. Folate supplementation can interfere with and reduce the effectiveness of antifolate based chemotherapy [19]. FA can also interact with prescription drugs including seizure medications, anti-inflammatory therapies, and malaria medications[15][5]. Other evidence points to FA supplementation causing genetic instability through epigenetic changes and selection of disease alleles [20]. High levels of folic acid have been linked to an immune weakening effect by decreasing natural killer cell cytotoxicity[15]. Furthermore, there is evidence that folate supplementation to pregnant women can lead to increased risk of insulin resistance and obesity in their children[15].

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Folate and cancer

The link between folate and cancer was established shortly after folic acid discovery and extraction [1]. Folate impact on cancer is complex and at times contradictory [18], [19]. After FA was discovered in the 1940's, based on limited evidence, researchers attempted to administer FA to children with leukemia leading to accelerated progression and poor outcomes [13]. Researchers then learned that the proliferation of leukemia cells is dependent on the availability of the tetrahydrofolate metabolites leading to the successful use of folate antagonists such as aminopterin to produce full remission of children with acute leukemia [13]. Conversely, evidence from a number of studies suggest that folate deficiency can increase the risk of cancer in normal tissues while adequate folate levels has a protective effect on the risk of developing malignancies of various tissues [13][21].

There are multiple theories on how folate deficiency can induce carcinogenesis. These range from folate deficiency causing DNA strand breaks due to uracil misincorporation, alteration of DNA methylation patterns and gene expression, genomic instability and chromosomal aberrations [22]–[24]. Others point to impaired DNA repair pathways under folate deficiency, thus reducing DNA damage threshold and increasing the risk of transformation and mutations with or without the addition of DNA damage or insult [25]–[28].

More recently, FA fortification was linked to a significant decrease in neuroblastoma among children, however no change was detected in the incidence of infant acute lymphoblastic leukemia and hepatoblastoma [13]. A case control study from Germany shows that mothers supplementing with vitamins containing FA resulted in a decreased risk of non-Hodgkin's lymphoma and some types of leukemia in their offspring, however, there was an increased risk of neuroblastoma [29]. Another study where pregnant women were supplemented with 5mg/day FA

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showed a 70% increase in all cancer mortality and a doubling of breast cancer mortality risk[30]. Kim et al. observed that FA fortification in women who are pregnant or of childbearing age can potentially reduce the risk of certain childhood cancers in the offspring, however, this comes at the risk of promoting the growth and progression of undiagnosed, preneoplastic or neoplastic lesions [19].

Folate and colorectal cancer

Evidence from recent meta-analyses suggest a significant reduction of adenomas and colorectal cancer (CRC) when comparing subjects of the highest folate status with the lowest [13]. The Nurse's Health Study showed a 75% reduction in colorectal cancer in women taking multivitamins with folate [31]. The ASPIRIN/FOLATE polyp prevention study published in 2003 reported that FA supplementation at 1 mg/d had no effect on the recurrence of adenomas. However, there was a 67% increase in highly malignant advanced lesions at the second follow up [32]. Furthermore, there was a significant increase of other malignancies such as prostate cancer in the folate supplemented group. Despite a decrease in homocysteine among the FA subjects, cardiovascular risk was not decreased among the FA supplemented group [32].

Animal studies reveal a dual modulating effect of folate on colorectal cancer whereby the timing and dosage of folate can have contrasting results [33]. The results show that folate deficiency increases the risk of transformation, while modest supplementation reduces cancer risk, and high or supraphysiological doses increase it [13]. Furthermore there is evidence that folate supplementation can increase CRC risk after the establishment of microscopic neoplastic foci [5]. A possible explanation to this modulation is that folate deficiency in normal tissues can lead to uracil misincorporation due to the decrease in thymidylate synthesis causing DNA strand breaks, impaired repair and genomic instability [33]. Furthermore, folate deficiency can lead to aberrant

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DNA methylation due to the possible decrease of S-adenosylmethionine leading to DNA methylation abnormalities [13]. A folate adequate diet is expected to correct these aberrations leading to a decrease in cancer risk. However, in preneoplastic or fully transformed lesions, folate deficiency can inhibit the growth requirements of a rapidly proliferating tissue, leading to decreased tumor growth and progression. At the same time, FA supplementation can provide the tumor with nucleotides leading to faster growth [34]. As evident from several studies, FA supplementation to individuals who might harbor microscopic undiagnosed preneoplastic lesions can lead to adverse outcomes by fueling cancer growth [32]. It is estimated that 25-50% of people in the US and Canada may carry asymptomatic adenomas by the age of 50 with the prevalence increasing with age [13]. Recent large studies reported a nonsignificant trend in total and colorectal cancer risk due to FA supplementation [13]. In the post fortification era, it is estimated that folate fortification has added about 200 ug/d FA through enriched products leading to a total intake of approximately 400 ug/day for non-supplement users and over 800 ug/day in individuals taking additional supplementation [13]. Other studies pointed out that these are likely to be underestimates indicating that a significant percentage of the population has high serum folates[13]. Analysis performed my Mason et al. shows that the United States and Canada had a reversal in the downward trend in colorectal cancer shortly after the FA mandate. The authors hypothesized that FA is wholly or partly responsible for the observed reversal in the trend, however, because of the study design, they could not determine causality [35].

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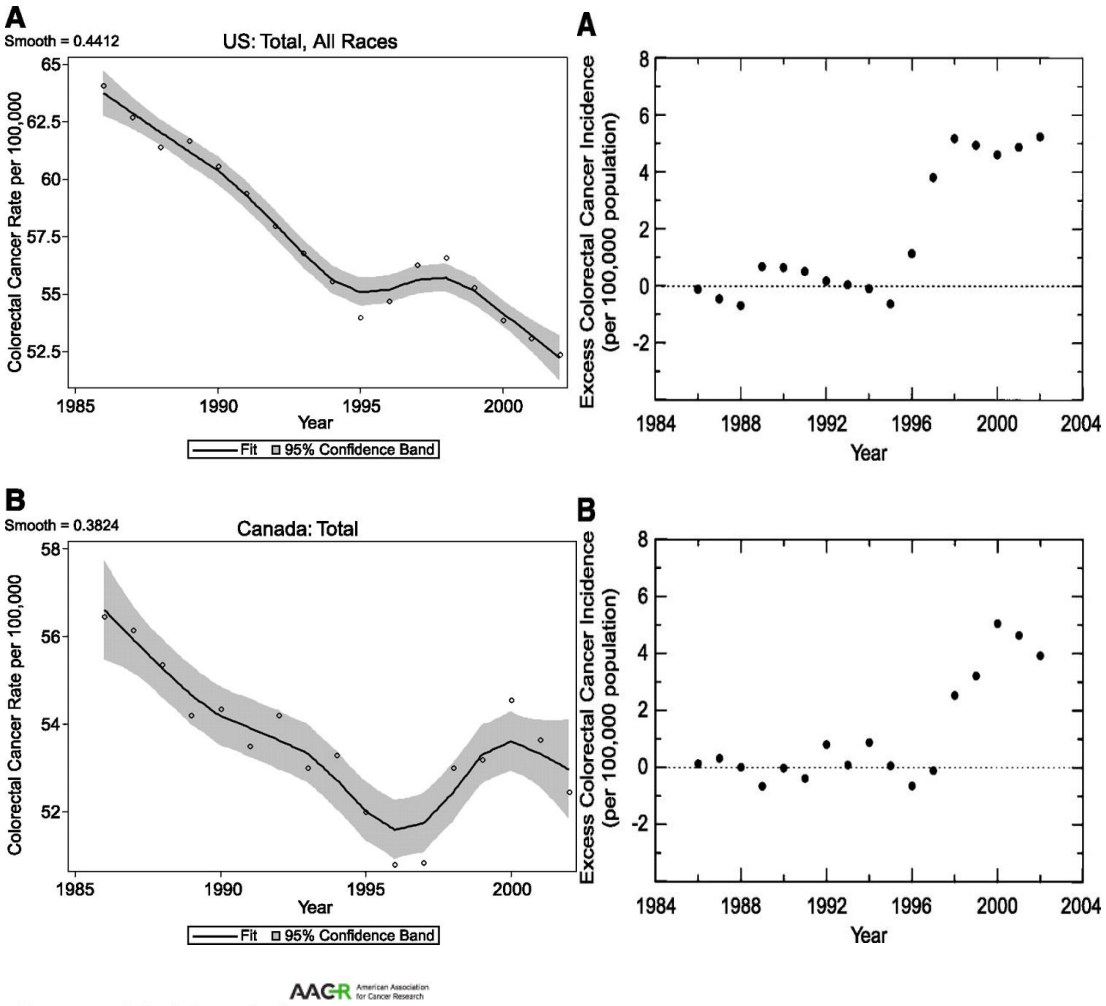

AACR American Association for Cancer Research
Cancer Epidemiology, Biomarkers & Prevention

CRC incidence Post Fortification by Mason et al.[35]

Folate and mTOR

Our early work with folate looked at the impact of folate deficiency on the initiation and progression of colon cancer in a polymerase β haploinsufficient (β-pol$^{+/-}$) mouse model. We had originally established that folate restriction negatively impacts DNA base excision repair (BER), a critical pathway that is required to repair DNA oxidative damage and uracil misincorporation, by regulating the expression of its rate limiting enzyme β-pol [36], [37]. We then hypothesized that folate restriction in addition to β-pol haploinsufficiency will lead to an increased formation of aberrant crypt foci (ACF), a preneoplastic lesion, in response to the carcinogen dimethylhydrazine

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(DMH). To our surprise, folate restriction in these animals conferred protection under these conditions (fig1.1) [38]. Analysis of tissue samples via BrdU and TUNEL assays revealed a decrease in proliferation and an increase in apoptosis in the colon of folate restricted animals [38]. We became interested in understanding how folate restriction (FR) can be protective in our colon cancer models and if that protection can persist under different conditions and genetic backgrounds. Analysis of the literature showed that other labs were observing that reduced dietary folate can confer protection against cancer as well as reducing inflammatory bowel disease in various lab rodent models [39]–[41]. We were looking for a missing link that can integrate signals from one carbon metabolism and affect proliferation, apoptosis and cancer, explaining our findings. Our breakthrough came about when analyzing microarray expression data comparing folate restricted animals to folate adequate. We saw that folate restriction significantly altered the expression of genes in the mTOR pathway. Analysis of plasma amino acid levels provided us with further evidence of the modulating effect of FR on various amino acids, specifically methionine, isoleucine and leucine. As mTOR is central to the pathways we were investigating, we were compelled to provide a mechanistic analysis of the effect of folate restriction on mTOR and colorectal cancer.

The mammalian target of rapamycin also known as the mechanistic target of rapamycin mTOR is a conserved serine/threonine protein kinase that integrates signals such as growth factors, amino acids, energy levels, and nutrient availability to modulate cell growth, proliferation, autophagy, and metabolism. mTOR is found in 2 distinct complexes mTORC1 and mTORC2. mTORC1 is rapamycin sensitive and is regulated by multiple upstream signals such as growth factors, amino acid availability, stress and hypoxia, and energy availability [42], [43]. mTORC2 is largely rapamycin insensitive and has different regulators and downstream targets than

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complex1. mTOR became the focus of intensive research in recent years as this signaling pathway has been altered in various conditions such as cancer, diabetes, and aging [44]. More recently, mTOR has been found to be modulated by perturbation in one carbon metabolism and more specifically folate availability [45]. Folate was shown to activate the mTOR pathway via activation of the AKT pathway, phosphorylating mTOR and the downstream targets p70S6k1 and 4E-BP1 [46]. Furthermore, mTOR was found to be directly linked to one carbon metabolism. Researchers reported that mTORC1 could control the mitochondrial tetrahydrofolate cycle and induce purine synthesis as well as stimulate the generation of SAM [47], [48]. Rosario et al. observed that placing mice on folate free diet for 6 weeks produced marked inhibition of mTORC1 and mTORC2 in various tissues. Further inhibition of downstream targets of both complexes was also observed as well as decreased activity and expression of the amino acid transporters solute carrier (SLC) [49]. In other research, Rosario et al. suggested that mTOR may utilize the same amino acids sensing mechanism for folate sensing [50]. Similar findings linking folate levels to mTOR signaling, and amino acid transporter activity were confirmed in baboons and humans [49]–[51]. Furthermore, Rosario et al. provided evidence that mTOR can directly regulate folate transporters trafficking thus regulating cellular folate uptake [52].

mTOR signaling pathway:

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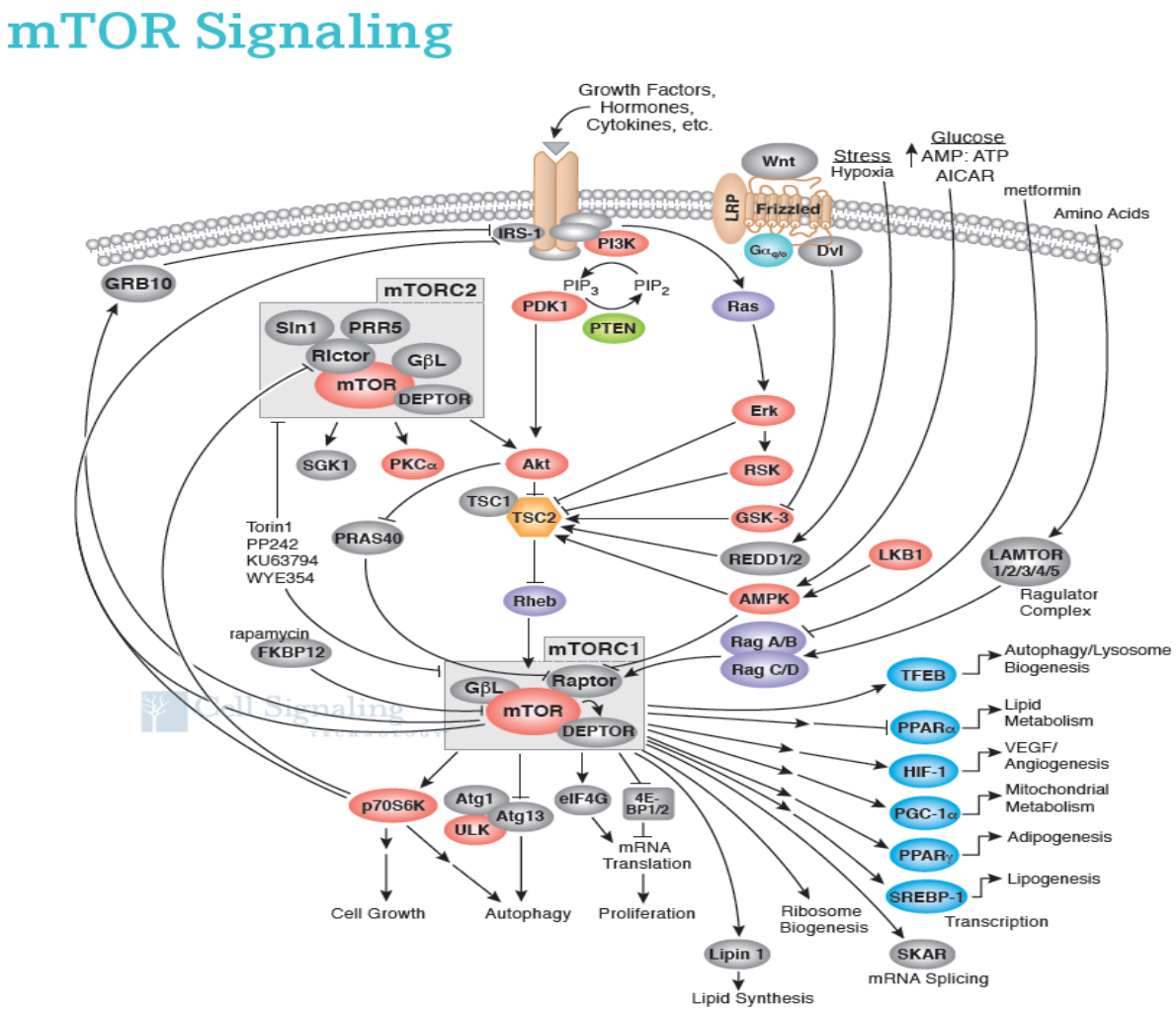

Folate studies

Our lab was interested in understanding how the timing and duration of folate restriction impacts colon carcinogenesis. Our initial studies in the β haploinsufficient (β-pol$^{+/-}$) mouse model yielded the surprising result of folate restriction conferring protection against colon cancer initiation as seen in the reduced numbers of ACF (figure 1.1). To further understand how a folate depleted diet impacts folate levels over time, we conducted a study with semi synthetic AIN93G-purified isoenergetic diet. Chow diet was not considered suitable for folate studies as it can contain variable amounts of folates that range from 2-15mg/kg of diet. Our folate adequate semi synthetic diet contained 2 mg of folic acid per kg of diet and our folate depleted diet had no added folate.

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Plasma folate decreased in the folate depleted (FD) group by the 3rd day on the diet and no further decrease was observed after the first week (figure 1.2). On average we observed a 90% decrease in plasma folate in animals fed FD diets (figure 1.3). The experiment was continued for 12 weeks and the animals did not exhibit any signs of anemia or pathologies. As a result, we termed this condition folate restriction rather than folate deficiency. Further pilot studies were conducted to study the effect of long-term folate restriction. After placing the animals on FD diets for 16 months, the animals did not display any signs of anemia and looked overall healthier than their FA counterparts. In a separate study, when we exposed long-term FD diet mice to the carcinogen dimethylhydrazine (DMH), the animals developed less ACF than the mice fed FA diet. This was in contradiction to our earlier studies and most published folate restriction studies[53]. It is important to point out that a review of the literature reveals that most studies investigating the effect of folate restriction on carcinogenesis place the animals on folate depleted diets for a relatively short duration before introducing the carcinogenic insult [54]. This short period of depletion does not allow the animals a chance to acclimate to folate restriction. To confirm the results, a larger experiment was designed to study the differential effect of the timing and duration of folate restriction on mice colon carcinogenesis. 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. As expected, we saw a significant (90%) decrease in folate levels in both the short term (FA/FR) and long term (FR) folate restricted groups as compared to controls (figure1.4). Remarkably, long term folate restriction conferred protection on animals that acclimated to folate restriction for 8 weeks. We observed that placing the animals on a folate restricted diet for one-week (FA/FR) before initiating DMH treatment resulted in a significant increase in ACF compared to the folate adequate (FA)

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animals. This finding was consistent with what is reported in the literature and our previous studies. However, animals on long term Folate restriction (FR) had less ACF in the colon compared to FA/FR animals (figure 1.5). This observation held true despite measured serum and tissue folate levels being comparably lower in both folate restricted groups (FA/FR) and (FR) compared to FA. We were faced with a paradox that begets the question: is all folate restriction disadvantageous with respect to CRC? And, how can the duration and the timing of folate restriction differentially impact the critical pathways that run amok in cancer? Furthermore, what other confounders are present in the standard models and study designs further skewing the results and leading to our incomplete understanding of the science?

To answer these questions, I began by examining the studies used to assess the effect of folate restriction on colon carcinogenesis. Many studies including ours resorted to the use of the antibiotic succinylsulfathiazole (SST) to reduce folate producing bacteria in the gut of the animals[36], [38]. SST radically alters the composition of the gut microbiome. We wondered if the use of SST can introduce a confounding element to our studies. The first part of my analysis addresses the potential of this antibiotic to alter critical pathways known to be affected by folate status such as mTOR, and oxidative stress. In this study, I analyzed the effect of SST on folate levels in folate adequate c57Bl/6 male mice fed folate supplemented diet. I did not observe any significant difference on growth and weight gain at 21 weeks. SST did not significantly affect folate levels in the plasma, liver and colon tissues of folate adequate mice, however it did alter energy metabolism and expression of key proteins in the mTOR signaling pathway in the liver. This research helps shed light on a possible confounding element when using SST to study folate depletion due to the potential overlap with multiple critical pathways such as mTOR.

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In the second part of the analysis, I will address the findings that the timing and the duration of folate restriction has a differential effect on colon carcinogenesis. As stated previously, I observed that long term folate restriction (FR) conferred protection to animals compared to short term (FA/FR). My research will attempt to elucidate a mechanistic analysis of how short- and long-term folate restriction differentially impact the critical pathway of mTOR, upstream regulators and downstream targets as well select gene expression analysis. This analysis will be done on animals with no exposure to the antibiotic SST, therefore, with an intact microbiome.

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Figure 1.1: *The effect of folate restriction on colon carcinogenesis in β-pol haploinsufficient mice.* Figure adapted from publication by Lucente et al [38]. 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. Bars indicate S.E. (n=6). Different letters indicate significant differences at $P < 0.05$.

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Figure 1.1: *The effect of folate restriction on colon carcinogenesis in β-pol haploinsufficient mice.*

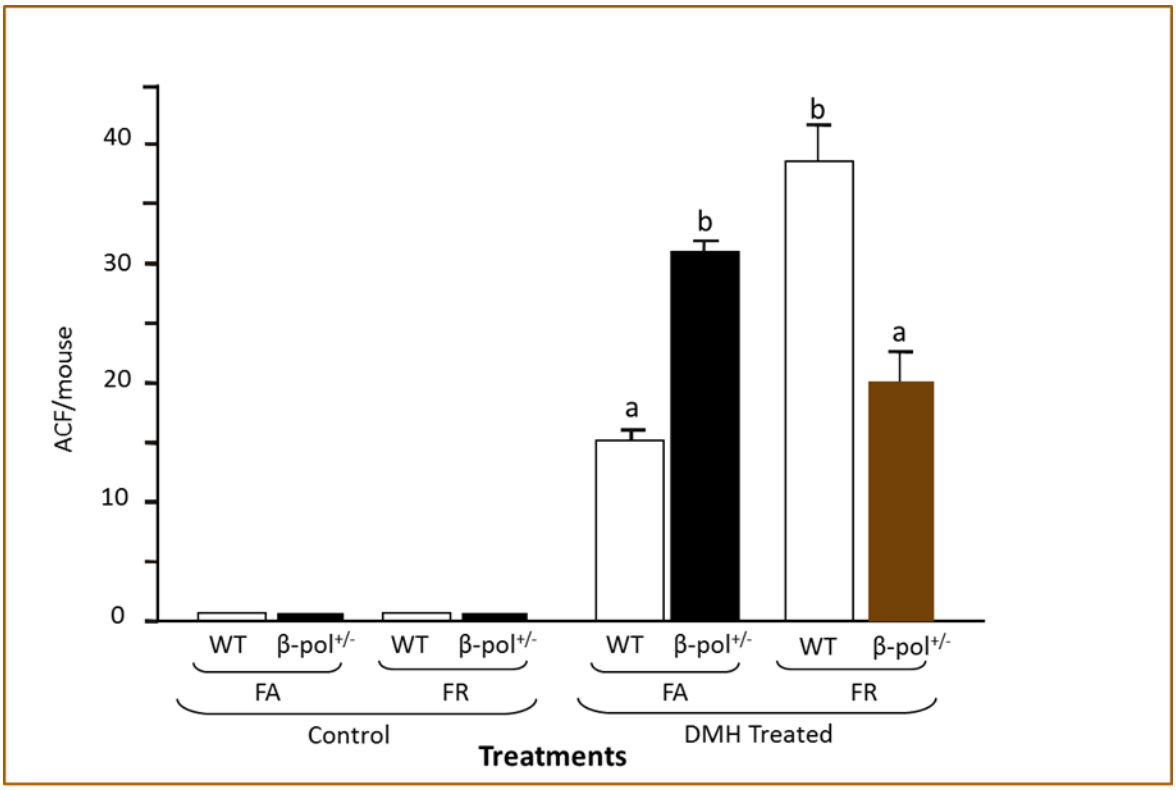

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Figure 1.2: *The effect dietary folate restriction on plasma folate levels at various timepoints.* C57BL/6 mice were fed either a folate adequate (FA) or folate depleted (FD) AIN93G-purified isoenergetic diet (Dyets, Inc., Lehigh Valley, PA) as previously 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]. (FA) represents the average value of folate levels for folate adequate fed mice normalized to 100%. The FD bars represent the average values of blood folate levels expressed as a percentage of FA levels at various time points. (n=6 per group). Bars represent S.E.M. Different letters indicate significant differences at $P < 0.05$.

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Figure 1.2: *The effect dietary folate restriction on plasma folate levels at various timepoints.*

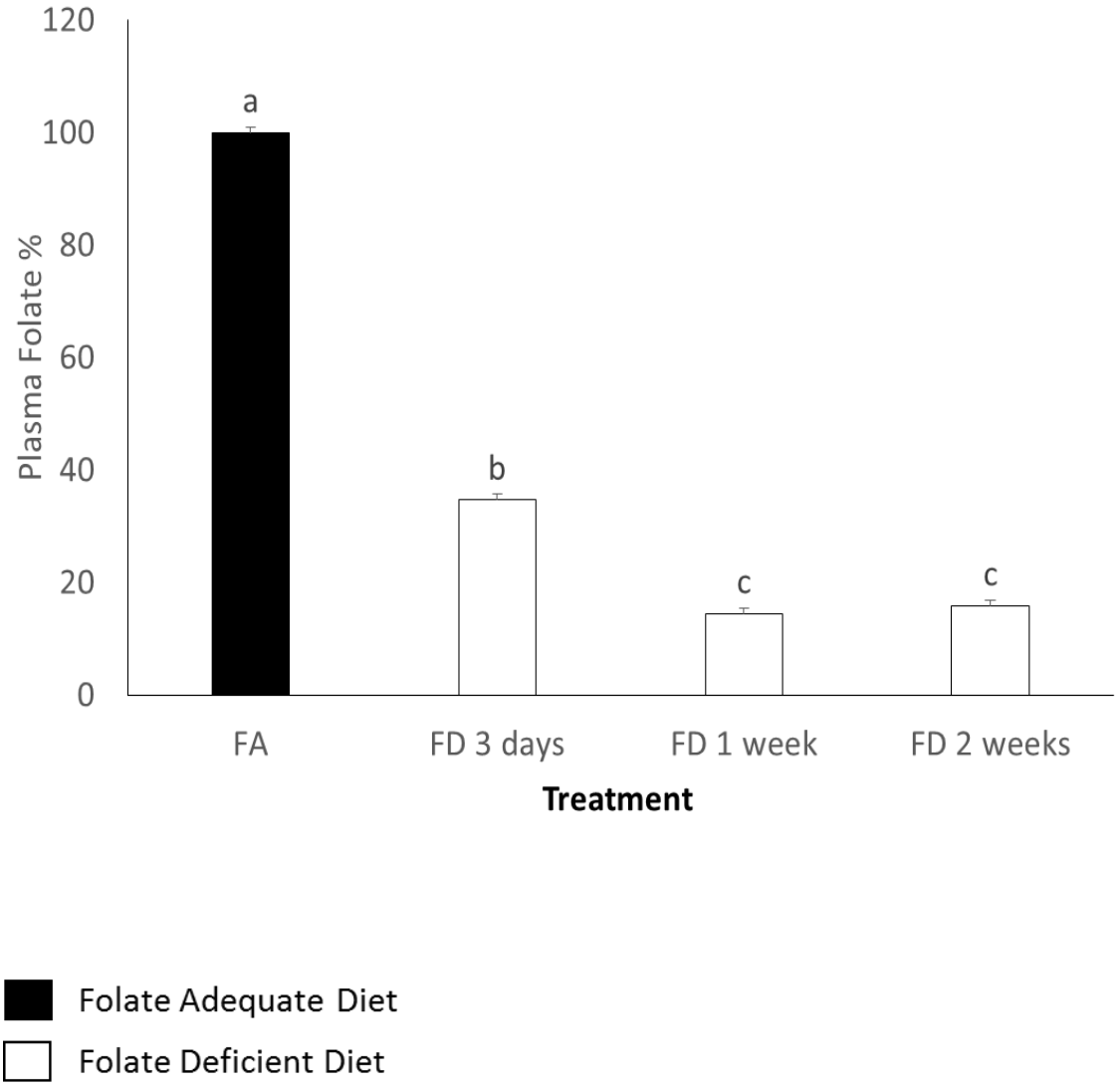

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Figure 1.3: *The effect of folate in the diet on plasma folate levels in C57BL/6 mice*.
The animals were fed either a folate adequate (FA) or folate depleted (FD) AIN93G-purified isoenergetic diet (Dyets, Inc., Lehigh Valley, PA). The FA group received 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 a lactobacillus casei microbiological assay. * Significant differences at $P < 0.05$.

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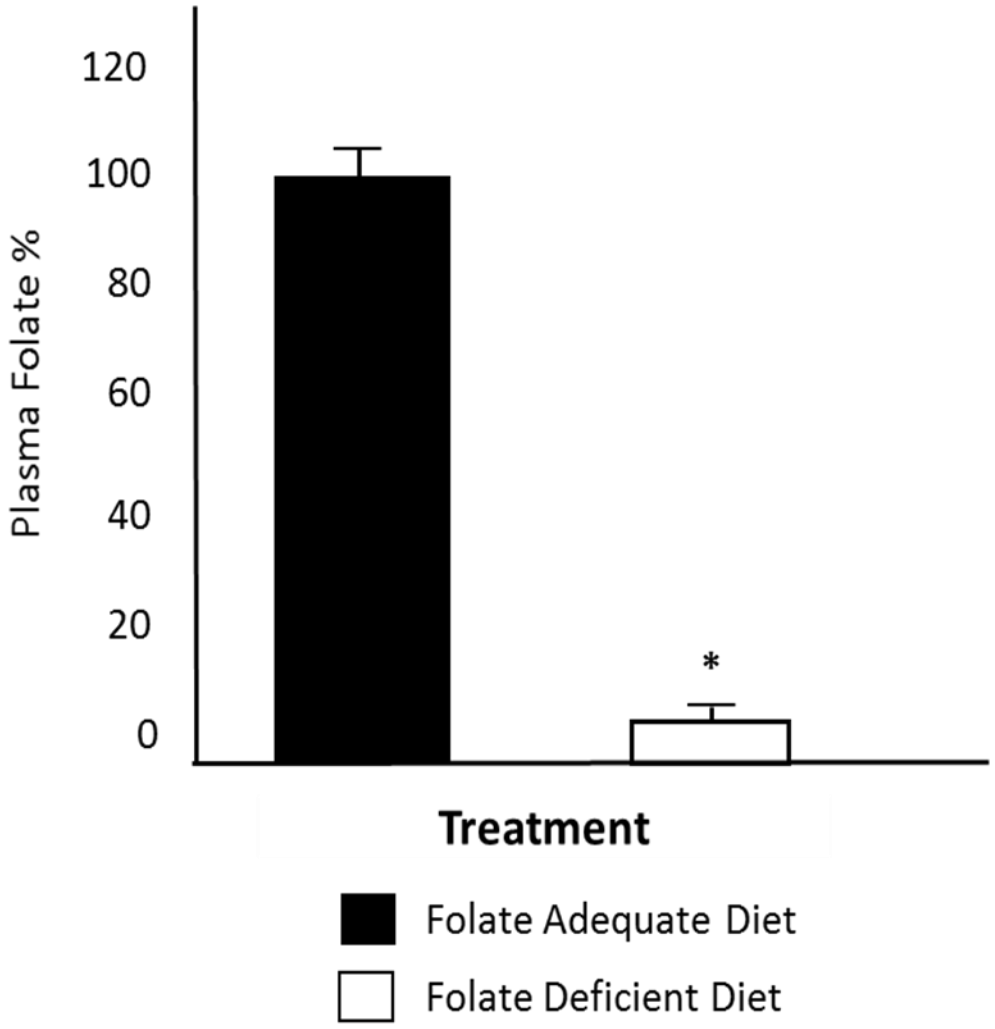
Figure 1.3: *The effect of folate in the diet on plasma folate levels in C57BL/6 mice.*



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CHAPTER 2: SUCCINYLSULFATHIAZOLE MODULATES mTOR SIGNALING

Introduction

The gut microbiome is a complex and diverse array of microorganisms that colonizes the digestive system. The gut microbiome of humans and other mammals have been extensively studied due to its profound effect on the health and disease of the host. Perturbations of the gut microbiome has been linked to various disease etiologies, including: metabolic diseases such as obesity and type 2 diabetes, autoimmune disorders, inflammation, allergies, cancer, and more[55]–[63]. The gut microbiota is responsible for producing a plethora of compounds and signaling molecules that facilitates the complex interactions with its host. These compounds include: branched chain fatty acids, organic acids, menaquinones (vitamin K), and some B vitamins[64], [65]. Specifically, the ability of certain bacteria in the gut to synthetize large amounts of folates is well documented[66], [67]. Others have shown that bacterial folate is easily absorbed and incorporated into the host folate stores in various tissues[6], [68]–[71]. The amount of folates produced by a healthy gut flora, specifically through fermentation in the large intestine, is diet dependent. In fact, diets rich in fermentable fibers have been shown to increase folate levels and decrease homocysteine in rats, while no effect was observed with non-fermentable fiber supplemented diets[72].

Other manipulation of the diet, such as the addition of antibiotics, can yield drastic changes to the gut microbiome[73]–[76]. Specifically, the addition of 1% succinylsulfathiazole (SST), a long-acting sulfonamide, can produce large shifts in the composition of the gut flora and its ability to synthetize folate[77], [78]. Succinylsulfathiazole is characterized by its poor absorption and localized antibacterial activity that is primarily limited to the gut[79]. It was used in the 1940s as gut antiseptic to lower the bacterial load and prevent infections after gut surgeries[77], [80], [81]. Similar to other sulfonamides in its class, SST works by inhibiting bacterial folate synthesis

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through competitive inhibition with P-aminobenzoic acid (PABA)[82]. SST persists in the gut much longer than absorbable antibiotics and has no known systemic toxicities[83]. It is hydrolyzed slowly in the large intestines by bacterial esterases to the active form sulfathiazole[79]. The effect of SST on gut microbial density and folate production is well documented in earlier studies dating to the 1940's[77], [80], [81]. Since then, SST has been replaced clinically by more efficacious and potent antibiotics. However, researchers have used it extensively in various studies to reduce or limit the folate producing bacteria in various lab models[36], [84]–[86].

In our studies, we have used SST along with dietary folate restriction to study the impact of folate depletion on colon cancer initiation[87]. Specifically, we were concerned that the use of SST to induce a folate deficient status in rodents can modulate some of the same pathways that are affected by folate depletion. In the present study, we were interested in examining the long-term effects of SST in the liver of C57BL6 mice fed folate supplemented diets. We analyzed the Impact of SST on folate levels in serum, colon, and Liver tissues. We were specifically interested in investigating the effect of the antibiotic on the mTOR signaling pathway, a major growth regulator that converges signals form growth factors, amino acids, glucose, stress, and energy levels, and impacts cellular growth, autophagy, and proliferation.

Experimental Procedures

Animals- c57Bl/6 male specific pathogen free mice were purchased from Charles Rivers at 6 weeks of age (n=3). Animals were maintained 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. They were fed the standard mouse chow and water ad libitum and were maintained on a 12-hr light/dark cycle until the start of the experiment. Mice were anesthetized in a CO2 chamber and the abdominal cavity was opened for excising the colon and harvesting the

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liver tissue. Blood was obtained by cardiac puncture, centrifuged, and the serum separated. The harvested liver was flash frozen and stored in liquid nitrogen.

Diets- At 4 months of age, mice were randomly assigned to two dietary groups and fed AIN93G-purified isoenergetic diets (Dyets, Inc., Lehigh Valley, PA)[88]. Diets were stored at -20°C. The control group received a folate adequate diet (2mg FA/kg diet for 21 weeks). The experimental group received a folate adequate diet supplemented with 1% succinyl sulfathiazole (2mg FA/kg diet + 1% succinyl sulfathiazole for 21 weeks).

Folate Assay- Serum, liver and colon mucosa were collected upon sacrifice. Folate was measured using the Lactobacillus casei microbiological assay of folic acid derivatives as described by Horne et. Al[89]. 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 nmol/g.

Isolation of Whole cell extract- Whole cell extracts were isolated using RIPA buffer. Briefly, 50 mg of liver tissue was homogenized using RIPA buffer (1% Igepal, 0.5% Sodium deoxycholate, 0.1% SDS, 1X PBS) combined with HALT protease inhibitor cocktail (Thermo Fisher). Whole cell extracts collected were stored at -80°C for further analysis.

Western Blot Analysis- Protein expression analysis was performed using the V3 western blot system from Bio-Rad. 50 µg of protein was loaded onto criterion TGX stain free precast Gels (Bio-Rad, Hercules, CA). Gels were imaged using chemidoc XRS+ system then wet transferred onto nitrocellulose membranes. Membranes and Gels were imaged to verify complete transfer. Manufacturer recommended dilutions of anti-sera developed against various anti-bodies (cell signaling) 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

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quantified using chemidoc XRS+ imager and Image lab software (Bio-Rad, Hercules, CA) after incubation in SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). Data are expressed as adjusted band volume normalized to total lane proteins. Phosphorylated forms of proteins were normalized to total and expressed as ratio.

Gene expression profiling- The mRNA expression levels of various genes 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 poly-A primers. Expression of each gene was quantified using real time PCR with specific primers for the gene. The gene transcript was normalized to GAPDH. External standards for all genes were prepared by subcloning the amplicons, synthesized using the specific primers into PGEM-T easy vector. Primer Sequences are listed in **Table 2.1**.

ATP Assay- ADP/ATP ratio was assessed using a luciferase based bioluminescent assay kit (Abcam). Briefly, 10 mg of liver tissue was quickly homogenized with nucleotide releasing buffer. Using a microplate reader, the homogenate was assayed per protocol to determine ATP induced luminescence. Then ADP converting enzyme was added and luminescence was further determined. Data are expressed at ADP/ATP ratio.

NAD/NADH Assay- NAD^+/NADH was assayed using a fluorometric microplate reader assay (Abcam). 20 mg of liver tissue was homogenized in provided lysis buffer and supernatant was collected. Total NAD, NAD^+ and NADH were assayed per protocol. Fluorescence was measured at EX/EM=540/590 nm. Data are expressed at NAD^+ to NADH ratio.

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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

Antibiotic in the Diet and its Effect on Weight gain, Serum, Liver and Colon Folate Levels:

The purpose of this study was to analyze the potential confounding effect of antibiotics used to induce folate depletion in mouse folate studies. Since antibiotics have been used non-therapeutically in animal husbandry to promote weight gain and growth[90], we were interested in examining the long-term (21 weeks) effect of SST on animal weights. We monitored weight gain weekly for the duration of the study, however there was no difference after 21 weeks on the diets (**Figure 2.1 a**). The animals grew normally and did not exhibit any gross anomalies. We also did not observe any differences in food consumption (**Figure 2.1 b**) between the control and experimental groups.

We were interested in determining whether long term SST alone influences folate levels in the animals. As expected, we did not observe any significant difference in tissue specific folate levels between animals fed control (folate supplemented) diet and treated (folate supplemented + 1% SST) diet (**Figure 2.1 c**). Liver folate levels were highest (control: 48.1 ng/ml, treatment: 53.7 ng/ml) compared to serum (control: 32.7 ng/ml, treatment: 34.1 ng/ml), and colon folate (control: 4.2 ng/ml, treatment: 2.8 ng/ml). This is consistent with findings of previous studies. SST is known to competitively inhibit folate uptake in the colon due to structural similarities to folic acid, however we did not see a significant difference in colon folate levels in the AB treated group. In contrast, animals fed a folate restricted diet with 1% SST for 21 weeks exhibited a significant

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decrease of folate levels in plasma, liver and colon (**Figure 2.1 c**). Thus, we show here that SST alone does not significantly affect liver, serum and colon folate levels.

Our lab has previously shown that folate depletion provides protection against colon carcinogenesis in β-pol haploinsufficient mice[87]. We have also seen that folate status in the diet alters expression of genes in mTOR signaling (**Figure 2.2**); a major nutrient sensing pathway that has been implicated in cancer and aging. Since SST is widely used in folate depletion mouse studies, it became critical to understand the effect of SST alone on mTOR signaling.

Antibiotic Effect on the PI3K/AKT/mTOR Signaling Pathway:

mTOR, mammalian target of rapamycin, is a conserved serine/threonine protein kinase downstream of the PI3K/AKT signaling pathway[43]. It is present in two multiprotein complexes (mTORC1 and mTORC2) and integrates signals from multiple upstream factors such as insulin, hormones, cytokines, growth factors, stress, hypoxia, glucose, energy and amino acid levels (**Figure 2.3**). mTORC1 specifically is a key player in regulating multiple downstream processes such as cell growth, proliferation and autophagy[91]. mTOR signaling has been shown to be dysregulated in various cancers and is a key target in multiple life extension strategies[44], [92], [93]. Since our array data previously showed that mTOR signaling is reduced under FR (**Figure 2.2**) it became crucial to determine whether SST has a confounding effect on the expression of proteins in this pathway.

PDK1 (3-phosphoinositide-dependent protein kinase 1) is an upstream activator of mTORC1 in the IRS-1/PI3K/AKT pathway. It phosphorylates AKT at the Thr308 residue, inhibiting the TSC1/TSC2 complex and activating mTORC1[94]. mTORC2 on the other hand phosphorylates AKT (Ser473) as a feedback mechanism activating mTORC1. SST alone significantly decreases expression of PDK1 protein in our folate adequate model (**Figure 2.4 a**),

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has no effect on AKT expression (**Figure 2.4 b**) and significantly decreases phosphorylation of mTORC1 at the ser2448 residue (**Figure 2.4 c-d**).

We examined the role of SST and its effect on mTORC1 regulation of protein synthesis and autophagy (**Figure 2.3**). The main characterized substrates of mTORC1 in regulating translation are S6K1 (ribosomal p70 S6 protein kinase) and 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1)[94]. In line with decreased mTORC1 expression, we saw that SST significantly decreases 4E-BP1 phosphorylation (**Figure 2.4 e**) but does not influence S6K1 phosphorylation (**Figure 2.4 f**). We looked at the effect of SST on the expression of autophagy related proteins Beclin, ATG5 and LCIII and we did not observe any differences (**Figure 2.4 g-i**). Observing that SST alters expression of proteins in the PI3K/AKT/mTOR signaling pathway, we were interested in understanding whether it alters other upstream regulators of TOR signaling, specifically regulators of stress and energy metabolism.

The effect of SST on upstream regulators of mTOR:

The mTOR signaling pathway is directly altered in response to hypoxia, DNA damage and energy status[91]. REDD1 (regulated in development and DNA damage response 1) is induced following exposure to hypoxia, DNA damage and other environmental stresses such as energy stress and reactive oxygen species[95], [96]. An increase in REDD1 levels lead to the activation of TSC1/TSC2 complex and inhibition of mTORC1 (**Figure 2.3**). Interestingly, REDD1 protein expression in the liver significantly increases in response to SST (**Figure 2.5 a**), consistent with the inhibition of mTOR seen (**Figure 2.4 c**).

The mTOR signaling pathway has also been shown to be altered in response to energy status[97]. AMPK, a metabolic fuel gauge that detects changes in intracellular AMP/ATP ratio, directly inhibits mTORC1[98]. It is activated in response to nutrient depletion and acts to maintain

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energy stores by switching on pathways that produce ATP and switching off pathways that consume ATP[97]. AMPK also enhances SIRT1 activity by increasing cellular levels of NAD^+ [99]. NAD cycles between the oxidized (NAD^+) and reduced forms (NADH), partaking a central role in cellular metabolism and energy production. We observed a significant decrease in ADP/ATP ratio (**Figure 2.5 b**) and AMPK phosphorylation (**Figure 2.5 c**) in response to SST. However, we did not see an effect on SIRT1 expression (**Figure 2.6**) or NAD^+/NADH levels (**Figure 2.5 d**) in the SST fed group. Our study sheds light on SST affecting expression of multiple regulators of mTOR independent of folate status in the diet.

Discussion

Recently, various laboratories began uncovering the complex interactions between the gut microbiome and the host [56], [58]–[61], [64], shedding new light on the importance of these microorganisms on the overall health. Antibiotic use in laboratory models can introduce significant confounding elements to an experiment due in part to the perturbation of the intestinal microbiome[74], [75], [100].

In our current study, the antibiotic alone did not influence serum and liver folate levels. We did however see a non-significant decrease in colon folate in the SST group, possibly due to the localized effect of the antibiotic on the gut flora. Since SST is used to deplete folate in folate restriction studies directly impacting one Carbon metabolism[36], [84]–[86], we were interested in looking at the effect of the antibiotic alone on the expression of genes in the transulfuration pathway. SST did not have a significant effect on the expression of cystathione-beta-synthase (CBS) the rate limiting enzyme in the transulfuration pathway or on the expression of anti-oxidant genes (TRX, TRXR, PRDX, OGG1, GPX1, GPX3, SOD and CAT) (**Figure 2.6**).

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Analysis of cellular energy levels revealed an increase in energy availability (decrease of ADP/ATP ratio) in SST fed mice. This was consistent with decreased activation of the cellular energy gauge AMPK. Conversely, we did not observe a change in NAD^+/NADH ratio consistent with SIRT1 mRNA expression.

Analysis of protein expression of targets in the mTOR signaling pathway revealed decreased activation upstream (PDK1), at both phosphorylated residues of mTOR (ser2448, ser2481), and at the downstream target (4ebp1). We looked at the expression of a downstream target of S6K1 important in cell cycle regulation and proliferation, P16[94], but we did not see any significant difference (**Figure 2.6**). Interestingly, we saw increased activation of REDD1, an inhibitor of mTOR signaling that responds to stress and hypoxia. Paradoxically, increased energy levels leading to the inactivation of AMPK did not result in upregulating mTOR.

Since SST is poorly absorbed, we suspect that the effect we saw is mediated through the perturbation of the intestinal microflora, where further research is warranted. Recently, the gut microbiome has been linked to complex interactions that affect almost all physiological functions including but not limited to metabolism, immunity, inflammation, stress, and cancer [58], [60], [61], [79]. This research helps shed light on a possible confounding element when using SST to study folate depletion due to the potential overlap with multiple critical pathways such as mTOR.

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Table 2.1: Primer Sequences

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'
Gadd45g	AGTTCCGGAAAGCACAGCCAGGATG	GCCAGCACGCAAAAGGTCACATTGT
GAPDH	GCACAGTCAAGGCCGAGA	ATGGGGGCATCGGCAGA
Sirt1	TGATTGGCACCGATCCTCG	CCACAGCGTCATATCATCCAG
TRXR	CTATTCAGCAGAGCGGTTCC	ACATTCCAAGGCGACATAGG
GPX1	CGAAGTGAATGGTGAGAAGG	TCAAAGTTCCAGGCAATGTC
PRDX6	AGCAGGTCCGTAGAAAGATCG	TCAAAGAGAGCCAGTCAGTAGG
TRX	CTGATCGAGAGCAAGGAAGC	TCATCCACATCCACTTCAAGG
OGG1	CGGCTGGCATCCTAAGACATC	AACAGGCTTGGTTGGCGAAGG
GPX3	CCATTTGGCTTGGTCATTCTGG	TCCCCGTTCACATCTCCTTTC
CAT	CTACCCGGACACTCACCGCC	GGGGCACCACCCTGGTTGTCA
SOD1	AACCAGTTGTGTTGTCAGGAC	CCACCATGTTTCTTAGAGTGAGG
CBS	CACAGTGCTGACCAAATCCCCC	CACACTTGGCCAAGAGCTCAC
CTH	GCTAGAGGCAGCGATTACACC	GCAGACATGAAGGTGTTATCTACAACC

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Figure 2.1: *1% succinylsulfathiazole (SST) in the diet does not impact weight gain, food intake or folate levels in folate adequate c57BL/6 mice.*
a) Average mouse weights at the beginning of the experiment and after 21 weeks on respective AIN93G semi synthetic diets (Control= folate adequate diet (2 mg folate/kg diet), Treatment= folate adequate diet (2 mg folate/kg diet) + SST). b) Average food consumption at the beginning and at the end of the study. c) Folate levels (nmol/g) in the serum, liver and colon of mice fed control and treatment diets compared to folate levels in mice fed a folate restricted diet (0 mg folate/ kg diet) with 1% SST (FR +AB). (n=3). Bars, S.E.M, *P < 0.05.

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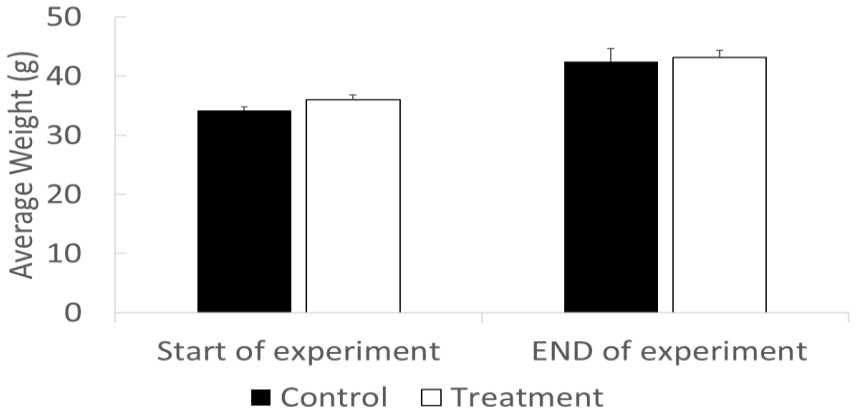
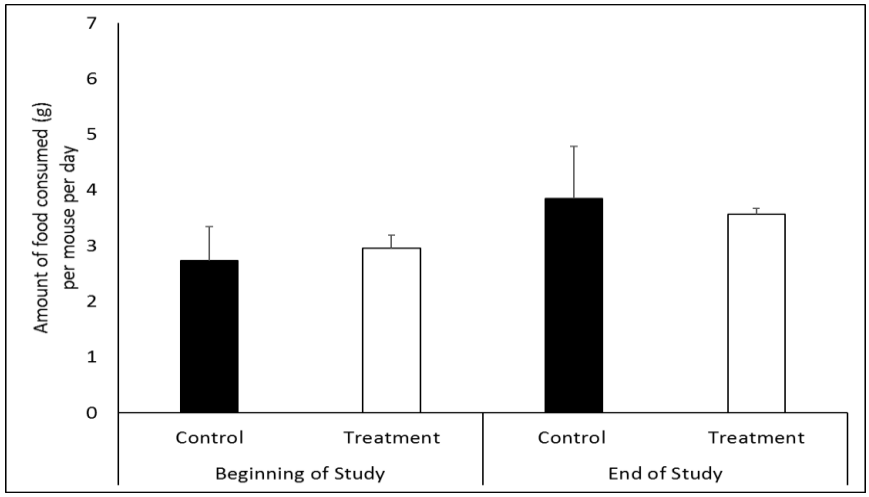
Figure 2.1: *1% succinylsulfathiazole (SST) in the diet does not impact weight gain, food intake or folate levels in folate adequate c57BL/6 mice.*

a



b



c

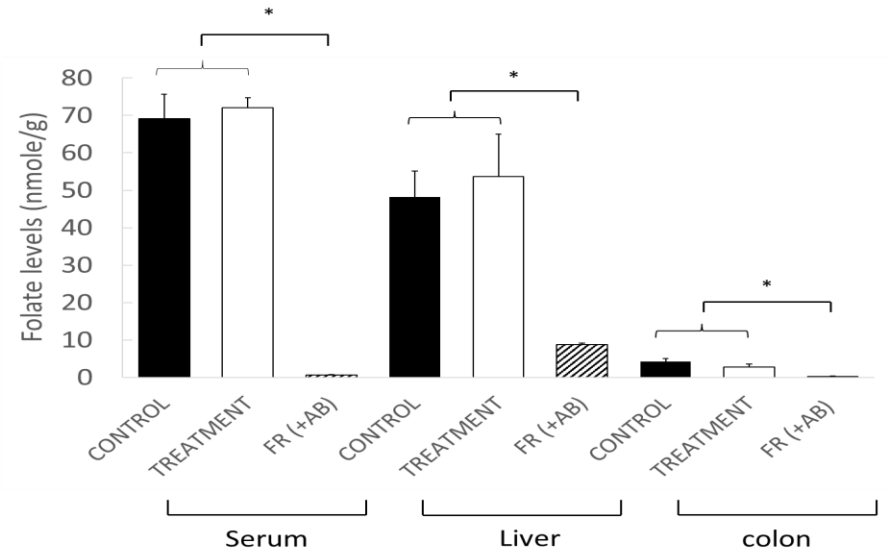


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Figure 2.2: Summary of the effect of folate restriction on expression of genes in the mTOR pathway.

Data adapted from Lucente et al [38]. Gene expression shown as fold change in β-pol$^{+/-}$ DMH/folate restricted and WT/folate restricted compared to WT/folate adequate. differentially expressed genes from colon mucosa were analyzed using the Agilent Whole Mouse Genome oligonucleotide microarray containing probes for over 41,000 well characterized genes and analyzed with Agilent Feature Extraction software, version A.5.1.1. The false discovery rate for FDWT was 5.1%, 1.7% for FDWT DMH treated, and 0.39% for FD β-pol+/− DMH-treated experimental groups. Gene ontology analysis was performed using the Gene Ontology Tree Machine (GOTM) (Bioinformatics, Vanderbilt University). Single gene set analysis was performed, where GOTM compares the distribution of single gene sets in each GO category to those in an existing reference gene list from the mouse genome, identifying GO categories with statistically significant enriched gene numbers as determined by the hypergeometric test ($p < 0.01$).n=4 per group, P<0.01 where value is dispayed; ns, no significant difference

Summary of the effect of folate restriction on mTOR pathway genes

Accession No.	Gene Symbol	Gene Name	β-pol$^{+/-}$ DMH/FR	WT FR
NM_133781	Cab39	Calcium binding protein 39	2.1	-1.2
NM_011492	Stk11	Serine/threonine kinase 11	1.2	-1.2
BC043920	**Frap1**	**FK506 binding protein 12-rapamycin associated protein 1**	**-1.8**	**-1.3**
AK029196	4921505C17Rik	RIKEN cDNA 4921505C17 gene	2.1	1.5
AK129326	4932417H02Rik	RIKEN cDNA 4932417H02 gene	-1.5	ns
NM_007918	Eif4ebp1	Eukaryotic translation initiation factor 4E binding protein 1	ns	ns

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Figure 2.3: The mTOR signaling pathway.

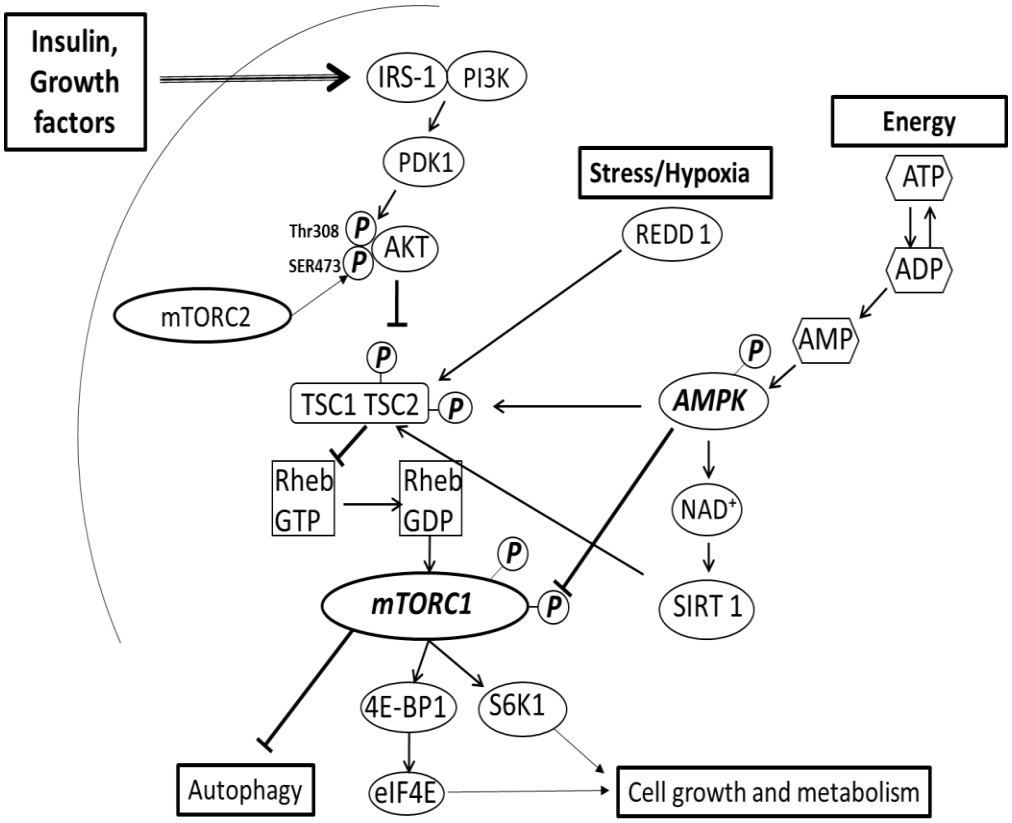

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Figure 2.4: Succinylsulfathiazole alters expression of proteins involved in the PI3K/AKT/mTOR signaling in the liver of c57BL/6 mice resulting in an overall decreased mTOR signaling.
Western blot images and quantification of: a-b) phosphorylation of upstream regulators of mTOR (PDK-1 (ser241) and AKT(ser473)), c-d) mTOR phosphorylation at ser2448 and ser2481 residues, e-f) phosphorylation of downstream targets of mTOR (4ebp1 (thr37/46) and s6k1(thr421/ser424)), and g-i) expression of autophagy related proteins (beclin, atg5 and cleaved lc3) in the liver of mice fed control and treatment diets. Protein expression analysis was performed using the V3 western blot system from Bio-Rad. Mice were placed on respective AIN93G semi synthetic diets for 21 weeks; Control= folate adequate diet (2 mg folate/kg diet), Treatment= folate adequate diet (2 mg folate/kg diet) + SST) n=3 per group; Bars, S.E.M., * $P < 0.05$.

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Figure 2.4: Succinylsulfathiazole alters expression of proteins involved in the PI3K/AKT/mTOR signaling in the liver of c57BL/6 mice resulting in an overall decreased mTOR signaling.

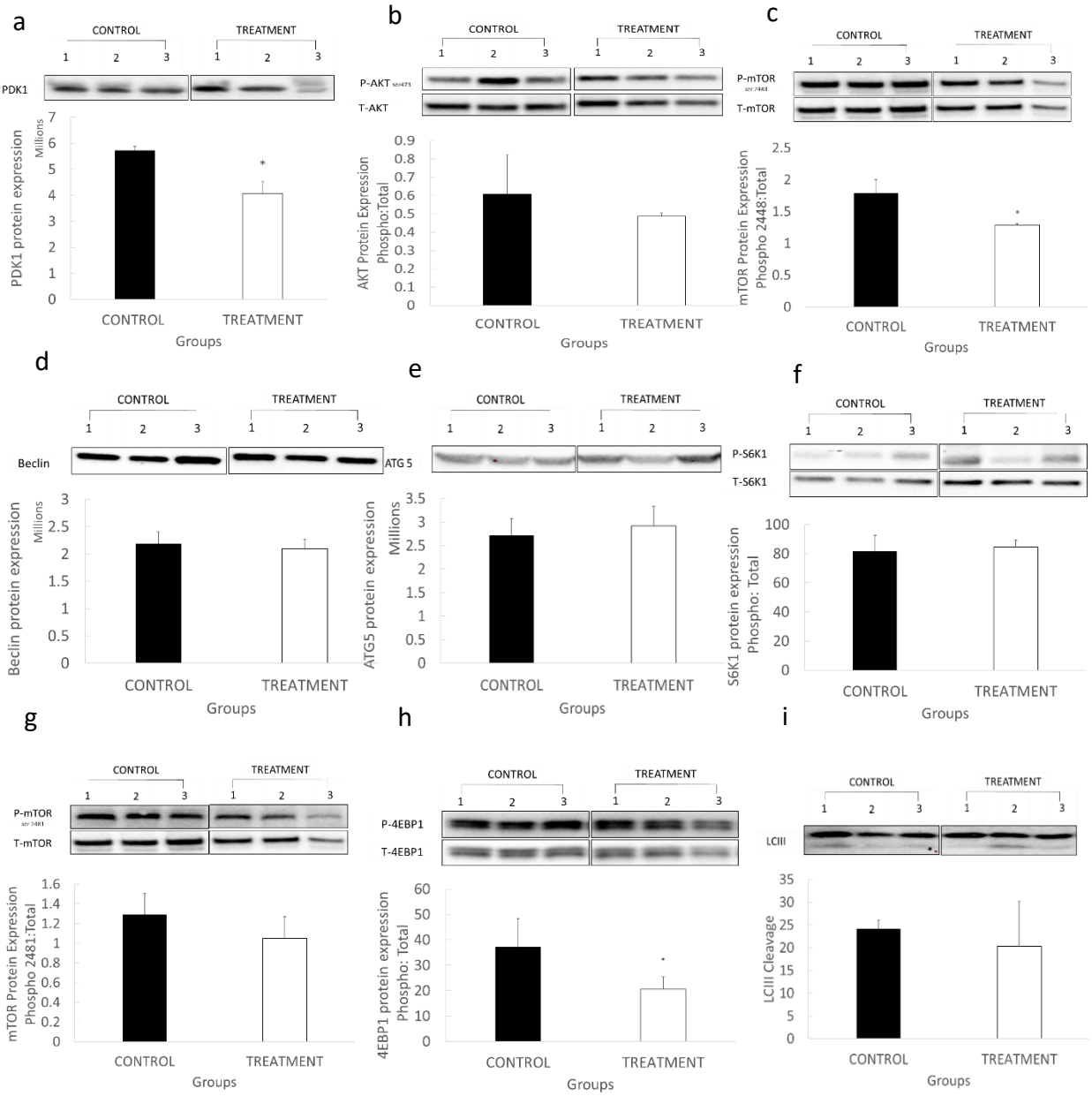

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Figure 2.5: Effect of SST on upstream regulators of mTOR in the liver of c57BL6 mice. a) Western blot image and quantification of REDD1 protein expression, b) ADP/ATP ratio, c) western blot image and quantification of AMPK (thr172) protein phosphorylation and d) NAD^+/NADH levels. n=3 per group,Bars S.E.M., * $P < 0.05$.

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Figure 2.5: Effect of SST on upstream regulators of mTOR in the liver of c57BL6 mice.

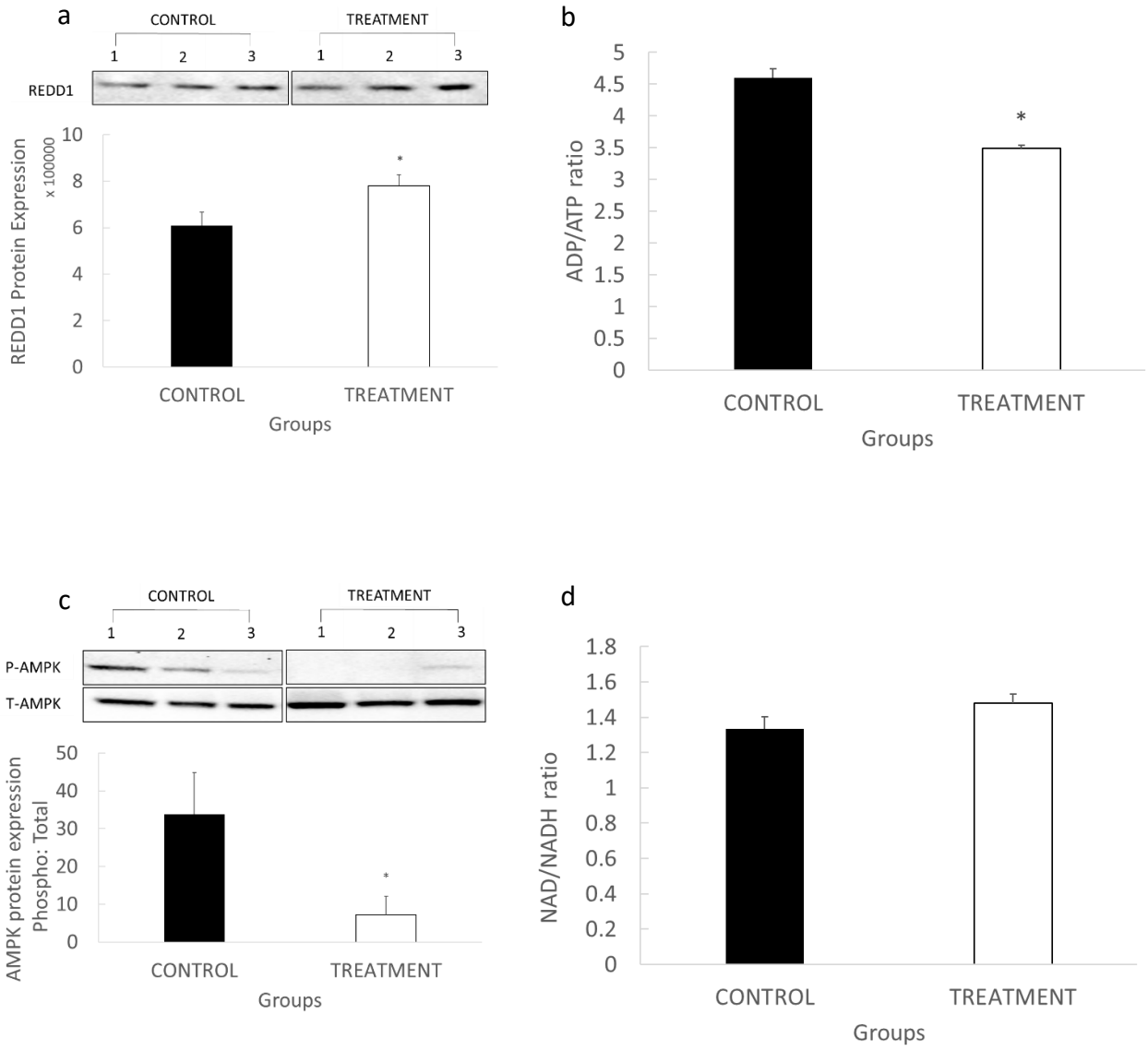

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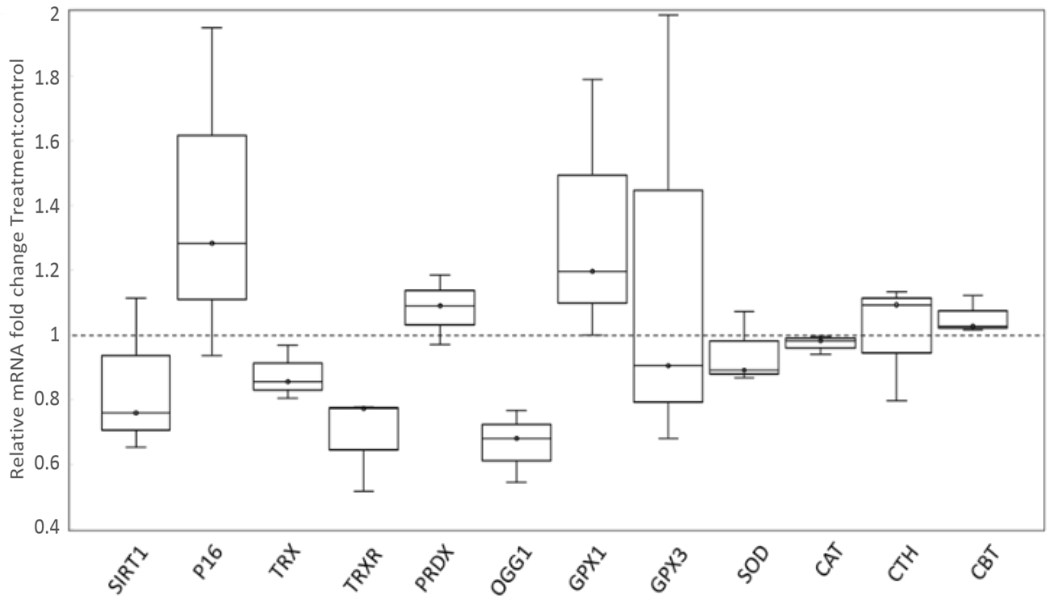
Figure 2.6: Effect of SST on relative expression of genes known to be altered by dietary folate status in the liver of mice (Treatment: Control).
Analysis of mRNA levels in liver of FA SST and FA mice. Mice were placed on respective AIN93G semi synthetic diets for 21 weeks; Treatment= folate adequate diet (2 mg folate/kg diet) + SST), Control= folate adequate diet (2 mg folate/kg diet). Expression of the genes was determined using a real time PCR technique and the level was normalized to Gapdh expression as described under "Experimental Procedures". Data was expressed as box plots representing fold ratio of treatment/control. No significant differences were observed. n=3 per group; Bars, S.E.M., * P < 0.05, Bars S.E.M.



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CHAPTER 3: Timing and Duration of Folate Restriction Differentially Impacts Colon Carcinogenesis

Introduction

Colorectal cancer (CRC) is the second most common reported cancer in western societies [101]. The majority of CRC are due to sporadic causes and are not linked to specific mutations [102]. Diet plays a crucial role in the risk and development of CRC [103]. Notably, folates, contained in vegetables and leafy greens have been associated with a protective effect [19]. Folate relevance came to light due to findings that folates reduce NTD, leading to the mandatory fortification of cereals and grains [11]. Folates are crucial to purines and pyrimidine synthesis, as well as remethylating homocysteine to methionine [104]. Low dietary folates can lead to uracil misincorporation in the DNA resulting in double strand breaks, and impaired methylation leading to genetic instability and inducing transformation [22], [105].

Research into the effect of folate restriction on colon carcinogenesis has yielded conflicting results. Considerable evidence from the literature suggests that high dietary folates reduced CRC risk while low folate induces it [33], [106]. However, there have been many discordant results reported in animal studies and from large epidemiological and case control studies [32], [33], [13], [5]. These studies raised concerns that the consequences of the mandatory folate fortification could lead to detrimental outcomes for a large subset of the population.

Our lab previously showed that a folate depleted diet in a β-pol haploinsufficient mouse model provided protection against CRC initiation as seen in reduced incidences of preneoplastic lesions (ACF) [87]. Others were able to show that folate restriction protected against intestinal tumorigenesis in various lab rodent models [39]–[41], [107]. In this study, we show that long term folate restriction protects against initiation of tumorigenesis in male c57Bl/6 mice. We observed

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that placing mice on one week of folate restricted diet before initiating carcinogenic (FA/FR) treatment produced more ACF compared to animals allowed to acclimate to folate restriction for 8 weeks (FR). Analysis of the mTOR signaling pathway and DNA methyltransferases revealed a divergent effect between long-term folate restriction FR compared to short-term FA/FR. Our results indicate that the timing and duration of folate restriction has a differential effect on colon tumorigenesis.

Experimental procedures

Animals- c57Bl/6 male specific pathogen free mice were purchased from Charles Rivers at 6 weeks of age. Animals were maintained 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. They were fed the standard mouse chow and water ad libitum and were maintained on a 12-hr light/dark cycle until the start of the experiment. Mice were anesthetized in a CO2 chamber and the abdominal cavity was opened for excising the colon and harvesting the liver tissue. Blood was obtained by cardiac puncture, centrifuged, and the serum separated. The harvested liver was flash frozen and stored in liquid nitrogen.

Diets- At 4 months of age, mice were randomly assigned to two dietary groups and fed AIN93G-purified isoenergetic diets (Dyets, Inc., Lehigh Valley, PA)[88]. Diets were stored at -20°C. The control group received a folate adequate diet (2mg FA/kg diet for 8 weeks). The experimental groups: (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), and (FR) received a folate deficient diet (0 mg folic acid/kg diet for 8 weeks). Control and experimental groups were further divided equally into diets supplemented with either 1% of the antibiotic succinylsulfathiazole or antibiotic free diets.

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Carcinogen treatment- 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/liter of $NaHCO_3$ (Fisher Scientific) once a week for 6 weeks. The animals were monitored for signs of toxicity and remained on their respective diets for an additional 7 weeks.

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. 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 [36], [87]

Folate Assay- Serum, liver and colon mucosa were collected upon sacrifice. Folate was measured using the Lactobacillus casei microbiological assay of folic acid derivatives as described by Horne et. Al[89]. 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 nmol/g.

Isolation of Whole cell extract- Whole cell extracts were isolated using RIPA buffer. Briefly, 50 mg of liver tissue was homogenized using RIPA buffer (1% Igepal, 0.5% Sodium deoxycholate, 0.1% SDS, 1X PBS) combined with HALT protease inhibitor cocktail (Thermo Fisher). Whole cell extracts collected were stored at -80°C for further analysis.

Western Blot Analysis- Protein expression analysis was performed using the V3 western blot system from Bio-Rad. 50 µg of protein was loaded onto criterion TGX stain free precast Gels (Bio-Rad, Hercules, CA). Gels were imaged using chemidoc XRS+ system then wet transferred onto nitrocellulose membranes. Membranes and Gels were imaged to verify complete transfer. Manufacturer recommended dilutions of anti-sera developed against various anti-bodies (cell

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signaling) 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 chemidoc XRS+ imager and Image lab software (Bio-Rad, Hercules, CA) after incubation in SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). Data are expressed as adjusted band volume normalized to total lane proteins. Phosphorylated forms of proteins were normalized to total and expressed as ratio.

Gene expression profiling- The mRNA expression levels of various genes 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 poly-A primers. Expression of each gene was quantified using real time PCR with specific primers for the gene. The gene transcript was normalized to GAPDH. External standards for all genes were prepared by subcloning the amplicons, synthesized using the specific primers into PGEM-T easy vector.

ATP Assay- ADP/ATP ratio was assessed using a luciferase based bioluminescent assay kit (Abcam). Briefly, 10 mg of liver tissue was quickly homogenized with nucleotide releasing buffer. Using a microplate reader, the homogenate was assayed per protocol to determine ATP induced luminescence. Then ADP converting enzyme was added and luminescence was further determined. Data are expressed at ADP/ATP ratio.

NAD/NADH Assay- NAD^+/NADH was assayed using a fluorometric microplate reader assay (Abcam). 20 mg of liver tissue was homogenized in provided lysis buffer and supernatant was collected. Total NAD, NAD^+ and NADH were assayed per protocol. Fluorescence was measured at EX/EM=540/590 nm. Data are expressed at NAD^+ to NADH ratio.

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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

Study Design and Rationale

Folate requirements in mice is not well defined. It is estimated that the minimum satisfactory requirement for folic acid based on early mouse studies to be around 0.5 mg/kg diet [108]. Modern rodent diets add a minimum of 2 mg/kg diet folic acid to the standard formulations [109]. Chow diets contain variable amounts of folic acid ranging from 2-15 mg/kg diet and averaging about 8 mg folic acid/kg diet. Mice fed a folate deficient diet experience a quick reduction in serum folate levels by as much as 60% after 3 days and is further reduced by 90% after 1 week. Maintaining the animals on a folate deficient diet does not yield any additional reduction in serum folates after 12 weeks (fig1.3). As a result, rodent studies investigating the effect of folate restriction typically do not maintain the animals on folate deficient diets for extended periods. A long-term folate restriction aging study conducted by our lab was able to maintain the mice on a folate depleted diet for over 32 months with the mice exhibiting better health and survival than their folate supplemented counterparts (unpublished data). We hypothesized that the animals might be getting trace amounts of folates from the casein-based AIN-93G diet as well as a result of gut bacterial fermentation and coprophagy. Since the animals did not exhibit any signs of anemia in any of our previous experiments, and no further reduction of serum folate levels was observed after the first week, we termed the condition folate restriction (FR) rather than folate deficiency (FD). For the purpose of this study, we chose a semi synthetic AIN-93G diet allowing us to manipulate the formulation while delivering consistency. The control

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group received a folate adequate diet (2mg FA/kg diet for 8 weeks). The experimental groups: (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, and (FR) received a folate deficient diet (0 mg folic acid/kg diet for 8 weeks). Control and experimental groups were further divided equally into diets supplemented with either 1% of the antibiotic succinylsulfathiazole SST (ABx+) or antibiotic free diets (ABx-) (fig 3.1). Succinylsulfathiazole (SST), a long-acting sulfonamide, works by inhibiting bacterial folate synthesis through competitive inhibition with P-aminobenzoic acid (PABA)[82]. After 8 weeks on the respective diets, mice were treated IP with 1,2-dimethylhydrazine (DMH) once a week for 6 weeks. DMH, an alkylating agent, is a potent colon and liver carcinogen that is extensively used to model colon carcinogenesis in rodents [110]–[114]. Animals remained on their respective diets until they were sacrificed at 21 weeks. There was no difference in food consumption and animal weights between experimental groups and their respective controls at various time points in the experiment (fig3.2).

Effect of folate restriction on folate levels in plasma, liver, and colon.

Folate levels were measured using the Lactobacillus casei microbiological assay of folic acid derivatives as described by Horne et. Al[89]. As expected, serum folate levels decreased significantly as a result of both short (FA/FR) and long-term (FR) diets compared to folate adequate (FA) diets in both control (CONT) and DMH treated animals (DMH) (fig3.3). The reduction of serum levels was not significantly different between FA/FR and FR animals. Animals fed diets containing 1% SST (ABx+) showed a greater decrease in serum folate levels when compared to mice without the antibiotic (ABx-). DMH had no effect on serum folate levels across all groups except for the FR DMH ABx+ group, where folate levels were too low to be assayed. Likewise, we observed an equal and significant reduction in liver folate levels under both FA/FR

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and FR conditions compared to FA. The addition of SST further reduced folate levels in the folate restricted control groups and more significantly in the DMH treated animals (fig3.4). Analysis of the folate levels in the colons of control animals revealed a similar pattern to the liver and serum. Folate levels significantly decreased to the same extent in both FA/FR and FR animals compared to FA (fig3.5). Animals fed folate deficient diets supplemented with SST experienced a more pronounced decrease in folate levels in the colons. Folate data were not collected from DMH animals as the colons were stained for ACF screening. A marked decrease of folates was observed in the serum of mice fed a folate deficient diet compared to the liver and colon tissues. It was clear from our analysis that the addition of the antibiotic SST produced a distinct and significant effect on folate levels in all tissues examined owing to its ability to significantly reduce the number of folate producing bacteria in the gut. As established previously, since SST perturbs the intestinal microbiome and potentially confounds our analysis, we conducted all further mechanistic analysis on non-SST supplemented samples.

Long-term folate restriction protects against preneoplastic lesions initiation

A quick review of the literature regarding the association between folates and colon carcinogenesis reveals an abundance of data linking low folates to adverse outcomes and cancer initiation [9], [18], [32], [115]. This view, however, has been challenged by emerging studies revealing that the relationship between folates and cancer is much more complex than initially perceived [116]. There is mounting evidence that low folate may protect against spontaneous and chemically induced cancer [107], [117], [118]. Various epidemiological studies have linked elevated folates with a higher incidence of several cancers including colon cancer[20], [35], [119]. In this experiment we wanted to assess the effect of the timing and duration of folate restriction on colon cancer initiation. The colons of mice treated with DMH were screened for the development

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of aberrant crypt foci (ACF), a preneoplastic lesion representing the initial stages of cancer initiation and development. We established in earlier experiments that folate restriction alone was insufficient to induce spontaneous preneoplastic lesions in our mouse model. Weekly DMH injections at 30mg/kg body weight for 6 weeks was adequate to induce ACF in c57Bl/6 mice. 7 weeks after the final injection, the mice were sacrificed, and the colons were stained and scored for ACF. Consistent with most published studies, we observed that placing the animals on a folate restricted diet for one week (FA/FR) before initiating the carcinogenic insult resulted in significantly more ACF compared to animals fed a folate supplemented diet. However, mice placed on 8 weeks of a folate (FR) depleted diet were protected as evident in significantly less ACF developed compared to (FA/FR) (Fig3.6). We even observed a non-significant decrease in the number of ACF when comparing FR to FA. Furthermore, upon sacrifice, we observed major pathologies in (FA) DMH animals in the form of large gastric tumors, liver nodules, and atrophied and inflamed colons (fig3.7). Our results show that the timing and duration of folate restriction has a differential effect on colon cancer initiation. Despite an equal decrease in serum, as well as tissue folate levels under short (FA/FR) and long-term (FR) folate restriction, FR conferred protection to c57bl/6 mice that acclimated to a folate restricted diet.

FR modulates the mTOR pathway

Previously published studies from our lab revealed that combining β-pol haploinsufficiency with folate restriction reduces ACF formation [87]. This was concurrent with decreased proliferation and increased apoptosis in the FR mice. Further studies from our lab confirmed that long term folate restricted mice had reduced proliferation in the colon compared to FA. Microarray analysis of the colon provided us with important clues that could uncover the responsible underlying mechanisms. Interestingly, the expression of the mechanistic target of

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rapamycin (mTOR) was significantly reduced by FR [87] (table 3.2). We became interested in investigating mTOR as a potential driver behind the effects of folate restriction as a major switch balancing growth and proliferation with autophagy and apoptosis.

Western blot analysis revealed reduced activation of mTOR in the liver under both FA/FR and FR as evident in reduced phosphorylation of the ser2448 residue (fig3.11). mTOR is phosphorylated at ser2448 via the IGF/PI3K/AKT signaling pathway. In DMH treated animals, FA/FR and FR show a significantly reduced level of mTOR ser2448 phosphorylation compared to FA mice. No difference is observed in the level of ser2448 phosphorylation between FA/FR and FR. No difference was observed at the auto phosphorylation site of mTOR, ser2481. This residue is considered rapamycin insensitive and is not regulated by the IGF/PI3K/AKT signaling cascade [120][121] (fig3.12). To investigate if mTOR is downregulated through inhibition of the IGF pathway, we looked at the phosphorylation of PDK1. Phosphorylation of PDK at Ser241 is crucial for the activation of the AKT pathway and the downstream mTOR target S6k1 [122]. Surprisingly, in DMH treated animals, we observed a significant increase in phosphorylation of PDK1 in FR, but not in FA/FR as compared to FA mice. We then looked at REDD1 levels, a marker of hypoxia and DNA damage. REDD1 regulates mTOR through activation of the inhibitory TSC1/TSC2 complex leading to downregulation of mTOR. REDD1 expression was attenuated in both FA/FR and FR DMH treated animals as compared to FA DMH (fig3.14). Our finding indicates that mTOR inhibition is not mediated through IGF/PI3K/AKT or REDD1 signaling.

We had established in earlier experiments that folate restriction disrupts energy pools and can affect the energy gauge AMPK [123]. AMPK detects changes in the intracellular AMP/ATP ratio and is activated under nutrient depletion to inhibit anabolic pathways that consume ATP and induce ATP production [97]. AMPK is an important regulator of mTOR, activating the inhibitory

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TSC1/TSC2 complex as well as directly inhibiting mTOR by phosphorylating the critical binding subunit raptor [124]. AMPK can neutralize the PI3K activation of mTORC1 as well as enhance SIRT1 activity by increasing cellular levels of NAD+ (FIG 2.3). ADP/ATP ratio serves as a proxy indicator for energy availability. In both controls and DMH animals we saw a decrease in the ADP/ATP ratio in FA/FR compared to FA and FR indicating increased energy availability (fig 3.15). The ratio was significantly increased in both control and DMH FR animals compared to FA/FR indicating that long term folate restriction has a differential effect on energy pools. However, looking at AMPK activation through phosphorylation at T172, FA/FR experienced a significant increase in AMPK phosphorylation in DMH treated mice compared to controls (fig 3.16). FR induced increase in AMPK activation did not reach statistical significance (P=0.1). Analysis of the NAD+/NADH ratio revealed a non-significant increase (P=0.07) in FR DMH compared to FA DMH (fig 3.17).

SIRT1 mRNA expression was increased in both FA/FR and FR DMH compared to FA DMH (fig 3.18). Energy levels, as well as AMPK and SIRT1 activation could potentially explain the downregulation of mTORC1 under both FA/FR and FR DMH treated animals despite upregulation of the upstream PI3K/PDK/AKT pathway, however, the results could not explain the adaptive differential effect that confers protection on long term FR animals.

mTORC1 regulates protein synthesis and growth through the downstream targets 4E-BP1 and S6K1. FA/FR DMH resulted in a decrease in 4E-BP1 phosphorylation at T37-46 compared to FA DMH, while FR DMH resulted in an additional significant decrease in 4E-BP1 phosphorylation compared to FA/FR and FA DMH (fig 3.19). Analysis of S6K1 phosphorylation revealed a different picture. FA/FR DMH drastically increased activation and phosphorylation of S6K1 compared to FA and FR DMH (fig 3.20). S6k1 is a critical downstream target of mTOR that

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regulates multiple processes such as protein synthesis, cellular metabolism, cell cycle progression, adipogenesis as well as playing a critical role in cancer [125].

In summary, we saw that mTOR is downregulated under both FA/R and FR potentially due to the activation of AMPK and SIRT1. However, a divergence of effects was observed downstream of mTORC1. FR caused a more significant decrease in 4E-BP1 indicating a further reduction in cap dependent translation. While FA/FR resulted in a significant increase in S6K1 activation potentially reversing the growth inhibition mediated my repressed mTOR and 4E-BP1.

Effect of FR on methylation

To assess the impact of a short and long-term folate deficient diet on global DNA methylation, we examined the expression levels of the major DNMT's involved in generating and maintaining CpG methylation across the genome. In mammals, DNA methylation is conducted mainly by DNMT1, DNMT3A, and DNMT3B. During DNA replication and cell division, DNMT1 conserves and maintains the methylation profile. DNMT3A and DNMT3B, expressed mainly in undifferentiated cells, are involved in de novo DNA methylation as well as assisting DNMT1 in maintaining genomic DNA methylation (fig 3.22) [126], [127]. DNMT1 expression decreased significantly in FA/FR DMH compared to FA DMH and FR DMH (fig3.23). There was no difference between DNMT1 expression between FR DMH and FA DMH, and a non-significant decrease in DNMT1 was observed in FR controls compared to FA and FA/FR controls (fig3.23). Similarly, we saw the same trend with DNMT3A mRNA levels significantly decreased in FA/FR DMH, but not FR DMH, compared to FA DMH (fig3.24). A non-significant decrease in DNMT3A expression was also observed in FR controls vs FA controls (fig 3.24). Expression of DNMT3B was equally significantly decreased in both FA/FR DMH and FR DMH compared to FA DMH (fig 3.25). Interestingly, DNMT3B expression decreased significantly in FR control compared to

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both FA/FR and FA control. Contrary to reported rodent studies[128], long-term folate restriction (FR) resulted in decreased DNMT expression (non-significant in DNMT1, P=0.06 in DNMT3A, significant in DNMT3B) in our control groups. While long-term folate FR DMH had no effect on DNMT1 and DNMT3A, we observed a significant decrease in DNMT3B compared to FA DMH. On the other hand, short term folate restriction (FA/FR) did not alter DNMT's expression in control animals, but significantly reduced mRNA expression of all 3 DNMT's as compared to FA DMH.

Our results clearly show that FA/FR has a differential effect on the expression of DNMT's compared to FR. These findings could provide us with clues to further investigate the role of DNA methylation in the adaptive and protective effect of long term-folate restriction.

Discussion

The dichotomy of folic acid

Folate deficiency has been linked to multiple pathologies including: anemia, psychiatric and neurodevelopmental disorders, cardiovascular disease, adverse pregnancy outcomes, low birth weight, cancer, and NTD [14], [31], [129], [130]. With the exception of the reduction of NTD, evidence of folic acid supplementation having a protective effect against these conditions is inconsistent [131].

Synthetic Folic acid added to food has a higher bioavailability (70-85%) compared to folates naturally occurring in foods (50%) [10], [132]. The widespread consumption of additional supplements in the form of vitamins, sports drinks, etc. in addition to fortified foods have caused a significant percentage of the population to approach or exceed the tolerable upper daily limit of folates [10], [132]–[134]. In Fact, a survey conducted after the folate mandate revealed that 23% of the US population, 43% of children under the age of 5 and 38% of elderly persons had high serum folates. Folic acid, a synthetic variant of natural folate, is converted to the biological form

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(5-methyltetrahydrofolate) as it passes through the intestinal wall thorough a saturable conversion mechanism, leading to detectable levels of unmetabolized FA [135], [136]. In Fact, following the folate fortification mandate, unmetabolized folic acid was detected in nearly all serum samples from US children, adolescents, and adults [137]. Unmetabolized FA can bypass some regulation in the one carbon cycle by entering as THF rather than 5-methylTHF leading to potentially detrimental effects [138]. Some point to natural folates having a positive differential effect on health and disease compared to the synthetic FA [106], [139]. Over the past 2 decades, researchers have uncovered a trove of information regarding the effects of folates on health and disease. However, these reports have been often contradictory[106], [140], [141] and have ignited a heated debate regarding the risks of increasing levels of folates in the population. Of unique importance, is understanding how folates both natural and supplemented can modulate cancer risk.

Folate and cancer

Folate has a complicated history with cancer. Early publications, following the discovery of the role of folates in cancer and the subsequent successful use of anti-folate therapy, established the importance of folate in cancer [142], [143]. Accumulating evidence from various experimental, clinical and epidemiological studies, suggest that folate deficiency predisposes normal tissues to carcinogenesis, while folate supplementation suppresses it [34]. However, high doses leading to supraphysiological concentrations can enhance the growth and progression of cancers in normal tissues [13], [34]. In the presence of preneoplastic or neoplastic lesions, folate supplementation can enhance growth and aggressiveness of many cancers [19], [144]. Animal studies reveal a dual modulating effect of folate on colorectal cancer whereby the timing and dosage of folate can have contrasting results [33]. Folate deficiency in normal tissues can lead to uracil misincorporation due to the decrease in thymidylate synthesis causing DNA strand breaks, impaired repair and genomic

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instability [33], [54]. Furthermore, folate deficiency can lead to aberrant DNA methylation due to the possible decrease of S-adenosylmethionine [13]. A folate adequate diet is expected to correct these aberrations leading to a decrease in cancer risk. However, in preneoplastic or fully transformed lesions, folate deficiency can inhibit the growth requirements of a rapidly proliferating tissue, leading to decreased tumor growth and progression. At the same time, FA supplementation can provide the tumor with nucleotides leading to faster growth [34].

Animal studies in various rodent models have linked inadequate folate consumption with an increased risk of cancer, specifically CRC [53], [145], [146]. Others have reached an opposite conclusion, with many reporting that folate restriction inhibits colorectal tumorigenesis in multiple models [40], [107], [147]–[149]. The inconsistencies in the results published have made it difficult to draw a conclusion regarding the effect of folate status on colorectal cancer. With the absence of a standard model and a multitude of dietary interventions used, it is challenging to draw parallels across these studies. Rosati et al [54] published a comprehensive review of models and methods used in studying folate and colorectal cancer in rodents. The studies referenced differed in the use of animal models, diets, dietary interventions, FA supplementation, antibiotic use, and type of caging [54]. Of particular importance, the duration and the type of dietary interventions differed considerably. While folate depleted groups received 0 mg/kg folate, control or supplemented groups received varying concentrations ranging for 2 mg/kg to 20 mg/kg folate[54]. The duration of folate depletion, as well as the use of antibiotics and wire-bottom caging to reduce the microflora and coprophagy supplied folates respectively, impacted serum and tissue specific folate levels [54]. Data is further confounded by the differential response to folate depletion seen when comparing rats to mice, as well as between various genotypes of the same species [54].

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The results of our experiment show unequivocally that within the same mouse genotype, the duration of folate restriction has a differential effect on colon carcinogenesis initiation under controlled and identical conditions. The inclusion of the antibiotic SST in part of our experimental groups allowed us to potentially isolate the effect of the antibiotic on our results and was documented in chapter 2. We saw that SST further reduced folate levels significantly in most folate restricted groups (fig 3.3, 3.4, 3.5). This effect translated into a non-significant upward trend in ACF formation (fig 3.6). Since SST is known to produce a large shift and perturbation in the gut microbiome, potentially altering various signaling pathways, we decided to conduct further mechanistic analysis on the non-SST supplemented groups. Our mice were housed in normal cages, leading to the likelihood of coprophagy. Folate levels form this experiment and previous data from our lab confirm that the reduction of folate remains constant after the first week on a folate depleted diet (fig 1.2). Since no observation of anemia or growth inhibition was seen even when animals remained on the folate depleted diet for several months (unpublished lab data), it is safe to assume that this condition is not consistent with a severe folate depletion and therefore considered folate restriction. While we saw a significant (90%) reduction in serum folate levels on average, the effect on liver and colon was less pronounced, indicating less abundant circulating folates and greater enrichment and uptake by tissues. Despite comparable levels of folate in serum and tissue of both FA/FR and FR animals, the acclimation period to folate restriction conferred protection to these animals. To our knowledge, this constitutes novel findings that has not been replicated before. Animal studies that previously attempted to pinpoint the effect of folate restriction on initiation of colon carcinogenesis yielded contradictory results due to the differences in study design, duration, and timing of folate restriction among other variables [34], [54], [106]. Others showed that folate supplementation is protective initially but then becomes detrimental

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after the establishment of the neoplastic lesion [147]. Hanley et al [107] showed that a temporary methyl donor depleted diet conferred long-lasting protection against intestinal adenoma by inducing changes to the intestinal epithelium. Our data suggest that adapting the animals to folate restriction is likely to induce positive and long-lasting changes to the expression profile of various tissues. In fact, there is evidence that long term folate restriction can mimic caloric restriction and potentially increase lifespan [150], [151]. Methionine restriction, another life extension strategy, requires an adaptation period to exhibit an anti-cancer effect (manuscript in preparation) [152], [153]. Data from our lab show that long term folate restriction improved survival rate of mice and modulated mTOR signaling similar to calorie restriction [123].

In humans, a large cohort found that individuals consuming the greatest amount of folate had a smaller risk of CRC compared the ones consuming the lowest [154]. Other studies could not conclude that higher folate status is protective [155], while some reported that low folate is protective [117], [156]. Other studies have linked high FA consumption to increases in the rate of colon cancer [32], [35]. In a recent large human interventional study, folic acid supplementation at 1mg/day had no effect on the recurrence of adenomas[32]. On the contrary, upon follow up, the authors reported a significant increase in advanced lesion with high malignant potential in the FA groups as well as an increase in overall cancer rates, and no reduction in cardiovascular or stroke risk [32]. Similar results were published in other large interventional trials were FA did not improve cardiovascular events [157], and an increased trend in total cancer and colorectal cancer risk was observed [158]. While several studies indicate that the level of folate levels and intake after the fortification efforts may be twice as high as originally anticipated [134], Mason et al. hypothesized that the FA fortification could be wholly or partly responsible for the observed increase in the colorectal cancer incidence observed in the 1990s [35]. FA has been linked to

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increased risk of prostate and breast cancer [159]–[161]. In a study of FA supplementation in pregnancy, women receiving 5mg FA/day experienced an 70% increased total cancer mortality and a doubling of breast cancer mortality compared to women not taking any supplementation [144]. Furthermore, Troen et al [162] observed that higher levels of FA in elderly women correlated with reduced cytotoxicity of natural killer cells, a critical part of the immune system capable of detecting and destroying neoplastic cell clones. However, direct evidence of causality between high FA and cancer has not been established [163].

The discrepant results from various folate studies have ignited a robust debate regarding the risk versus benefit of FA supplementation. Some hypothesized that natural folate, not FA is associated with the protective effect against CRC [34], [119], [138]. While others questioned the validity of using serum folate versus dietary intake to assess folate exposure [164]. Other modifiers and confounding elements such as obesity, smoking, alcohol use, gene polymorphism, and B vitamins status could complicate the necessary analysis [138]. Furthermore, parents' folate status and the subsequent effect on epigenetic imprinting can impact the offspring's cancer risk later in life [138].

Attempting to unravel the complex relationship between folate and cancer can prove to be quite challenging. Additional layers of complexities are being uncovered as research is progressing into the matter and we are thrown into a heated discussion regarding the merits of supplementation. Our research provides another piece of evidence supporting the modulating and sometimes contradictory effect of folate on health and disease.

Folate and mTOR

mTOR is a conserved serine/threonine protein kinase that integrates signals such as growth factors, amino acids, energy levels, and nutrient availability to modulate cell growth, proliferation, autophagy, and metabolism (fig3.10). mTOR is found in 2 distinct complexes mTORC1 and

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mTORC2. mTORC1 is rapamycin sensitive and is regulated by multiple upstream signals such as growth factors, amino acid availability, stress and hypoxia, and energy availability [42], [43]. mTORc2 is largely rapamycin insensitive with different regulators and downstream targets and is involved in the mTORC1 signaling feedback as well as regulating the actin cytoskeleton [165]. mTOR controls protein synthesis and ribosome biogenesis through the downstream targets 4E-BP1 and P70-S6k1 [166]. mTOR is regulated by and receives input from various upstream regulators such as AKT, REDD1, AMPK and signaling pathways such as Wnt and Ras. mTOR became the focus of intensive research in recent years as this signaling pathway has been altered in various conditions such as cancer, diabetes, and aging [44]. More recently mTOR has been found to be modulated by perturbation in one carbon metabolism and more specifically folate availability [45].

Current research has been unraveling the intimate relationship between the one carbon cycle and mTOR. In fact, mTORC1 was found to induce purine synthesis by controlling the mitochondrial tetrahydrofolate cycle [47]. mTORC1 was also found to induce the generation of SAM [48]. Folate deficiency in mice inhibited both mTORC1 and mTORC2 signaling and downregulated amino acid transporters in a pregnant mouse model as well as human trophoblasts [51]. Rosario et al. suggests that mTOR senses folate through the proton coupled folate transporter by modulating the cell surface expression of folate receptor alpha and reduced folate carrier [49], [51], [167]. Folic acid can activate the PI3K/AKT pathway leading to the phosphorylation of mTOR and its downstream targets S6K1 and 4E-BP1 [46]. Furthermore, both mTOR and one carbon cycle are regulated through energy availability. NAD+/NADH ratio, maintained by mitochondrial oxidative phosphorylation, links energy levels and one carbon cycle through formate production from serine, as well as regulating mTOR through SIRT1 [9], [168]. Research

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in *C. elegans* revealed that low levels of folic acid supplementation inhibit mTOR and IGF1 [169]. In vascular smooth muscle cells, FA suppressed mTOR and S6K1 [170]. Others have shown that folate deprivation can induce cell cycle arrest in neuronal cells via inhibition of IGF-1 signaling [171]. The seemingly contradictory effects suggest that folate can differentially impact mTOR depending on the tissue type and conditions.

In our model, we saw that the timing and duration of folate restriction has a divergent effect on the mTOR signaling pathway. Although both FA/FR and FR downregulation of mTOR were not mediated by the PI3K/PDK/AKT pathway or REDD1, the perturbation of energy pools and subsequent activation of AMPK and SIRT1 provided us with a possible mechanistic justification for mTOR decreased phosphorylation. The equal decrease of mTOR activation under both FA/FR and FR could not explain the differential effect of these conditions on colon carcinogenesis. However, analysis of the downstream targets of mTOR, 4E-BP1 and S6K1, revealed a contrasting difference between the two conditions in DMH treated animals. FR decreased 4E-BP more significantly than FA/FR indicating a more pronounced inhibition on cap dependent translation, reducing cell growth and metabolism. Remarkably, FA/FR induced a significant increase in S6k1 phosphorylation, while FR had no effect. S6K1 activation could potentially override the inhibition on growth and proliferation perpetuated by mTOR [172]–[174]. In fact, mTOR/S6K1 signaling plays an important role in colorectal carcinoma [175]. S6K1 is able to phosphorylate targets as rpS6 (ribosomal protein S6), eIF4B (eukaryotic translation Initiation Factor 4B) and eEF2K (eukaryotic Elongation Factor 2 Kinase), promoting protein synthesis and cell growth [172], [176]. S6K1 can also be activated by TOR-insensitive signaling pathways such as PDK1, MAPK and SAPK (stress-activated protein kinase) [125], [173]. S6K1 activation contributes to viability, proliferation, migration and chemo-resistance of various cancers [177]–[179].

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The differential effect of short and long-term folate restriction on 4E-BP1 and S6K1 provided us with crucial evidence to help explain the disparity in the ACF results observed. However further investigation and research would be needed to confirm and validate the possibility that the protective effect of folate restriction is mediated through the mTOR pathway.

Folate and methylation

Folate is essential not only for purine and pyrimidine nucleotide synthesis, but also for maintenance of SAM levels, the universal donor of methyl groups to various methylation reactions including DNA methylation (fig3.9) [128]. Folate status has a direct and significant effect on DNA methylation. Perturbances in the folate pools stemming from low or high folate status can result in hypomethylation or hypermethylation leading to epigenetic instability [180]. DNA methylation occurs on CpG islands in the promoter regions of genes with the methylation status affecting gene expression, chromatin modification, and transcription [181]. A number of disease etiologies including cancer are linked to dysfunctional DNA methylation [131]. Methyl donors include: folate, B12, betaine, choline and methionine [104]. Critical to maintaining epigenetic imprinting to DNA, folates became the focus of fortification efforts to reduce the frequency of neural tube defects [182]. Subject to intense scientific debate, the incidence of many diseases including growth syndromes, neurological disorders, respiratory disorders, multiple sclerosis, autism, and cancer increased post fortification [131], [183]. Conversely, diets deficient in methyl donors have been shown to have deleterious effects [184].

Early animal experiments clearly show that a maternal diet high in methyl donors can alter epigenetic imprinting by silencing gene mutations in the agouti and Axin alleles [185], [186]. Studies in C57BL/6 mice linked high maternal dietary folate to alteration in the methylome of the offspring leading to changes in the expression of various genes involved in development as well

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as behavioral changes [187], [188]. Diets low in folate affected methylation patterns of genes associated with cancer, diabetes, autism and schizophrenia in the sperm of male mice [189]. Combination of the western high fat diet with FA supplementation in mice can potentially induce heritable susceptibility to diseases such as obesity, type 2 diabetes, and insulin resistance in the offspring, possibly through epigenetic modification of DNA and histone methylation [131], [190], [191]. Human studies linked maternal FA supplementation to changes in global methylation patterns as well as the methylation status of several critical genes affecting birth weight, insulin resistance, obesity, type 2 diabetes, asthma, and vocabulary development [132], [192]–[197].

DNA hypomethylation is considered an early event in carcinogenesis causing increased gene expression and DNA strand breaks [181]. In colorectal cancer (CRC), global genomic hypomethylation is well documented. Additionally, genomic instability, site specific hypermethylation of tumor suppressor, and mismatch repair genes lead to increased mutations [128]. Diets deficient in methyl group donors have been shown to upregulate DNA methyltransferase DNMT in the rat liver [198], [199]. In contrast, folic acid supplementation has been linked to an increase in DNMT activity [200]. Results from recent studies suggest that moderate short duration folate deficiency induces DNA hypermethylation in rodent liver, possibly due to a compensatory upregulation of DNMT [201]–[203]. Others noted that the genomic DNA methylation in the liver returned to baseline after 8 weeks on folate deficient diet [147]. The effect of folate restriction on methylation was not consistent across studies. Kim et al. review of the literature reported that folate restriction affected DNA methylation differently depending on the severity and length of folate restriction, as well as the use of carcinogenic treatments such as DMH or AOM [128]. Some argue that aberrant methylation alone could not consistently explain how

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folate restriction could promote carcinogenesis in adults, however, a transgenerational epigenetic effect could modulate cancer risk in the offsprings [138].

Our results show that long term folate restriction FR produced a more pronounced decrease in all DNMT's expression in control animals compared to FA/FR and FR. However, in the DMH treated animals, the expression levels of DNMT1 and DNMT3A in FR animals were equivalent to that of FA, while in FA/FR both were significantly reduced. This suggests that animals adapted to long term folate restriction and then exposed to DMH mimic FA in the expression of critical maintenance methyltransferases. This is consistent with the findings by song et al. [147] noting an adaptive rebound of genomic DNA methylation. The differential effect of FR compared to FA/FR on DNA methyltransferase expression represents a valid target for further analysis to fully characterize the impact on global methylation as well as on gene specific methylation patterns.

Our research provides yet another evidence of the increasingly complex and puzzling effects of folate. This data shows, for the first time in a controlled experiment, that adapting to long term folates restriction can provide a protective effect against colon cancer. We show that long-term folate restriction impacts critical pathways, such as mTOR and methylation, differently than short-term. While more research is needed to unravel the complex dynamics of this observed protection, our results provides us with important leads. The significance of these findings is that it overturns many of the rigid assumptions we had about the role of folates in CRC, revealing a much more complicated picture indeed.

It is quite clear that the discourse regarding the effects of folate on health and disease will not be settled any time soon. In fact, results from the last decades of research regarding the relationship between folate and cancer have added to the confusion and complexity surrounding the subject. This fact does not detract from the importance of the issue at hand. On the contrary, it

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delivers a great sense of urgency to pursue vital research and attempt to unravel the mystery. As a greater percentage of the population is exposed to elevated levels of folates (both natural and synthetic), a condition that may be considered anomalous from an evolutionary perspective, we are faced with a difficult conundrum. Do we continue with the efforts to supplement the whole population with FA to prevent a limited number of neural tube defects while risking a much larger and vulnerable segment of the population? Our incomplete understanding of the subject prevents us from providing a definitive answer. Furthermore, it could take decades more for a clear consensus to emerge. Further action could be delayed by bureaucracy and policy implementation. By that time, the potential deleterious effects would have impacted generations as well as their offspring.

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Figure 3.1: *Study design*
Sixty 4 months old C57BL/6 male mice were randomly assigned to 6 groups and fed AIN93G-purified isoenergetic diets (Dyets, Inc., Lehigh Valley, PA)[88]. The control group (FA) received a folate adequate diet (2mg FA/kg diet for 8 weeks) +/- ABx (antibiotic SST). The experimental groups: (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) +/- ABx (antibiotic SST), and (FR) received a folate deficient diet (0 mg folic acid/kg diet for 8 weeks) +/- ABx (antibiotic SST). After 8 weeks on the respective diets, 6-7 mice from each group were randomly chosen and treated IP with 1, 2-dimethylhydrazine HCl (DMH, 30 mg/kg body weight) in 10 mmol/liter of $NaHCO_3$ (Fisher Scientific) once a week for 6 weeks. The animals were monitored for signs of toxicity and remained on their respective diets for an additional 7 weeks before sacrifice. Control n=3, DMH n=6-7.

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Figure 3.1: *Study design*

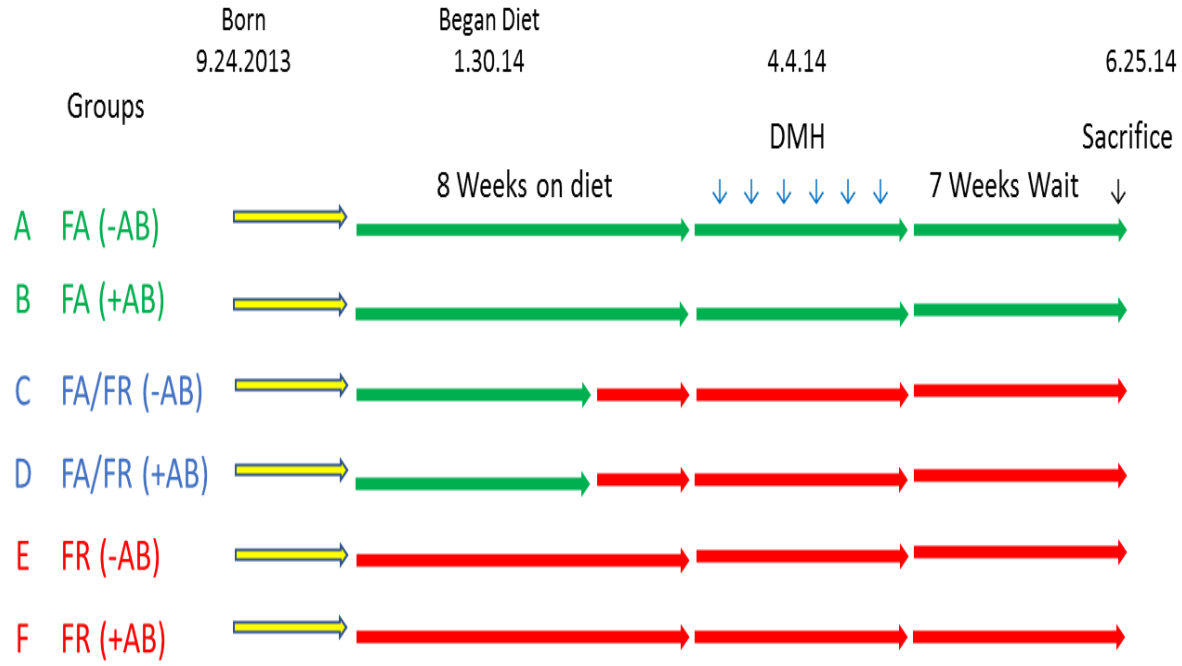

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Figure 3.2: *The effect of short-term and long-term folate restriction with/without antibiotic on animal weights in control and DMH treated animals*
Weights of FA, FA/FR, and FR mice +/- ABx (antibiotic SST) at the beginning of the experiment, after 8 weeks on respective diets, and at the time of sacrifice (21 weeks). Control n=3, DMH n=6-7. Bars S.E.M

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Figure 3.2: *The effect of short-term and long-term folate restriction with/without antibiotic on animal weights in control and DMH treated animals*

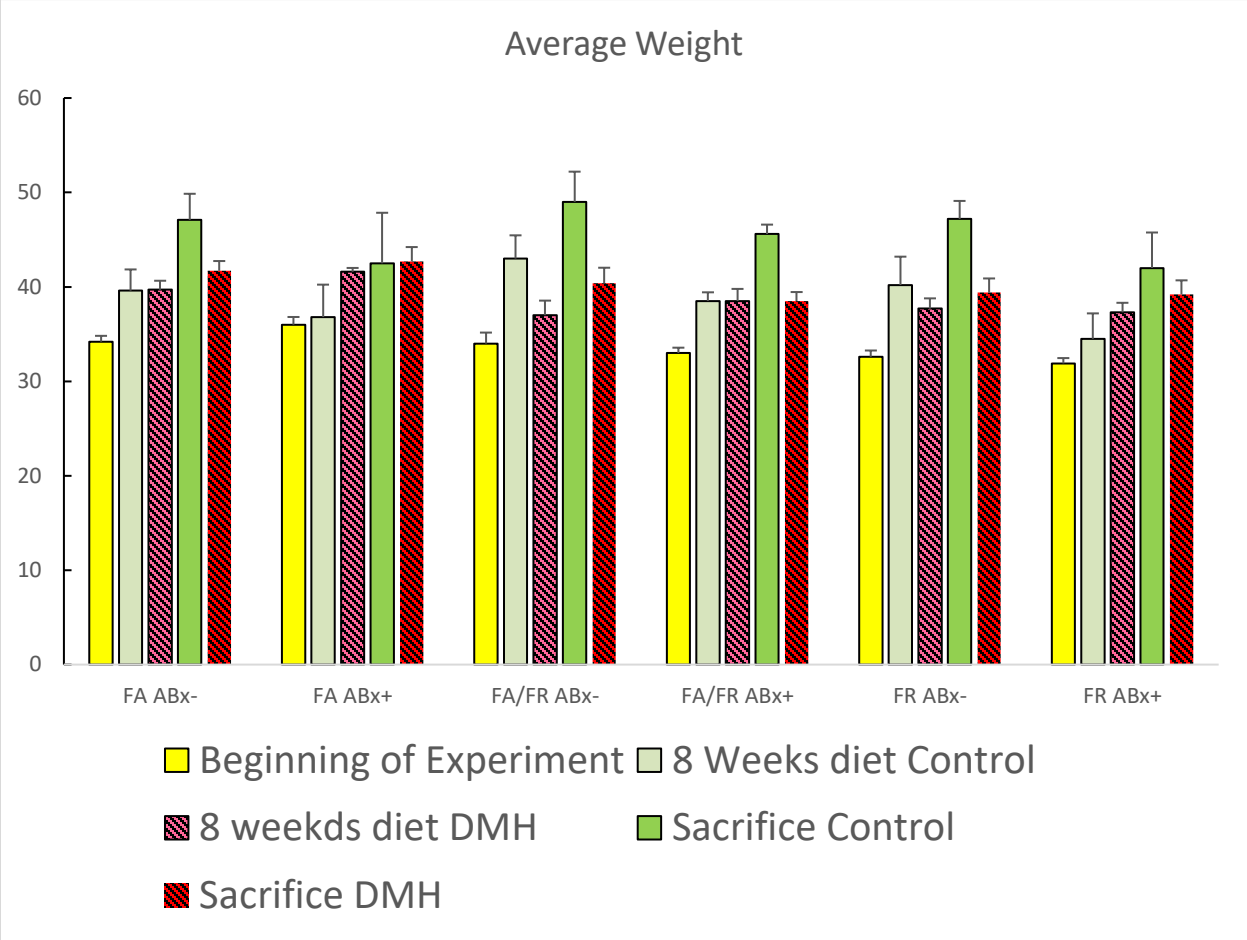

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Figure 3.3: *The effect of short-term and long-term folate restriction with/without antibiotic on serum folate levels in control and DMH treated animals*
Folate levels were analyzed using the Lactobacillus casei microbiological assay of folic acid derivatives as described by Horne et. Al[89]. Serum folate levels are expressed as ng/ml. FA: Folate adequate, FA/FR: short-term Folate restriction, FR: long-term Folate Restricted, ABx+: diet with 1% SST, ABx-: diet without 1% SST. Control n=3, DMH n=6-7. Different letters indicate significant differences at $P < 0.05$.

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Figure 3.3: *The effect of short-term and long-term folate restriction with/without antibiotic on serum folate levels in control and DMH treated animals*

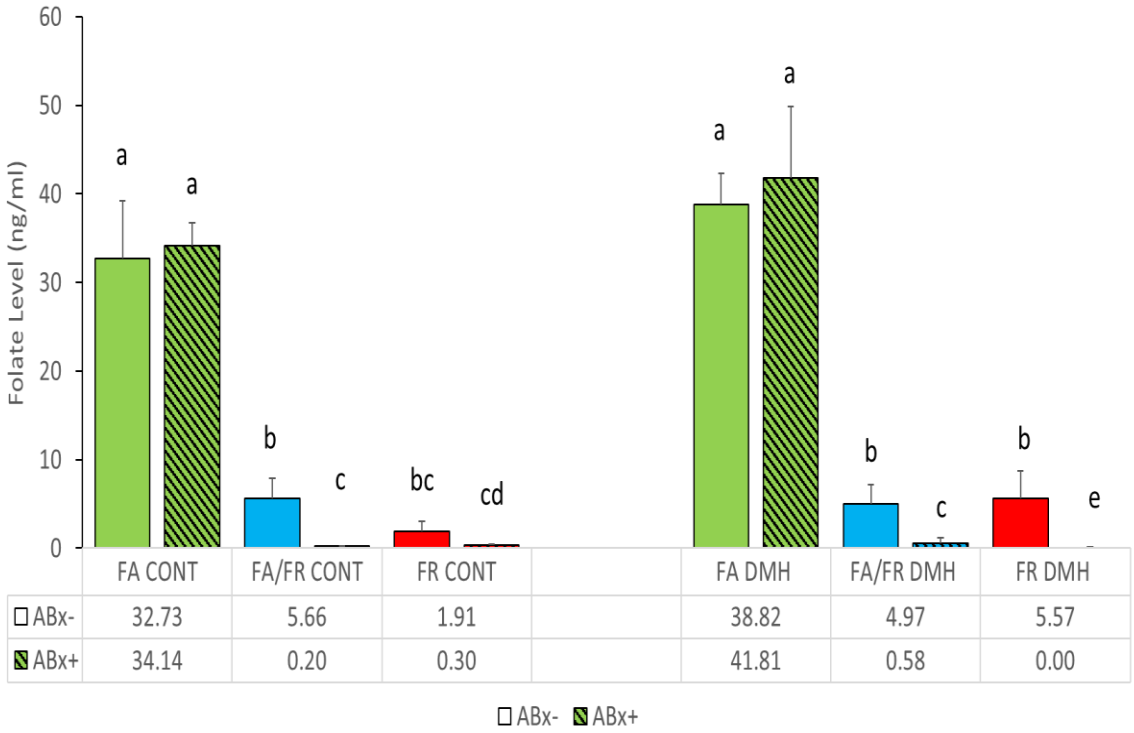

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Figure 3.4: *The effect of short-term and long-term folate restriction with/without antibiotic on liver folate levels in control and DMH treated animals*
Folate levels were analyzed using the Lactobacillus casei microbiological assay of folic acid derivatives as described by Horne et. Al[89]. Folate levels are expressed as nmol/g. FA: Folate adequate, FA/FR: short-term Folate restriction, FR: long-term Folate Restricted, ABx+: diet with 1% SST, ABx-: diet without 1% SST. Control n=3, DMH n=6-7. Different letters indicate significant differences at $P < 0.05$.

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Figure 3.4: *The effect of short-term and long-term folate restriction with/without antibiotic on liver folate levels in control and DMH treated animals*

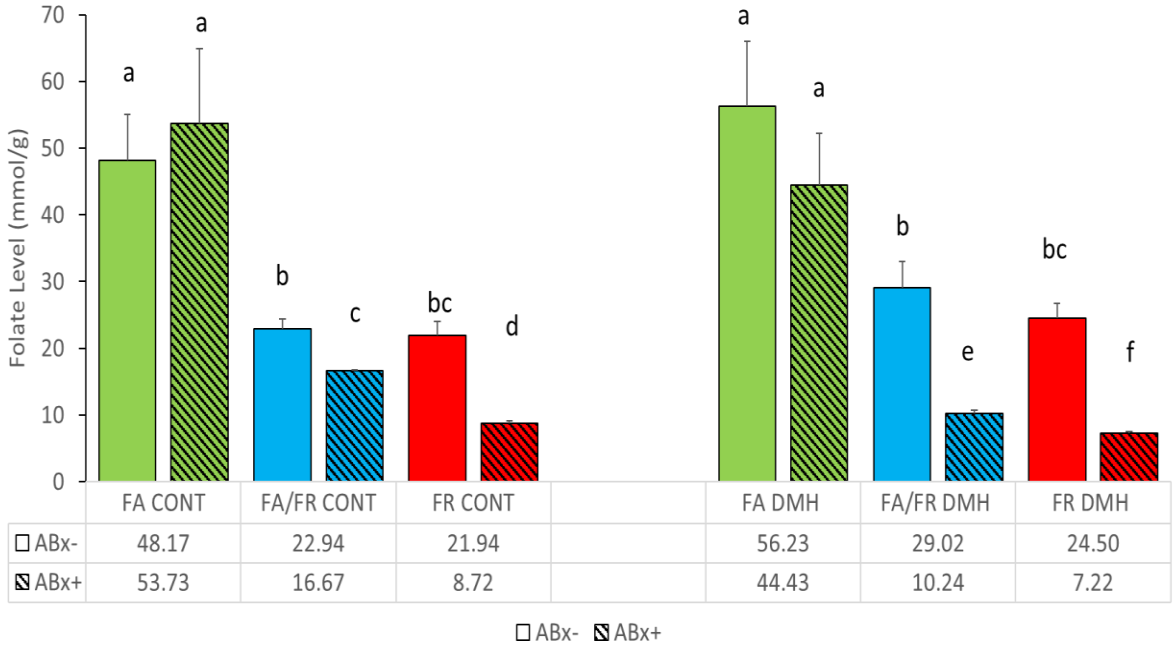

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Figure 3.5: *The effect of short-term and long-term folate restriction with/without antibiotic on colon folate levels in control animals*
Folate levels were analyzed using the Lactobacillus casei microbiological assay of folic acid derivatives as described by Horne et. Al[89]. Folate levels are expressed as nmol/g. FA: Folate adequate, FA/FR: short-term Folate restriction, FR: long-term Folate Restricted, ABx+: diet with 1% SST, ABx-: diet without 1% SST. n=3. Different letters indicate significant differences at $P < 0.05$.

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Figure 3.5: *The effect of short-term and long-term folate restriction with/without antibiotic on colon folate levels in control animals*

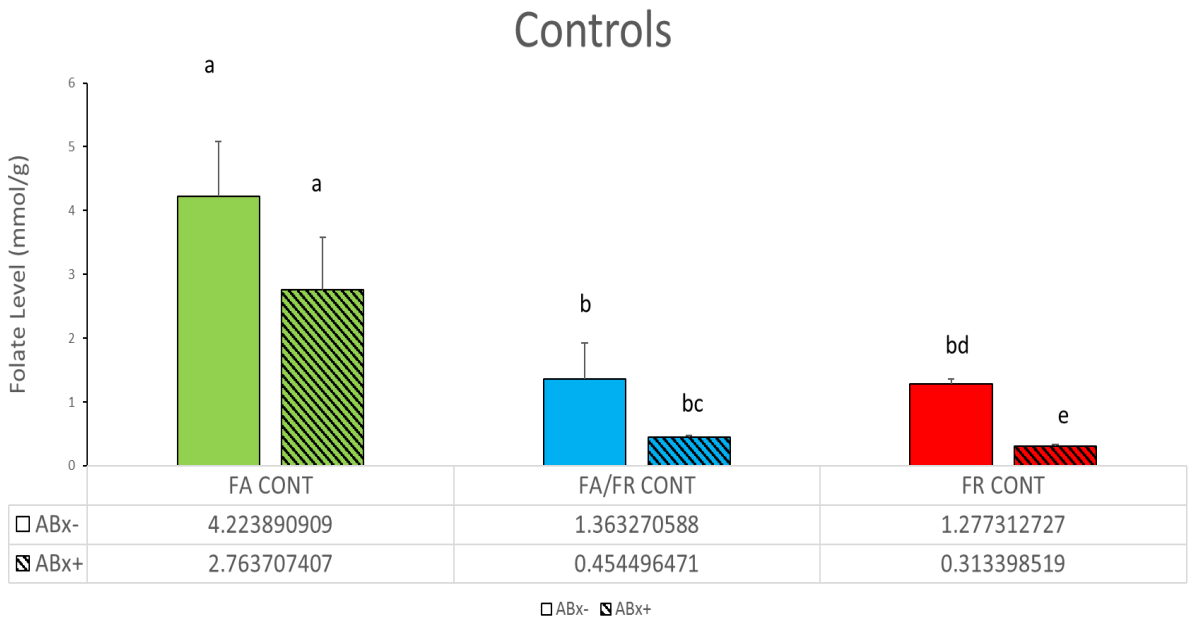

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Figure 3.6*: Long-term folate restriction protects against ACF formation*
Aberrant Crypt Foci (ACF) numbers in the colons of DMH treated mice were determined by light microscopy at 10x magnification in a blinded manner as described in material and methods. Image I represent a normal colon with no pathologies. Image III shows a preneoplastic aberrant crypt foci that displays as a raised area in the center. Highlighted samples refer to animals that exhibited large cancerous growths and gross abnormalities in the stomach and liver upon sacrifice. FA: Folate adequate, FA/FR: short-term Folate restriction, FR: long-term Folate Restricted, ABx+: diet with 1% SST, ABx-: diet without 1% SST. n=6-7. Different letters indicate significant differences at $P < 0.05$.

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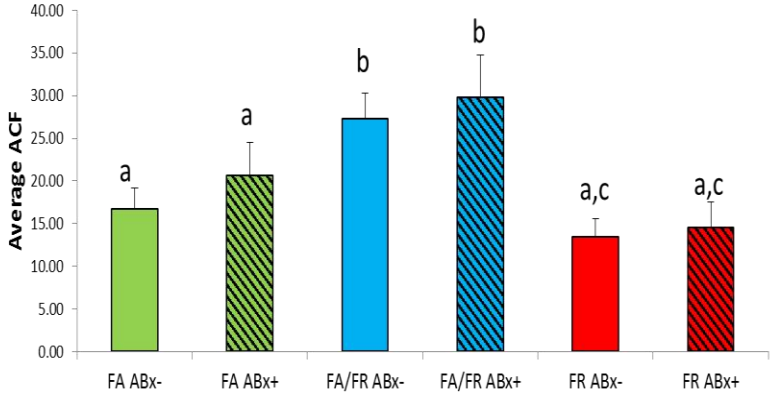
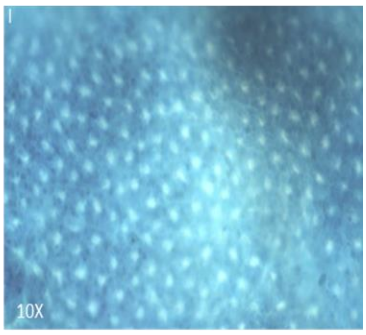
Figure 3.6: *long-term folate restriction protects against ACF formation*

ACF DATA

	A	B	C	D	E	F
	FA ABx-	FA ABx+	FA/FR ABx-	FA/FR ABx+	FR ABx-	FR ABx+
1	12	15	32	21	14	26
2	25	37	30	18	11	9
3	11	19	24	46	21	13
4	10	12	36	37	7	10
5	16	20	24	35	16	10
6	23	21	18	22	12	19
AVG	16.71	20.67	27.33	29.83	13.50	14.50
SDV	6.05	8.69	6.53	11.13	4.76	6.72
SEM	2.47	3.88	2.92	4.97	2.13	3.00





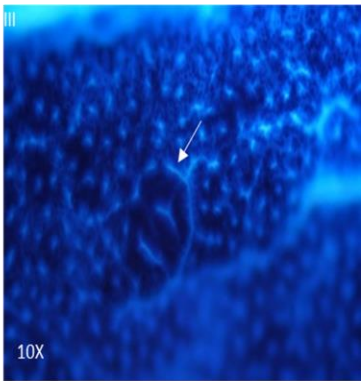


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Figure 3.7: *Gross abnormalities in FA DMH animals*
The following images refer to cancerous growths and pathologies uncovered in animals in the Folate supplemented (FA) DMH animals.

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Figure 3.7: *Gross abnormalities in FA DMH animals*

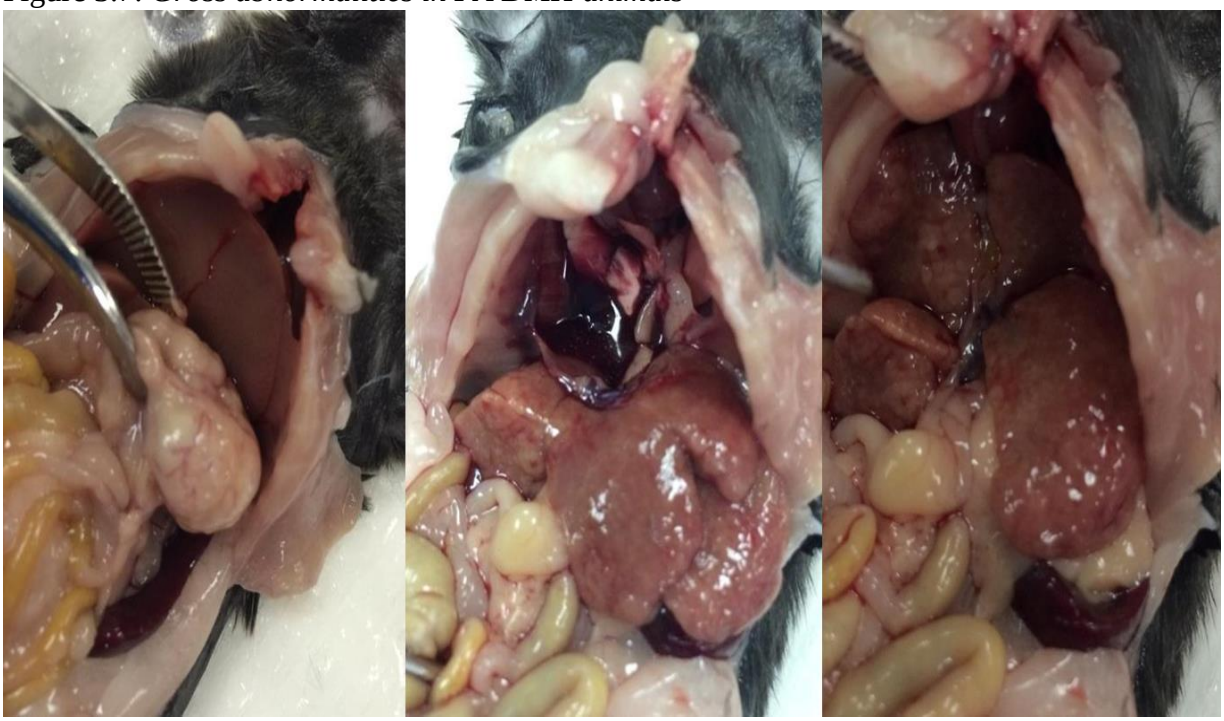

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Figure 3.8: *The timing and duration of folate restriction has a differential effect on the occurrence of preneoplastic lesions ACF*
Top figure: Folate levels were analyzed using the Lactobacillus casei microbiological assay of folic acid derivatives as described by Horne et. Al[89]. Serum folate levels are expressed as ng/ml. Bottom figure: Aberrant Crypt Foci (ACF) numbers in the colons of DMH treated mice were determined by light microscopy at 10x magnification in a blinded manner.
FA: Folate adequate, FA/FR: short-term Folate restriction, FR: long-term Folate Restricted, ABx+: diet with 1% SST, ABx-: diet without 1% SST. DMH n=6-7. Different letters indicate significant differences at $P < 0.05$.

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Figure 3.8: *The timing and duration of folate restriction has a differential effect on the occurrence of preneoplastic lesions ACF*

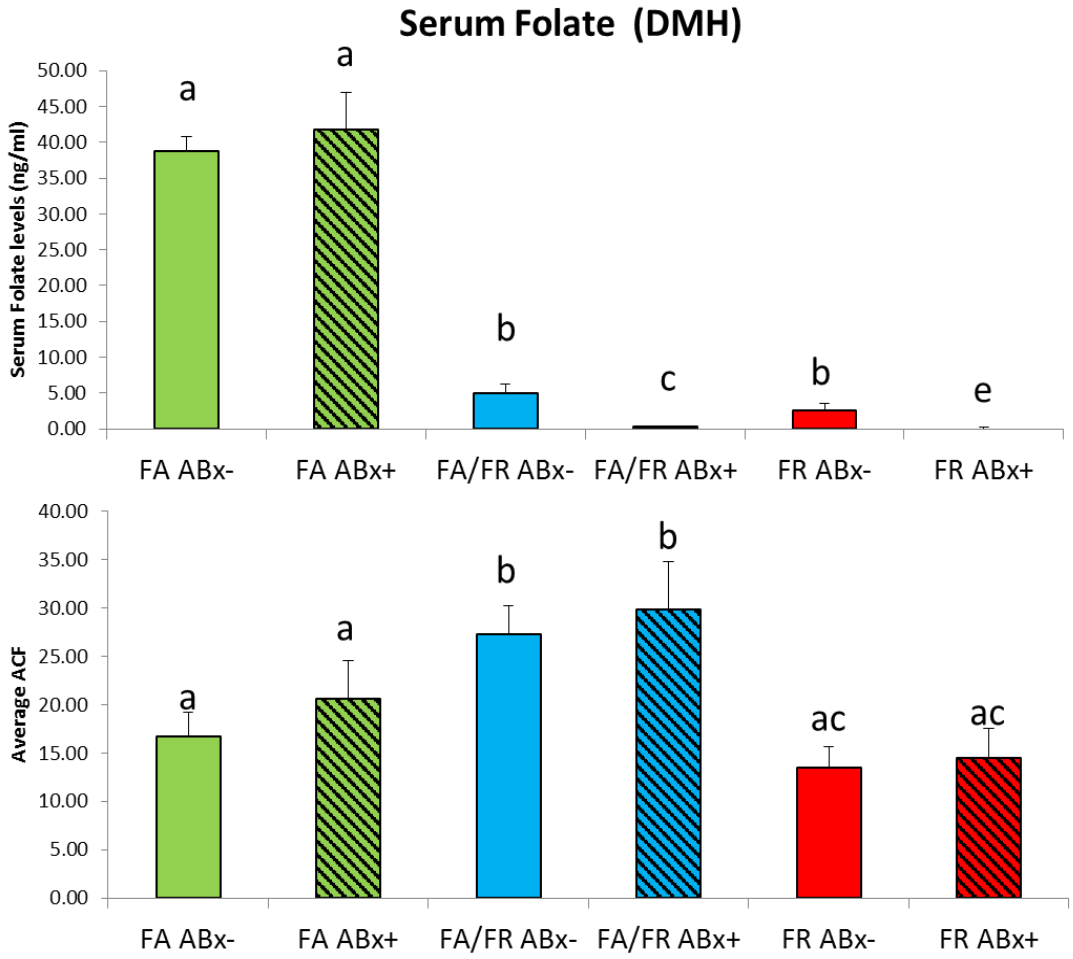

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Figure 3.9: *Folate metabolism and one carbon cycle*

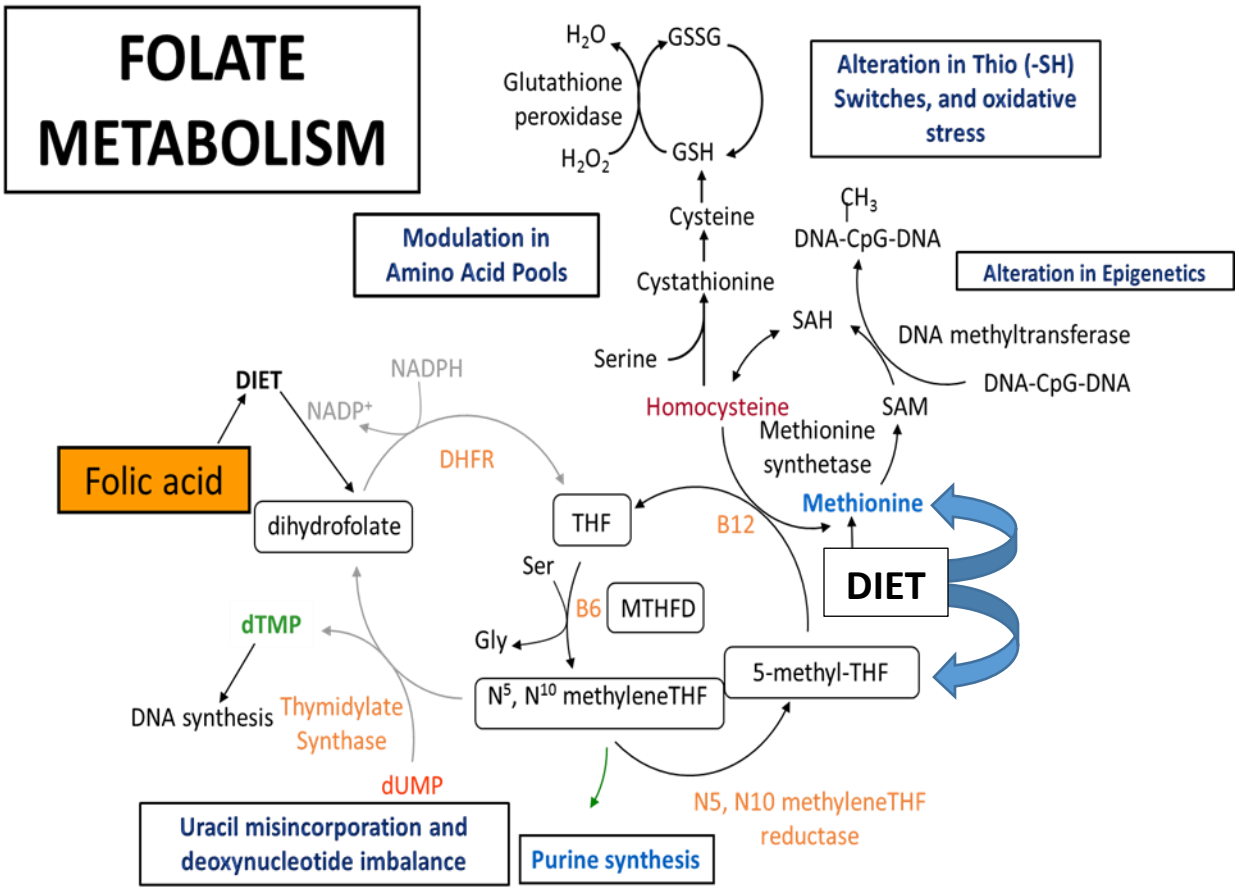

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Table 3.1: *Folate restriction alters plasma amino acids*
Plasma was collected from C57BL/6 mice fed (FA) folate adequate diet (2 mg folic acid/kg diet) and (FR) folate restricted diet (0 mg folic acid/kg diet) upon sacrifice. Data obtained from 4 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.

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

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Table 3.2: *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. ns: not significant

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

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Figure 3.10: *Folate restriction reduces mTOR phosphorylation at the serine 2448 residue*
The level of mTOR protein phosphorylation at serine 2448 was analyzed using western blotting as described in materials and methods. Data is expressed as adjusted band volume normalized to total lane proteins. Levels of phosphorylated protein were determined first, then the membrane lightly stripped and tested for respective total protein. Phosphorylated levels of protein were normalized to total and expressed as ratio. FA: Folate adequate diet, FA/FR: short-term Folate restricted diet, FR: long-term Folate restricted diet, CON: control mice, DMH: mice treated with dimethylhydrazine, ABx-: diet without 1% SST. Control n=3, DMH n=6-7. * Significant differences at $P < 0.05$.

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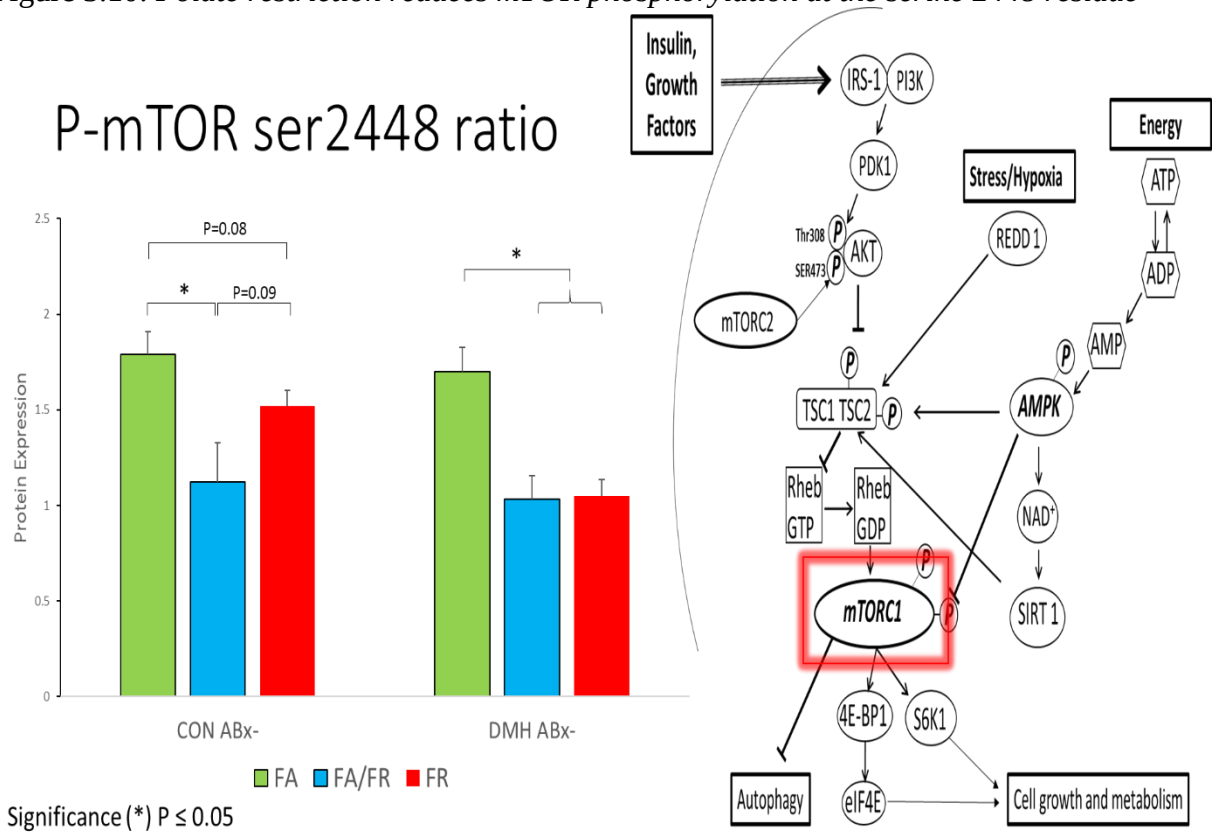
Figure 3.10: *Folate restriction reduces mTOR phosphorylation at the serine 2448 residue*



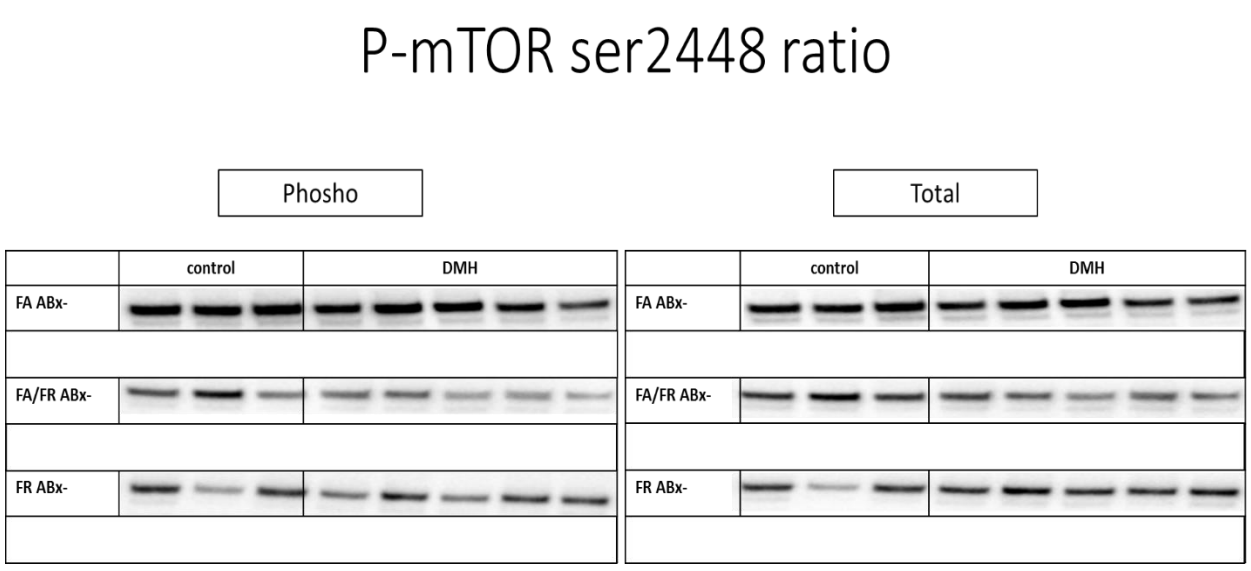


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Figure 3.11: *Effect of folate restriction on mTOR auto phosphorylation at the serine 2481 residue* The level of mTOR protein phosphorylation at serine 2481 was analyzed using western blotting as described in materials and methods. Data is expressed as adjusted band volume normalized to total lane proteins. Levels of phosphorylated protein were determined first, then the membrane lightly stripped and tested for respective total protein. Phosphorylated levels of protein were normalized to total and expressed as ratio. FA: Folate adequate diet, FA/FR: short-term Folate restricted diet, FR: long-term Folate restricted diet, CON: control mice, DMH: mice treated with dimethylhydrazine, ABx-: diet without 1% SST. Control n=3, DMH n=6-7. * Significant differences at $P < 0.05$.

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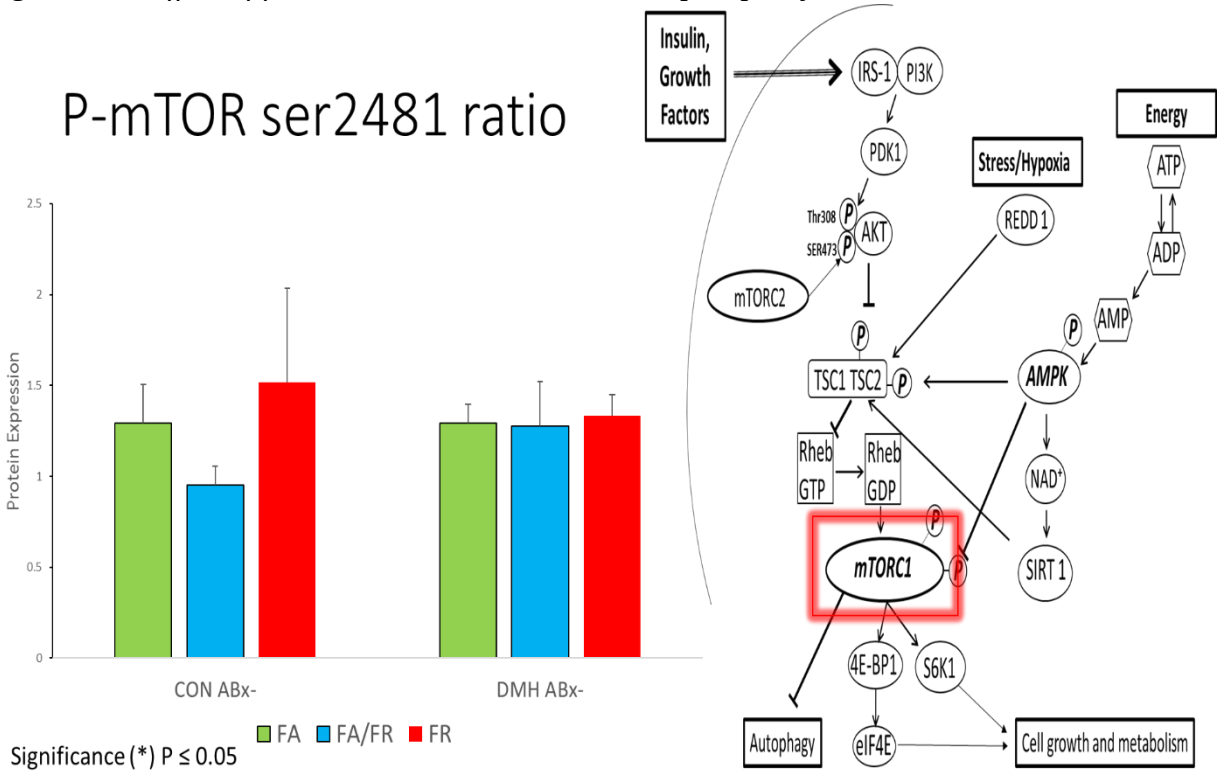
Figure 3.11: *Effect of folate restriction on mTOR auto phosphorylation at the serine 2481 residue*



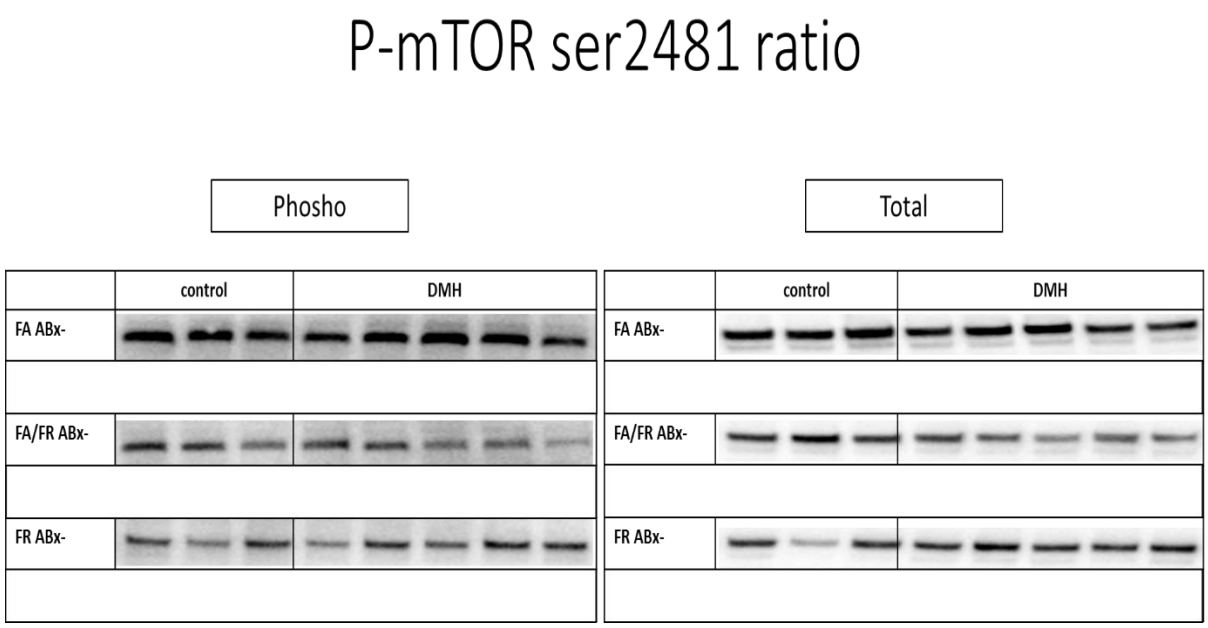


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Figure 3.12: *Effect of folate restriction on PDK1 phosphorylation at the serine 241 residue*
The level of PDK1 phosohoryation was analyzed using western blotting as described in materials and methods. Data is expressed as adjusted band volume normalized to total lane proteins. Levels of phosphorylated protein were determined first, then the membrane lightly stripped and tested for respective total protein. Phosphorylated levels of protein were normalized to total and expressed as ratio. FA: Folate adequate diet, FA/FR: short-term Folate restricted diet, FR: long-term Folate restricted diet, CON: control mice, DMH: mice treated with dimethylhydrazine, ABx-: diet without 1% SST. Control n=3, DMH n=6-7. * Significant differences at $P < 0.05$.

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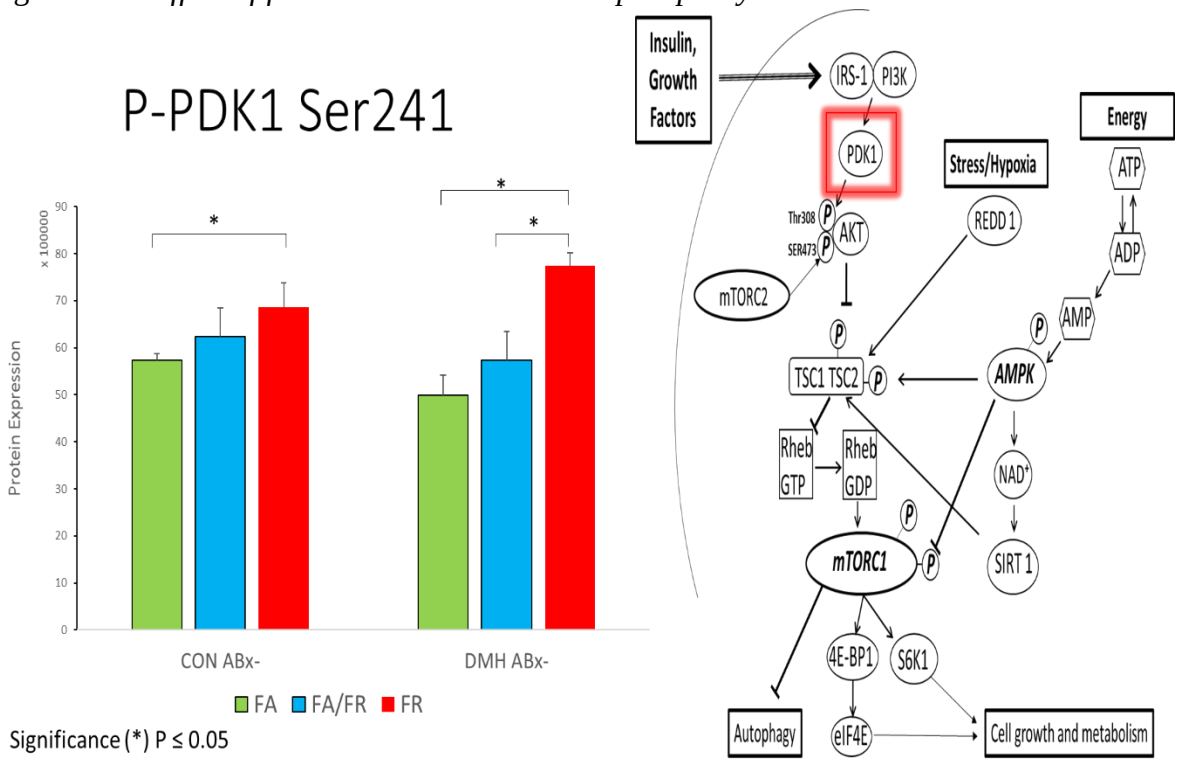
Figure 3.12: *Effect of folate restriction on PDK1 phosphorylation at the serine 241 residue*



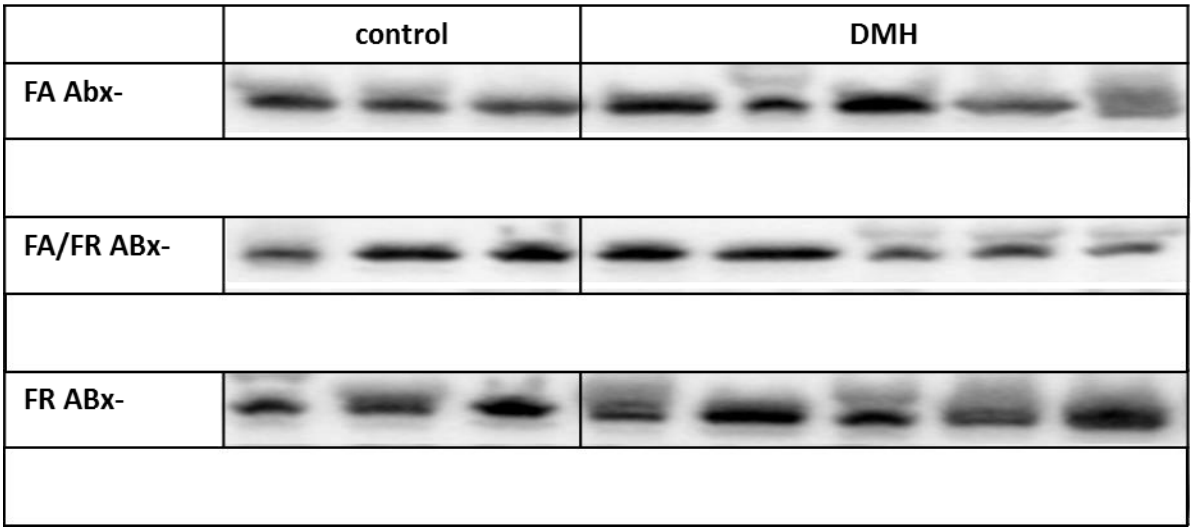


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Figure 3.13: *Effect of folate restriction on REDD1 protein expression*
The level of REDD1 protein expression was analyzed using western blotting as described in materials and methods. Data is expressed as adjusted band volume normalized to total lane proteins. FA: Folate adequate diet, FA/FR: short-term Folate restricted diet, FR: long-term Folate restricted diet, CON: control mice, DMH: mice treated with dimethylhydrazine, ABx-: diet without 1% SST. Control n=3, DMH n=6-7. * Significant differences at $P < 0.05$.

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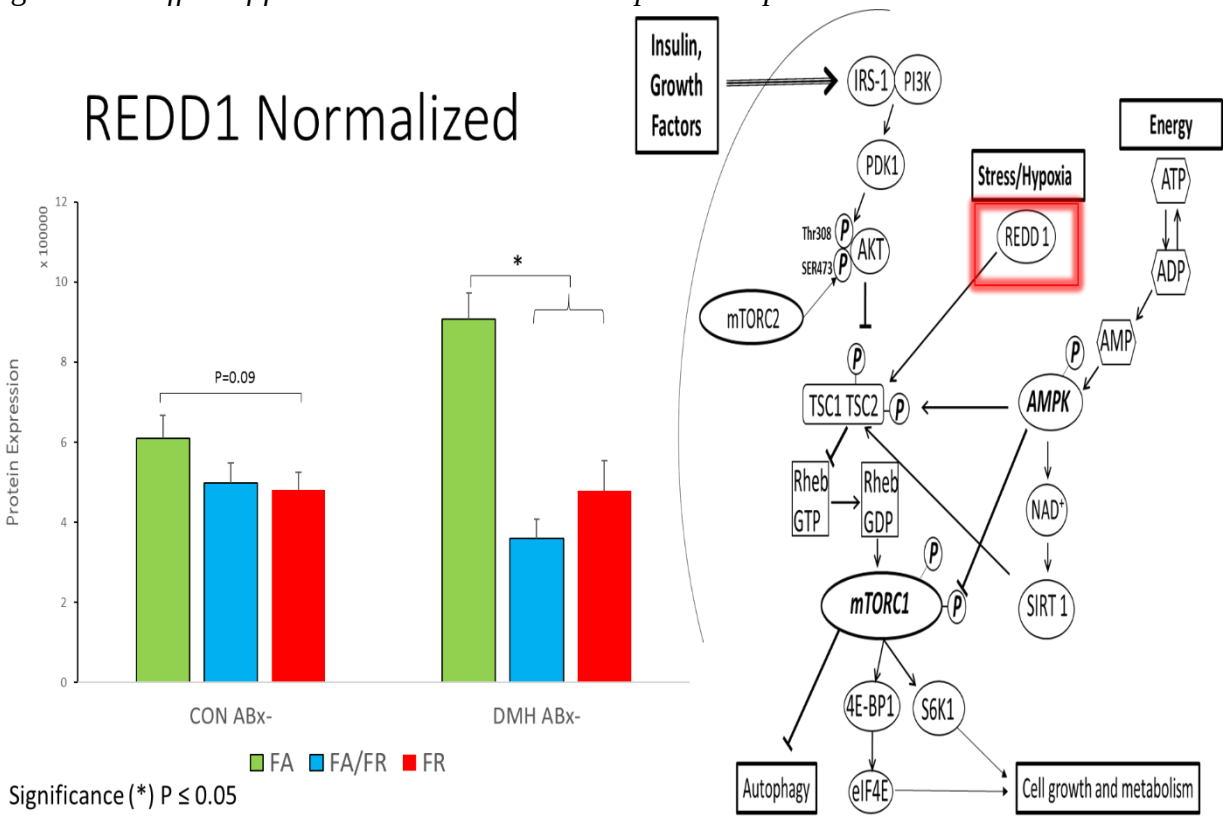
Figure 3.13: *Effect of folate restriction on REDD1 protein expression*



	control	DMH
FA ABx-		
FA/FR ABx-		
FR ABx-		

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Figure 3.14: *Effect of folate restriction on energy levels (ADP/ATP ratio)*
ADP/ATP ratio was assessed using a luciferase based bioluminescent assay kit (Abcam). 10 mg of liver tissue was quickly homogenized with nucleotide releasing buffer. Using a microplate reader, the homogenate was assayed per protocol to determine ATP induced luminescence. Then ADP converting enzyme was added and luminescence was further determined. Data are expressed at ADP/ATP ratio. FA: Folate adequate diet, FA/FR: short-term Folate restricted diet, FR: long-term Folate restricted diet, CON: control mice, DMH: mice treated with dimethylhydrazine, ABx-: diet without 1% SST. Control n=3, DMH n=6-7. * Significant differences at $P < 0.05$.

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Figure 3.14: *Effect of folate restriction on energy levels (ADP/ATP ratio)*

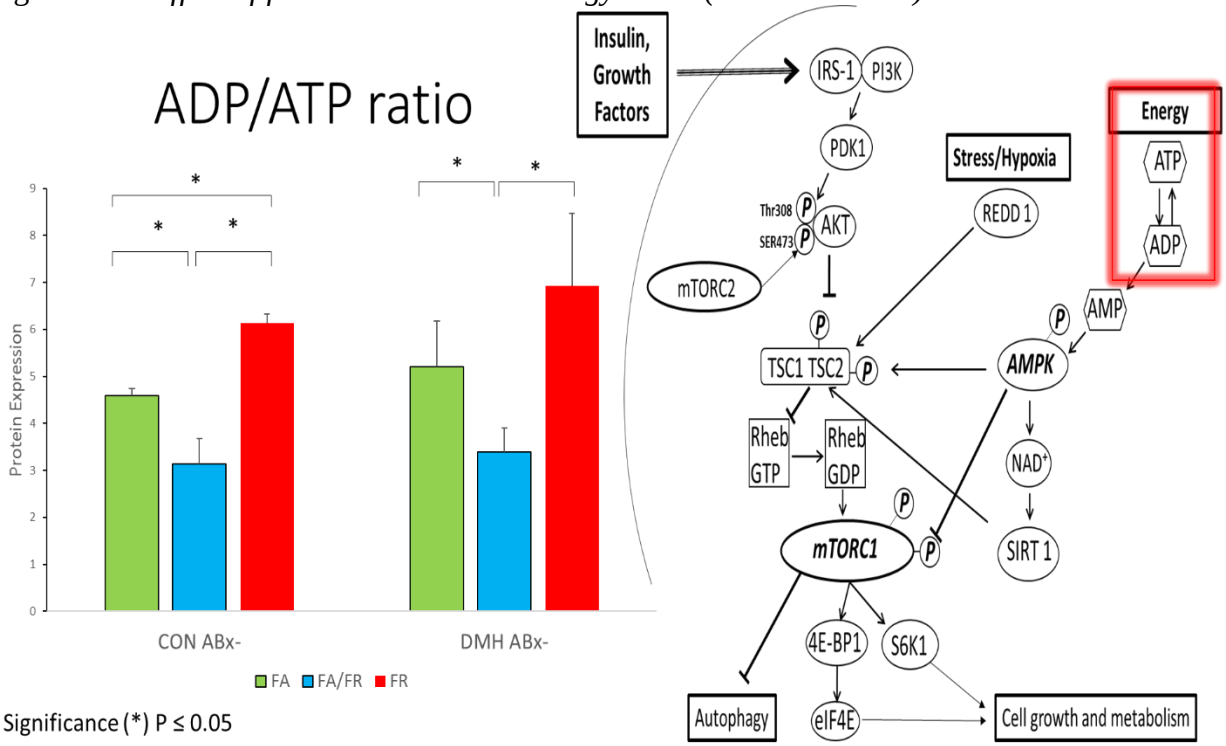

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Figure 3.15: *Effect of folate restriction on AMPK phosphorylation at T172 residue*
The level of AMPK protein phosphorylation at threonine 172 was analyzed using western blotting as described in materials and methods. Data is expressed as adjusted band volume normalized to total lane proteins. Levels of phosphorylated protein were determined first, then the membrane lightly stripped and tested for respective total protein. Phosphorylated levels of protein were normalized to total and expressed as ratio. FA: Folate adequate diet, FA/FR: short-term Folate restricted diet, FR: long-term Folate restricted diet, CON: control mice, DMH: mice treated with dimethylhydrazine, ABx-: diet without 1% SST. Control n=3, DMH n=6-7. * Significant differences at $P < 0.05$.

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Figure 3.15: *Effect of folate restriction on AMPK phosphorylation at T172 residue*

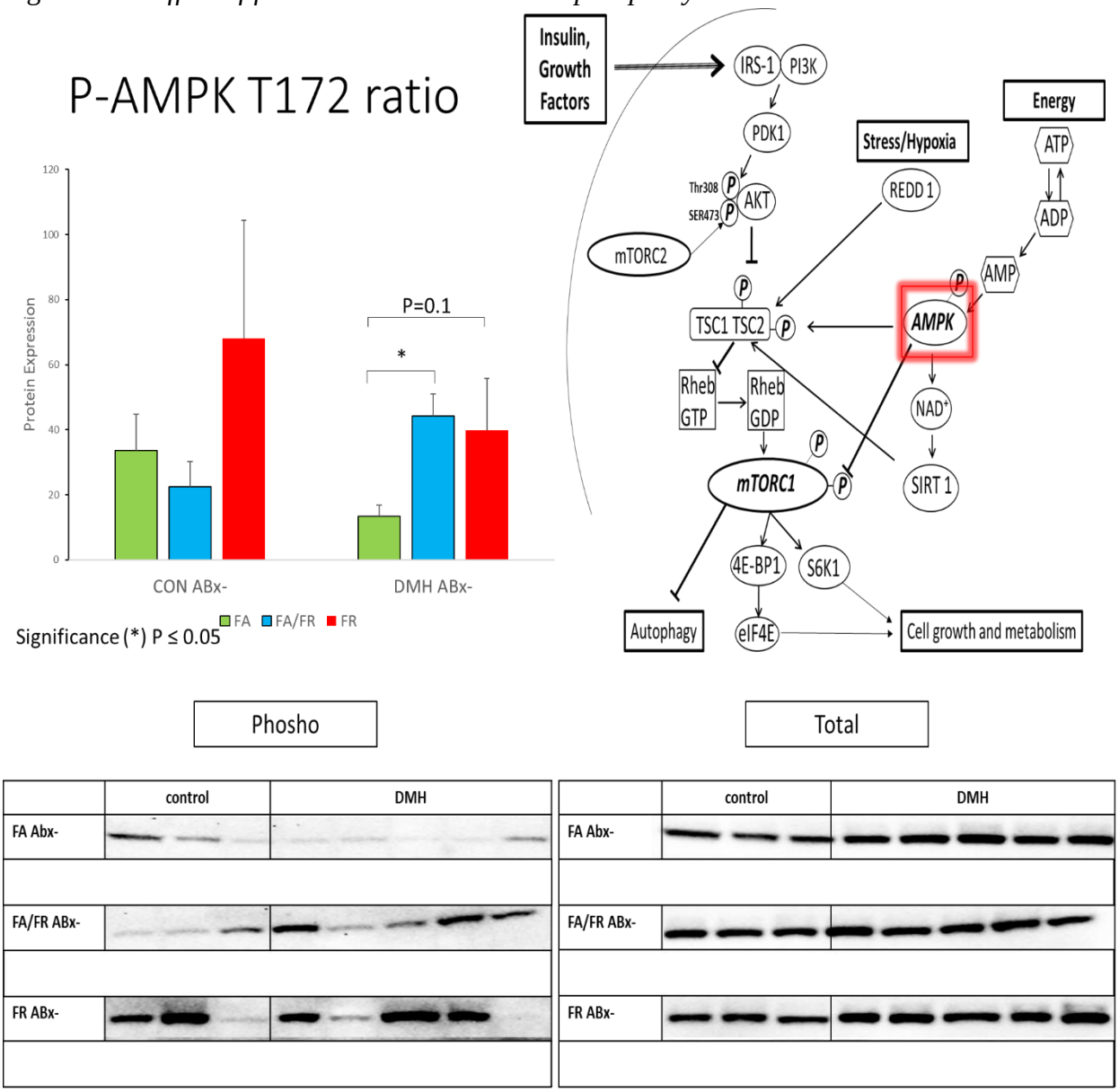

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Figure 3.16: E*ffect of folate restriction on energy levels (NAD+/NADH ratio)*
NAD^+/NADH ratio was assayed using a fluorometric microplate reader assay (Abcam). 20 mg of liver tissue was homogenized in provided lysis buffer and supernatant was collected. Total NAD, NAD^+ and NADH were assayed per protocol. Fluorescence was measured at EX/EM=540/590 nm. Data is expressed at NAD^+ to NADH ratio. FA: Folate adequate diet, FA/FR: short-term Folate restricted diet, FR: long-term Folate restricted diet, CON: control mice, DMH: mice treated with dimethylhydrazine, ABx-: diet without 1% SST. Control n=3, DMH n=6-7. * Significant differences at $P < 0.05$.

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Figure 3.16: *Effect of folate restriction on energy levels (NAD+/NADH ratio)*

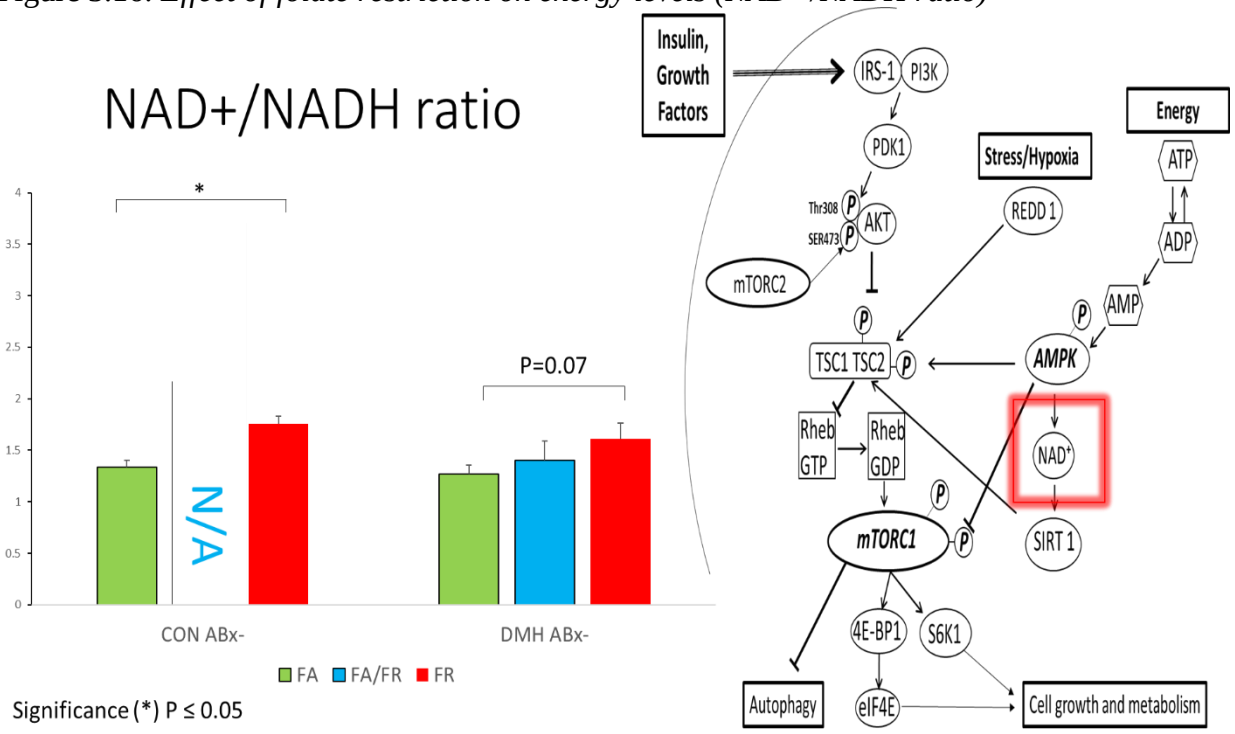

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Figure 3.17: *Effect of folate restriction on SIRT1 mRNA expression*
Levels of SIRT1 mRNA were analyzed in the liver. Expression of the gene was determined using real time PCR technique and the level was normalized to Gapdh expression as described in materials and methods. FA: Folate adequate diet, FA/FR: short-term Folate restricted diet, FR: long-term Folate restricted diet, CON: control mice, DMH: mice treated with dimethylhydrazine, ABx-: diet without 1% SST. Control n=3, DMH n=6-7. * Significant differences at $P < 0.05$.

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Figure 3.17: *Effect of folate restriction on SIRT1 mRNA expression*

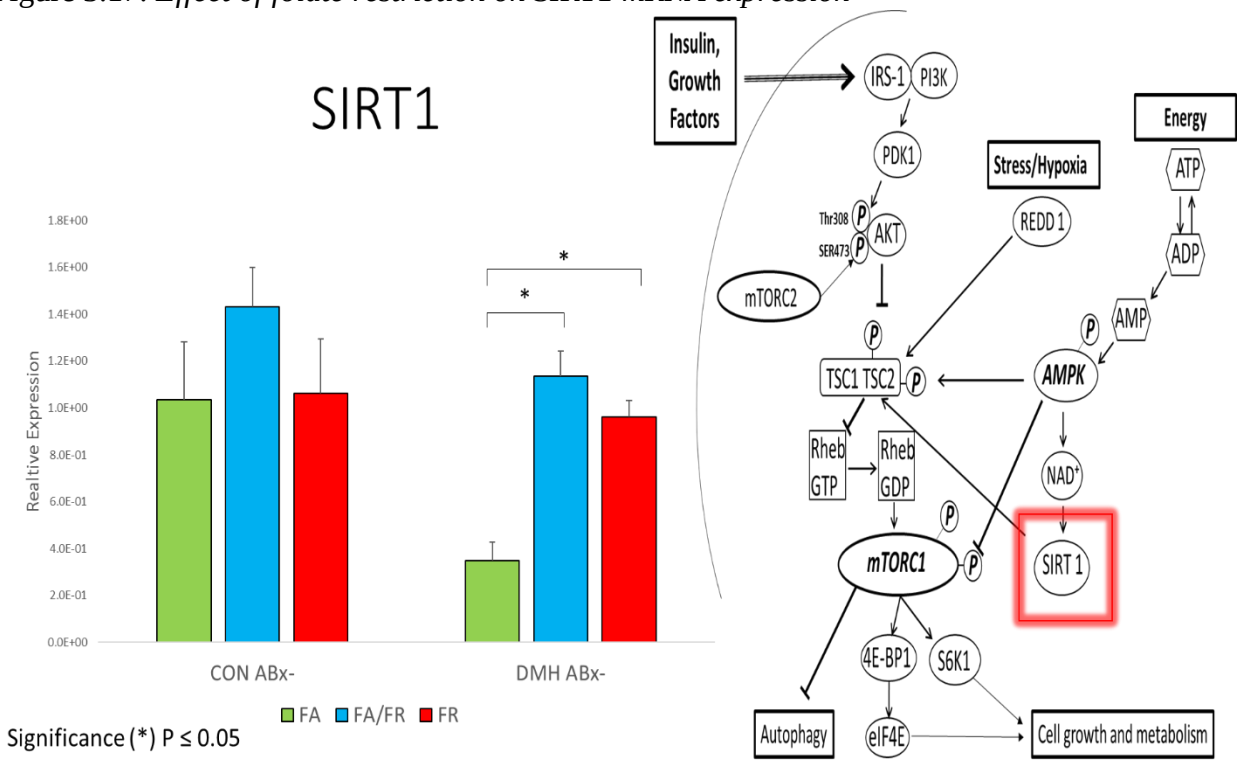

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Figure 3.18: *Effect of folate restriction on 4E-BP phosphorylation at T37-46 residues*
The level of 4E-BP1 protein phosphorylation at T37-46 was analyzed using western blotting as described in materials and methods. Data is expressed as adjusted band volume normalized to total lane proteins. Levels of phosphorylated protein were determined first, then the membrane lightly stripped and tested for respective total protein. Phosphorylated levels of protein were normalized to total and expressed as ratio. FA: Folate adequate diet, FA/FR: short-term Folate restricted diet, FR: long-term Folate restricted diet, CON: control mice, DMH: mice treated with dimethylhydrazine, ABx-: diet without 1% SST.Control n=3, DMH n=6-7. * Significant differences at $P < 0.05$.

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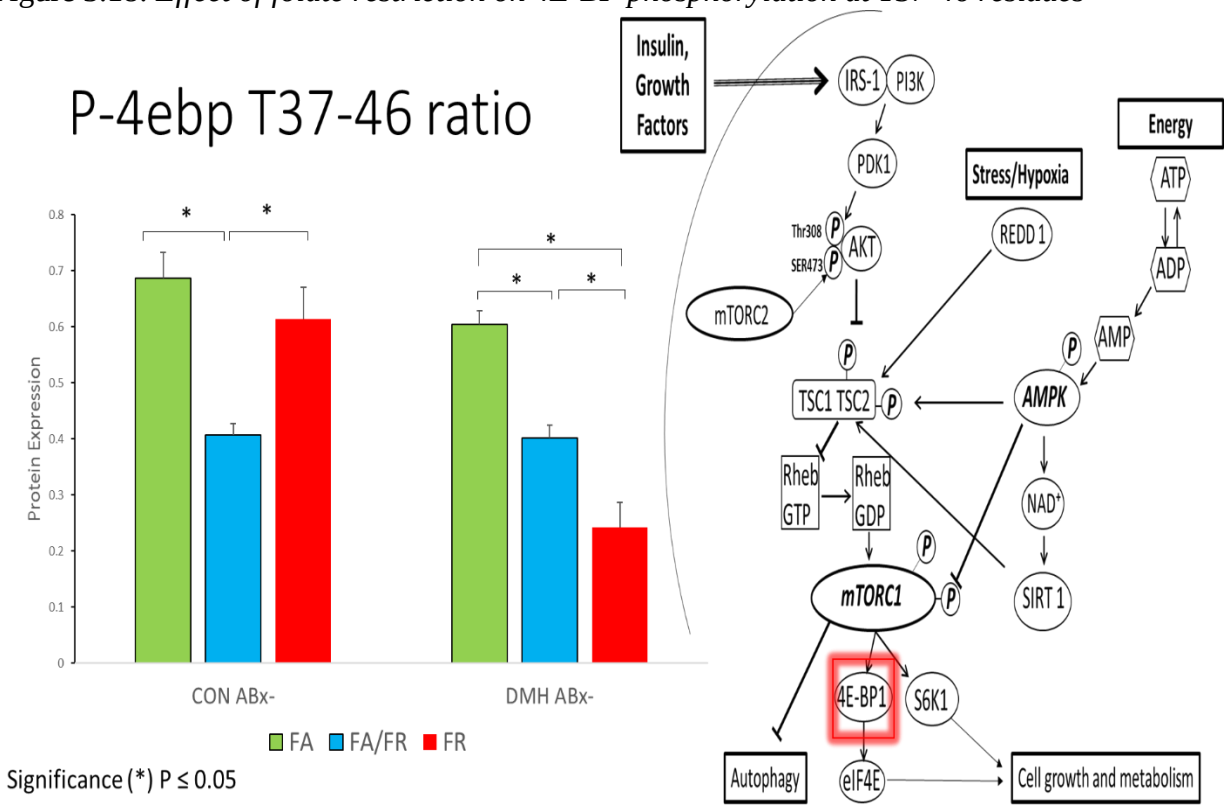
Figure 3.18: *Effect of folate restriction on 4E-BP phosphorylation at T37-46 residues*



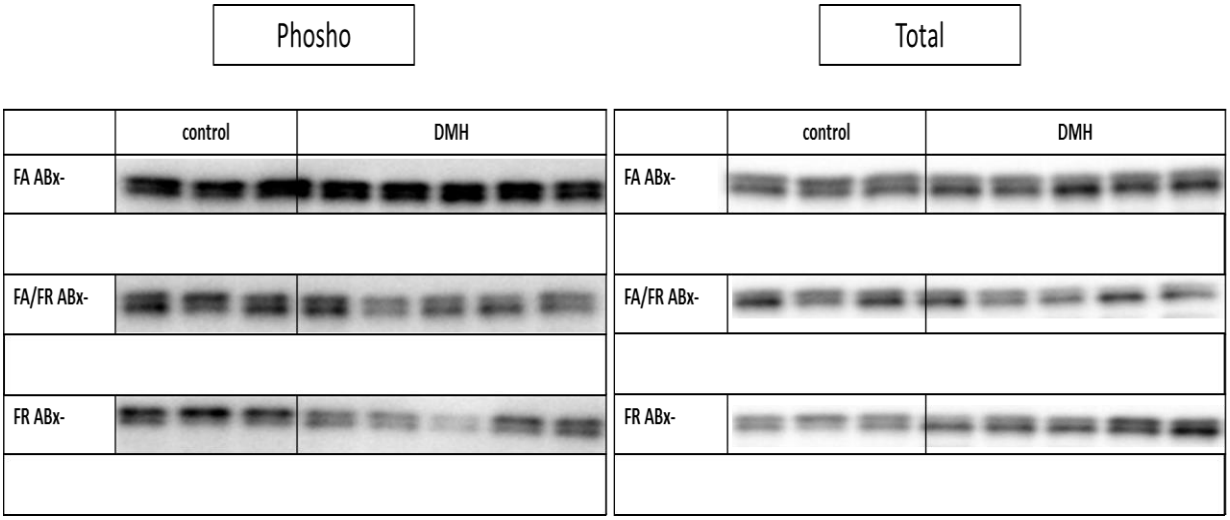


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Figure 3.19: *Effect of folate restriction on P-70 S6K1 phosphorylation*
The level of P-70 S6K1 protein phosphorylation was analyzed using western blotting as described in materials and methods. Data is expressed as adjusted band volume normalized to total lane proteins. Levels of phosphorylated protein were determined first, then the membrane lightly stripped and tested for respective total protein. Phosphorylated levels of protein were normalized to total and expressed as ratio. FA: Folate adequate diet, FA/FR: short-term Folate restricted diet, FR: long-term Folate restricted diet, CON: control mice, DMH: mice treated with dimethylhydrazine, ABx-: diet without 1% SST. Control n=3, DMH n=6-7. * Significant differences at $P < 0.05$.

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Figure 3.19: *Effect of folate restriction on P-70 S6K1 phosphorylation*

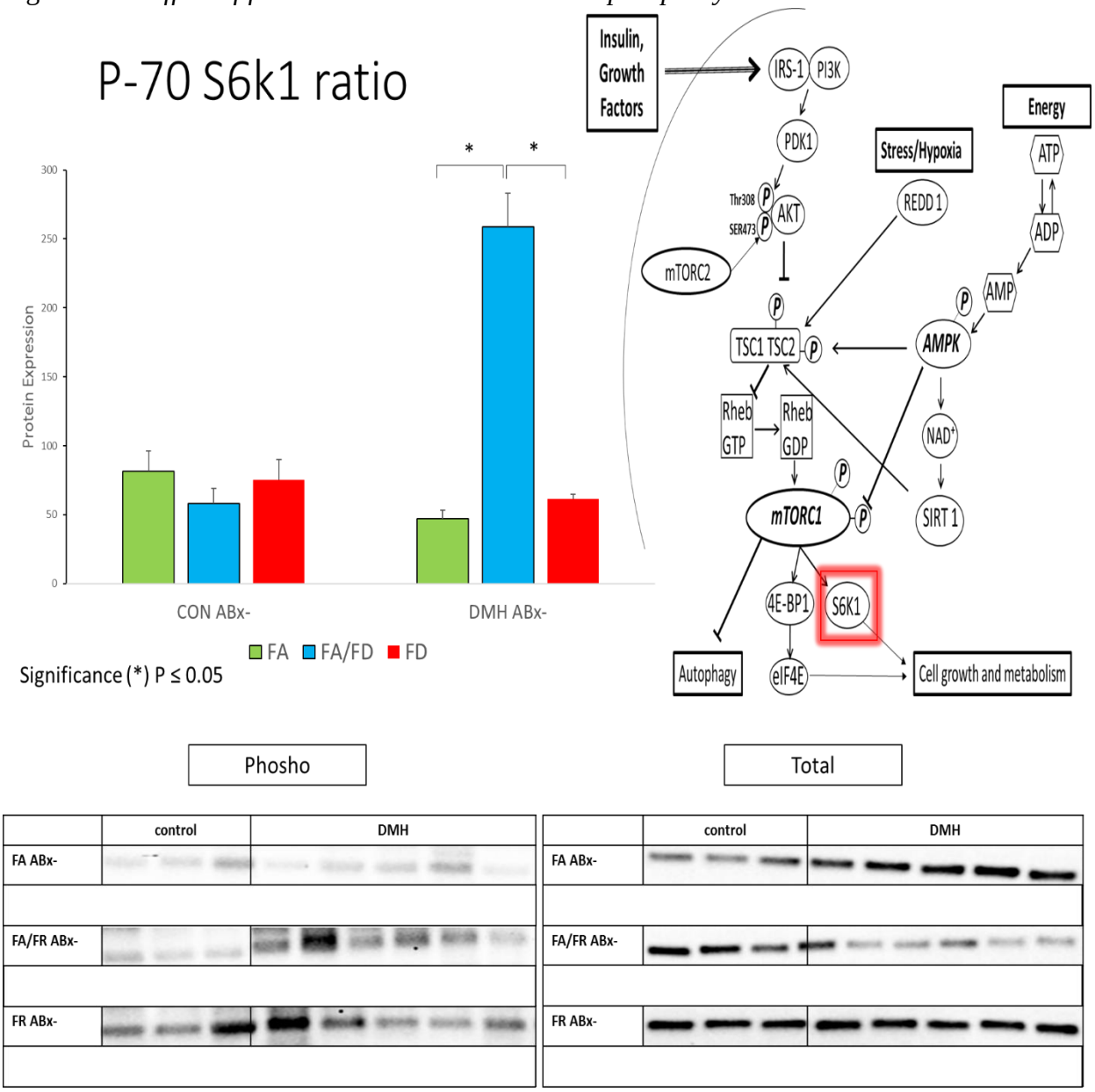

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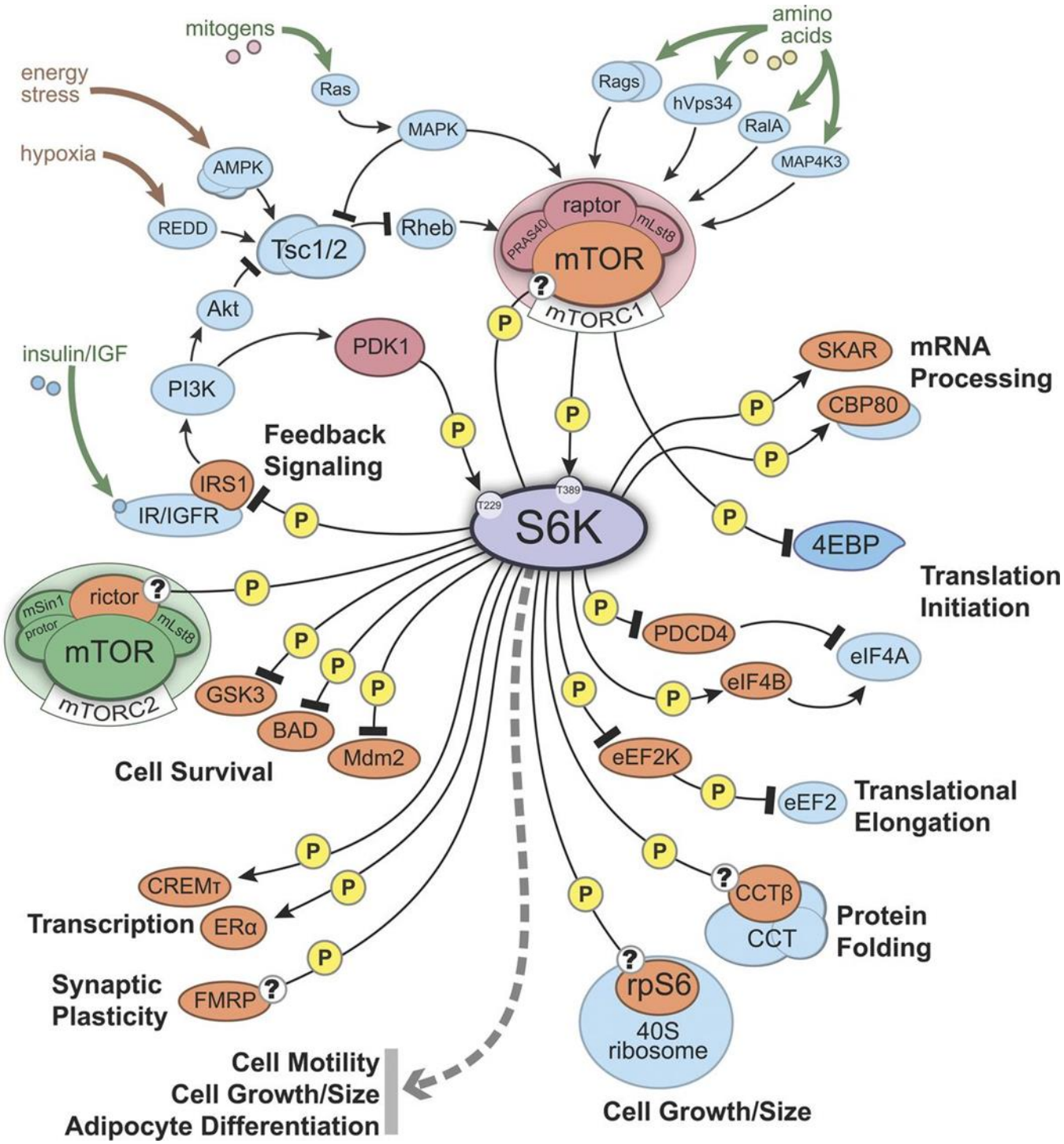
Figure 3.20: *P-70 S6K1 signaling cascade and feedback mechanisms*



Source:[204]

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Figure 3.21: *DNA methyltransferases role in methylation*

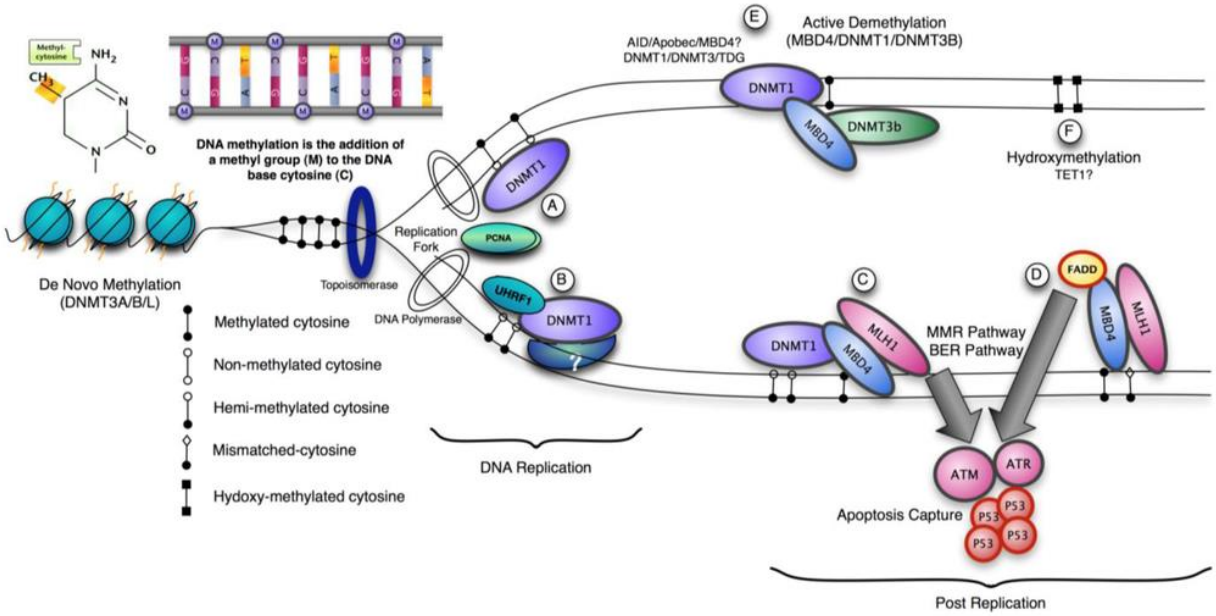

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Figure 3.22: *Effect of folate restriction on DNMT1 mRNA expression*
Expression of DNMT1 mRNA was analyzed in the liver tissues. Expression of the gene was determined using Real Time PCR technique and the level was normalized to Gapdh expression as described in materials and methods. Experiments were conducted in technical triplicates. FA: Folate adequate diet, FA/FR: short-term Folate restricted diet, FR: long-term Folate restricted diet, CON: control mice, DMH: mice treated with dimethylhydrazine, ABx-: diet without 1% SST. Control n=3, DMH n=6-7. * Significant differences at $P < 0.05$

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Figure 3.22: *Effect of folate restriction on DNMT1 mRNA expression*

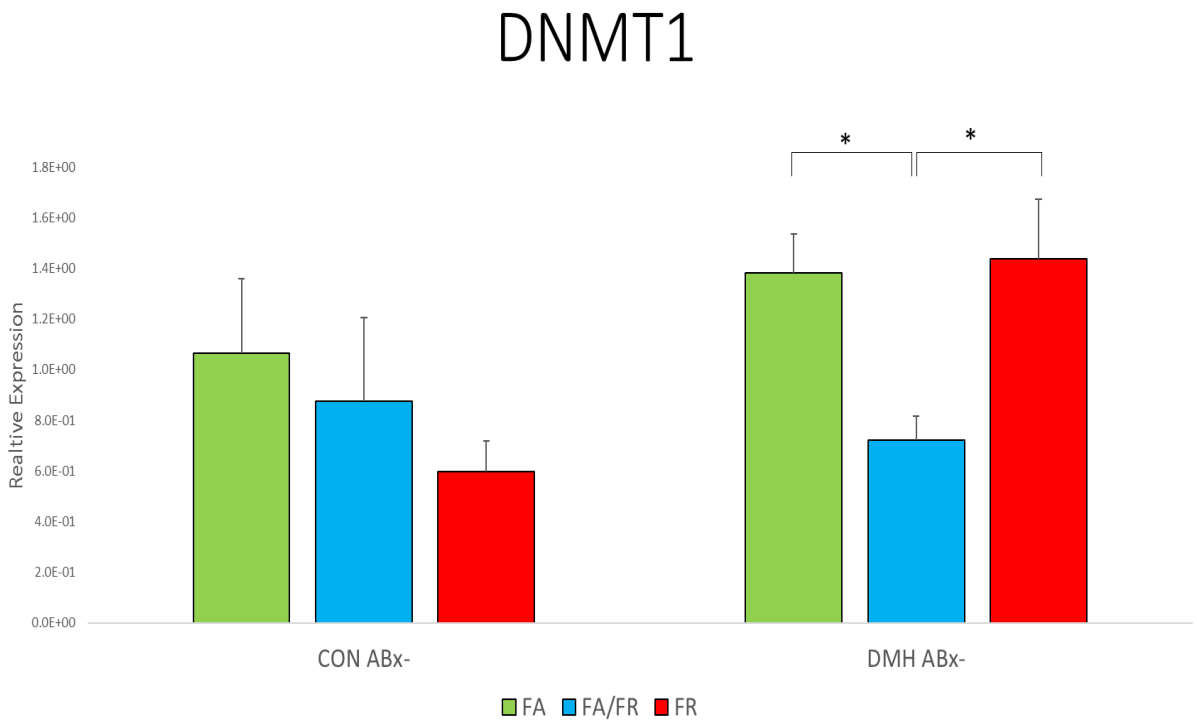

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Figure 3.23: *Effect of folate restriction on DNMT3a mRNA expression*
Expression of DNMT3a mRNA was analyzed in the liver tissues. Expression of the gene was determined using Real Time PCR technique and the level was normalized to Gapdh expression as described in materials and methods. Experiments were conducted in technical triplicates. FA: Folate adequate diet, FA/FR: short-term Folate restricted diet, FR: long-term Folate restricted diet, CON: control mice, DMH: mice treated with dimethylhydrazine, ABx-: diet without 1% SST. Control n=3, DMH n=6-7. * Significant differences at $P < 0.05$

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Figure 3.23: *Effect of folate restriction on DNMT3a mRNA expression*

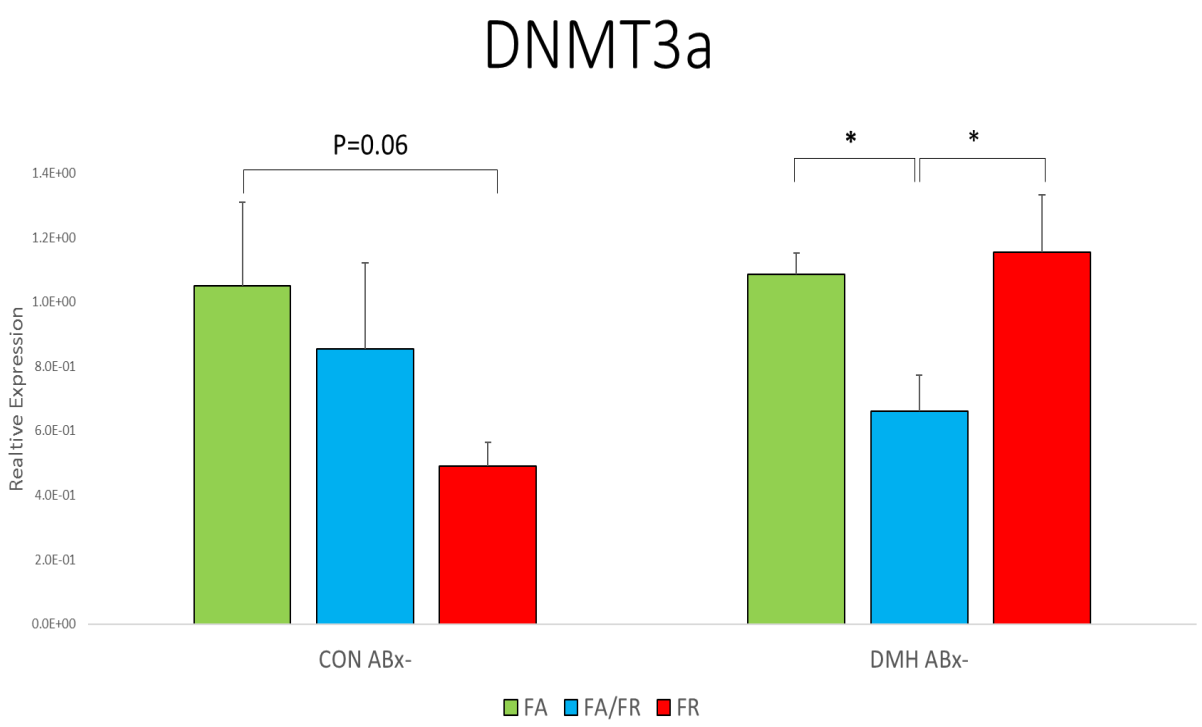

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Figure 3.24: *Effect of folate restriction on DNMT3b mRNA expression*
Expression of DNMT3b mRNA was analyzed in the liver tissues. Expression of the gene was determined using Real Time PCR technique and the level was normalized to Gapdh expression as described in materials and methods. Experiments were conducted in technical triplicates. FA: Folate adequate diet, FA/FR: short-term Folate restricted diet, FR: long-term Folate restricted diet, CON: control mice, DMH: mice treated with dimethylhydrazine, ABx-: diet without 1% SST. Control n=3, DMH n=6-7. * Significant differences at $P < 0.05$

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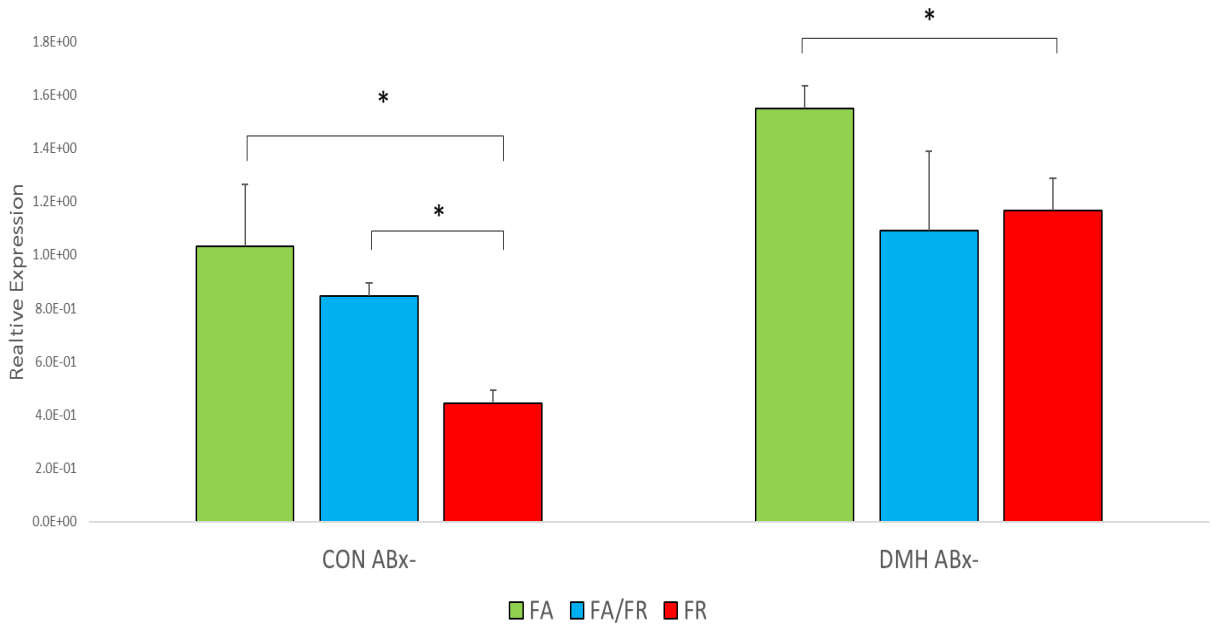
Figure 3.24: *Effect of folate restriction on DNMT3b mRNA expression*



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CHAPTER 4: SUMMARY AND FUTURE DIRECTIONS

The relation between folate and cancer is growing increasingly complex as research is uncovering new facts that contradict the existing paradigm. For the past several decades the collective evidence supported the increased intake of folates to improve health outcomes and protect against various cancers, specifically colon cancer [26]. Folate's role in nucleotide synthesis was well known since the 40's and that knowledge led to the rise of the antifolate class of drugs to treat various cancers [142]. Adequate folate is also considered essential for maintaining genetic stability and protecting tissues from transformation. However, overly abundant consumption of folates can encourage the growth of dysplastic neoplastic lesions [106]. The mandatory fortification of grains and cereals with folates to reduce NTD's since the 90's led to a substantial increase in the population's folate levels [10].

Animal studies assessing the risks and benefits of folate supplementation provided contradictory findings [54]. Our initial findings uncovering a protective effect of dietary folate restriction on colon carcinogenesis in our β-pol haploinsufficient model led us to expand our investigation to see if the protection persists in normal mouse genetic background [38]. Our initial pilot studies revealed that extended folate restriction, allowing the animals to acclimate, provided protection as seen in reduced ACF formation. We then embarked on this current study to further characterize and attempt to mechanistically dissect that protective effect. We randomized our experimental and control mice in groups with or without the antibiotic succinylsulfathiazole. SST is commonly used to reduce folate producing bacteria in the gut of the animals and can therefore induce a more severe folate depleted condition. We were also interested in isolating the effect of the antibiotic in a folate adequate condition to see if it can modify or confound the suspected pathways modulated by folate restriction and cancer. Surprisingly, SST had a significant effect on

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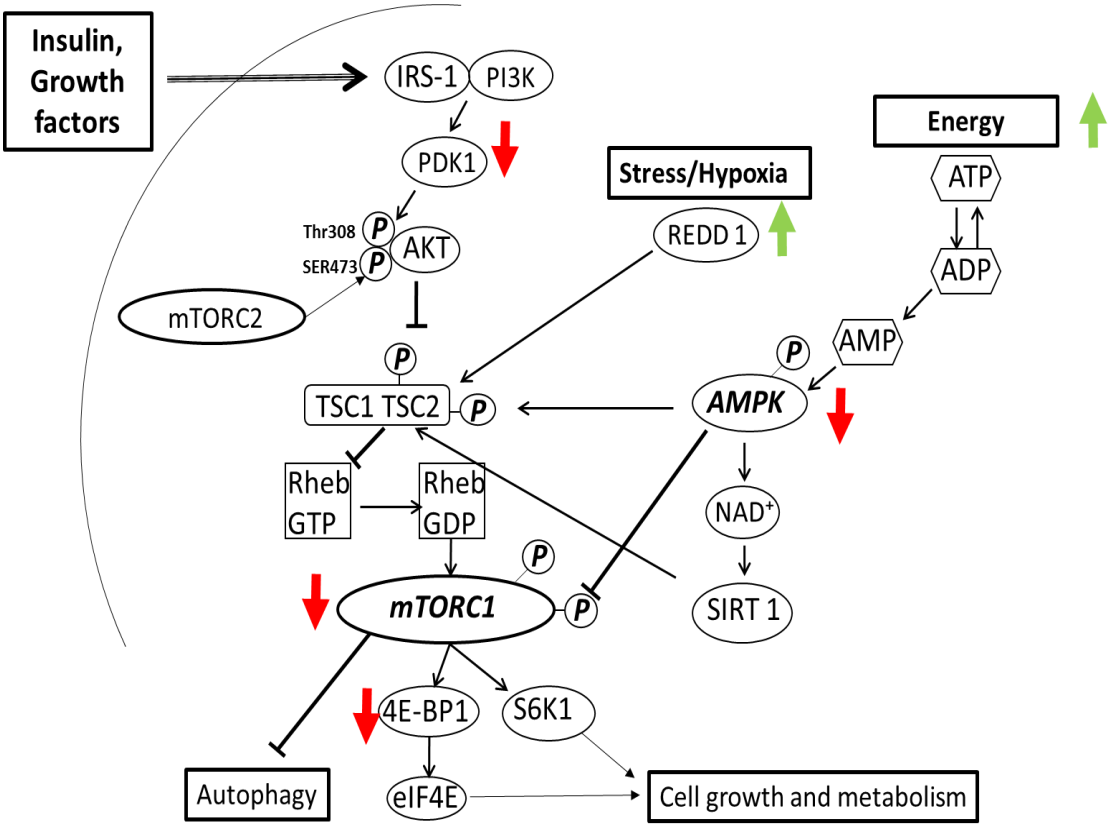
the mTOR pathway, reducing mTOR phosphorylation and the downstream target 4E-BP1. Furthermore, we saw a decrease in IGF1 activation upstream of mTOR as evident from reduced PDK1 phosphorylation. Paradoxically, SST increased energy levels leading to decreased activation of the mTOR inhibitor AMPK. However, REDD1, an inhibitor of mTOR that responds to stress and hypoxia was increased by SST.

Summary of the effect of SST on the mTOR pathway:



These findings allowed us to characterize the effect of SST on the critical mTOR pathway. The observed effects most likely stemmed from the perturbation of intestinal flora leading to the disruption of growth signaling and energy metabolism. SST groups were then omitted from further mechanistic analysis in the 2nd part of the study.

After establishing that an intact microflora is crucial for a sound analysis of the effect of folate restriction on colon carcinogenesis, we proceeded with analysis of the remaining groups that

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were not exposed to SST. The results clearly show that in c57/bl6 mice, carcinogenic treatment initiated one week after starting a folate depleted diet caused significantly more ACF than a folate depleted diet for 8 weeks when compared to FA control. This effect was observed despite an equal reduction in serum, liver and colon folate levels between FA/FR and FR mice. That brings us to the conclusion that folate restriction requires an adaptation period to exert a protective effect. Analysis of the mTOR pathway revealed that both FA/FR and FR reduced mTORC1 activation. However, we observed a paradoxical activation of the IGF1 pathway upstream of mTORC1 though increased activation of PDK1. REDD1 expression was decreased in both FA/FR and FR. mTORC1 downregulation was likely induced by perturbance in energy levels and metabolism as seen by the increased expression of AMPK and SIRT1. We observed a differential effect between FR and FA/FR downstream of mTOR. 4E-BP1 phosphorylation was significantly reduced in FR compared to FA/FR and FA, while S6K1 phosphorylation was significantly increased in FA/FR compared to FA and FR. These findings suggest that long term folate restriction FR may potentially inhibit growth and metabolism, a condition that is considered protective against cancer initiation and progression. While short term folate restriction resulted in a paradoxical increase in S6K1 activation, potentially overriding the growth inhibition of mTORC1 downregulation. Analysis of the DNA methyltransferases revealed that FA/FR resulted in decreased expression of DNMT1 and DNMT3a compared to FA, while FR had no effect. The decrease in the activity of methyltransferases in FA/FR warrants additional research to fully characterize the impact on genomic methylation patterns.

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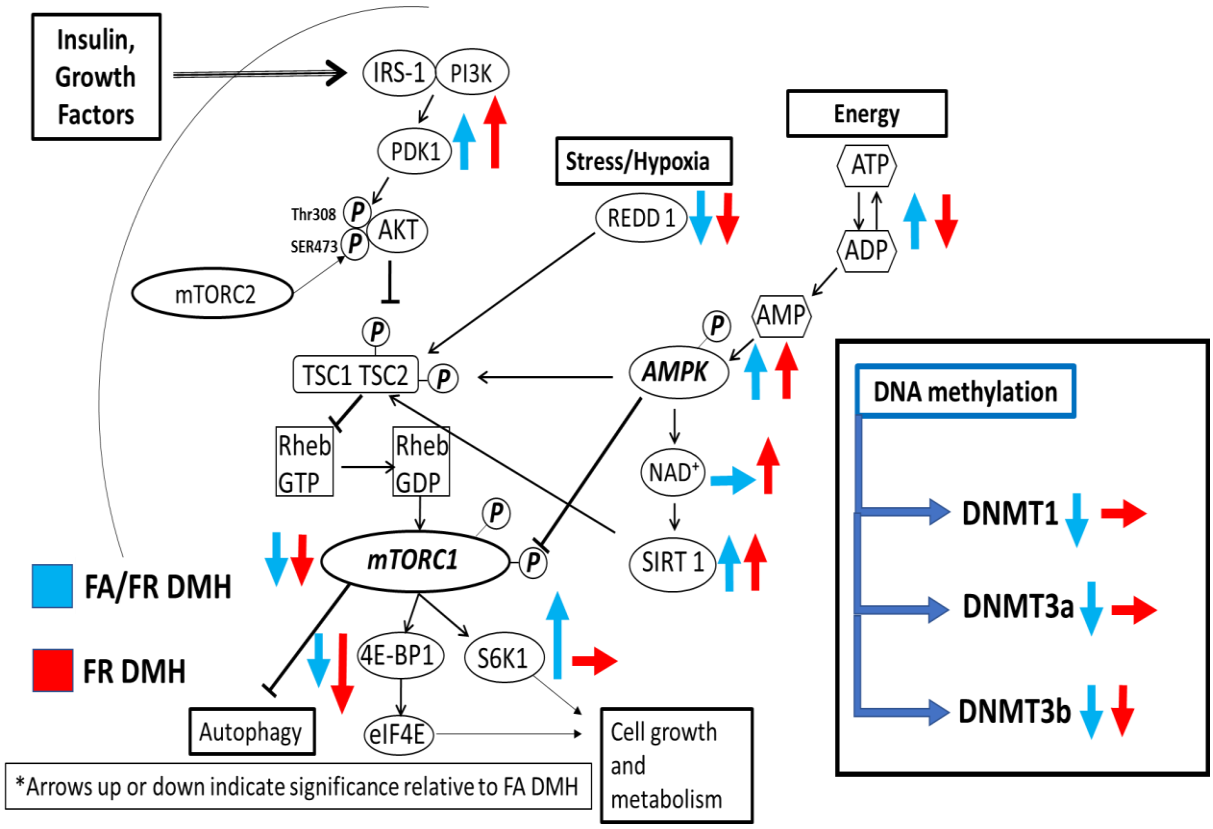
Summary of the differential effect of timing and duration of folate restriction on mTOR and DNA methyltransferases.



It is of utmost importance to continue to investigate the relationship between folate and cancer. It would be tempting to accept the opinion of the majority and presume that folate can do no harm. However, the magnitude of evidence that points in the other direction has become overwhelming. Future studies should focus on attempting to duplicate the results in other lab models and genetic backgrounds. Proteomics, metabolomic, and epigenetic studies should be conducted to further understand the dynamics and adaptation that link folate to various signaling pathways involved in cancer. Establishing accurate and standardized folate requirements for animal studies is necessary to draw parallels and compare results. Most importantly, establishing the proper human requirements without attempting to generalize and enforce the recommendation on all subsets of the population is warranted. The human population is heterogeneous with varying

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nutritional requirements and subject to diverse environmental and genetic influences. In the era of personalized medicine, we should scrutinize generalized public health policies and personalize nutrition in the same manner we personalize medicine.

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ABSTRACT

TIMING AND DURATION OF FOLATE RESTRICTION DIFFERENTIALLY IMPACTS COLON CARCINOGENESIS.

by

ALI FARDOUS

May 2020

Advisor: Dr. Ahmad R. Heydari

Major: Nutrition and Food Science

Degree: Doctor of Philosophy

Colorectal cancer (CRC) constitutes a major burden on the healthcare system as the second most commonly diagnosed cancer in the developed world. Dietary folate is considered an important modulator of colorectal cancer. Folate restriction has been implicated in increasing CRC incidence by disrupting nucleotide synthesis, Impacting DNA methylation and inducing genetic instability. Our research shows that the timing and duration of dietary folate restriction can differentially impact Colorectal cancer initiation. Acclimating mice to folate restriction for 8 weeks results in a reduced number of preneoplastic lesions compared to mice placed of folate restriction for 1 week prior to initiating the carcinogenic treatment. Analysis of the mTOR pathway revealed distinct differences in crucial target proteins between the two conditions. Furthermore, we noted differences in the expression of key DNA methyltransferases impacting global methylation.

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AUTOBIOGRAPHICAL STATEMENT

ALI FARDOUS

Education

April 2004 Bachelor of science in Pharmacy

Professional Experience

2004-2009 Pharmacist in charge, Rite Aid Pharmacy, Detroit MI
2009-2020 Stafff Phrmacist, Walmart Pharmacy, Taylor MI
2011-2014 Graduate Teaching Assistant, Depatment of Nutrition and Food Science
Wayne State University, Detroit, MI

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