Effect of Maternal Dietary B-Vitamin Intake on Offspring DNA Methylation Patterns, Gene Expression, Cytokinetics, and Intestinal Tumorigenesis in the $Apc^{+/1638N}$ Mouse Model

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Abstract

Epidemiological and laboratory data have repeatedly implicated one-carbon nutrients such as folate, riboflavin (vitamin B_2), vitamin B_6, and vitamin B_{12} as being protective against various cancers, most notably those of the colorectum. Deficiencies in one or all of these nutrients have been shown in a variety of experimental settings to lead to increases in genotoxic events associated with cancer such as dysregulation of DNA methylation as well as altered expression of several genes in the Wnt signaling pathway.

The role of maternal nutriture in determining offspring disease susceptibility is increasingly recognized. Therefore, we aimed to determine whether a relationship between maternal dietary B-vitamin intake and intestinal tumorigenesis in offspring was present in a genetically engineered model of colorectal cancer. It was our central hypothesis that supplemental levels of one-carbon nutrients in the maternal diet from preconception through weaning would decrease, while deficient levels would increase, dysregulation in DNA methylation patterns in Wnt pathway genes in the offspring that preserve apoptotic function leading to a reduction in tumorigenesis.

Three groups of 6-week old female C57BL/6 mice were fed diets either deficient, replete, or supplemented with folate, vitamin B_2, B_6, and B_{12} for 4 weeks, then mated with male *Apc*$^{+/1638N}$ mice. Females remained on their respective diets throughout mating, pregnancy and the suckling period (11 weeks total). Pups were genotyped at 3 weeks of age, and all wild type pups were sacrificed. After weaning, all *Apc*$^{+/1638N}$ pups were fed a replete diet (AIN-93) regardless of the maternal diet. At 32 weeks of age, 56% and 59% of *Apc*$^{+/1638N}$ pups born to deficient and replete mothers, respectively, exhibited tumors in the small intestine compared to 21% of pups born to supplemented mothers (p=0.031). Furthermore, a significantly (p=0.026) higher percentage of tumors collected from pups of deficient mothers (54%) displayed invasive features compared to tumors from pups of replete mothers (18%).

In the small intestine of 3-week old wild type pups as well as 32-week old *Apc*$^{+/1638N}$ pups, the expression of several negative regulators of Wnt signaling such as *Apc*, *Sfrp1*, *Wif1*, and *Wnt5a* was proportional to the concentration of B-vitamins in the maternal diet. Furthermore, the expression of *Sfrp1* was significantly and inversely correlated with the degree of methylation within the promoter region of the gene. Contrary to our expectations, an increase in apoptosis was observed among pups born to deficient dams in both genotypes. Along with an elevation of β-catenin in the small intestine, these data are consistent with the hypothesis that the maternal diet affects offspring tumorigenesis through a methylation induced de-repression of the Wnt pathway.

The results of the project described herein are among the first observations regarding maternal B-vitamin intake and offspring colorectal carcinogenesis and provide the most comprehensive dataset available to date regarding the potential mechanism mediating this effect.

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I. Statement of the Hypothesis to be Tested

Maternal dietary intake of one-carbon nutrients such as folate and vitamins B_2, B_6, and B_{12} may be a determinant of epigenetic modifications in the offspring and as such have the potential to impact later disease susceptibility. **The central hypothesis of this dissertation is that supplemental levels of one-carbon nutrients in the maternal diet from preconception through weaning will decrease, while deficient levels will increase, dysregulation in DNA methylation patterns in Wnt pathway genes in the offspring that preserve apoptotic function leading to a reduction in tumorigenesis.** This hypothesis was tested by pursuing the following specific aims:

Specific Aim 1: *To determine the impact of maternal one-carbon nutrient intake on tumor incidence and grade in the intestine of offspring in a genetic model of colorectal cancer.* We observed a significant reduction of tumor incidence among pups born to supplemented mothers, as well as an increased likelihood that tumors in deficient offspring displayed invasive characteristics.

Specific Aim 2: *To determine whether DNA methylation density in offspring at both the gene-specific and genomic levels is impacted by changes in maternal B-vitamin intake and whether such methylation changes are associated with alterations in gene expression.* A significant impact of maternal diet was observed on the expression of Wnt pathway inhibitory genes among offspring. The expression of one such gene, *Sfrp1*, was inversely correlated to the degree of methylation in the upstream portion of the gene.

Specific Aim 3: *To assess the effect of maternal diet on downstream Wnt signaling events and cytokinetics in the intestinal mucosa of offspring.* Contrary to our expectations, a significant increase in apoptosis was observed in pups born to deficient mothers.

II. Review of the Literature

A. Folate, B-Vitamins, and Carcinogenesis

Epidemiological and laboratory data have repeatedly implicated folate and other B-vitamins (vitamins B_2, B_6, and B_{12}) in the prevention of various cancers, most notably colorectal cancer. Mechanistically, these nutrients may impact carcinogenesis through their role in providing methyl groups for the synthesis of nucleotides and of S-adenoysl methionine (SAM), the universal donor for the methylation of DNA. Importantly, the inter-conversion of different biological forms of folate is dependent on vitamins B_2, B_6 and B_{12} as four folate-metabolizing enzymes require one of these vitamins as cofactors. Vitamin B_6 appears to be particularly important as several studies report a robust inverse relationship between blood concentrations or dietary intake and risk for CRC [1-4]. Folate inadequacy causes an intracellular accumulation of dUMP and depletion of dTMP [5, 6], which promotes uracil misincorporation into DNA [7-11] and results in DNA strand breakage [12, 13]. Related to its role in SAM synthesis, folate deficiency is reported to cause epigenetic instability by promoting genomic *hypo*methylation [14-16] and the seemingly paradoxical *hyper*methylation of specific gene promoters [17-19]. These molecular perturbations are also believed to be responsible for the resulting disruptions in gene expression in the p16, p53, and Wnt signaling pathways that occur secondary to folate insufficiency [16, 20, 21]. It is worth noting that a state of combined mild deficiency in all four of these nutrients results in greater disruptions in these procarcinogenic aberrations than does a deficiency in any one or two of these nutrients [16, 20, 22].

Timing has emerged as an important mediator in the relationship between one-carbon nutrients and carcinogenesis [23], In the case of folate, for example, supplementation before the appearance of a pre-neoplastic lesion may reduce, while supplementation after may promote tumorigenesis [24]. Similarly, one recent high-profile intervention trial has shown that folic acid supplementation may increase the likelihood of adenoma appearance in those who have previously had an adenoma removed [25] although another failed to detect a harmful effect [26]. Our current understanding is that an insufficiency of folate may promote carcinogenesis by causing DNA breakage and methylation aberrations while supplemental quantities of the vitamin may also promote tumorigenesis by supporting cellular proliferation of existing pre-neoplastic lesions through the provision of an abundant supply of nucleotides for DNA synthesis [27].

B. Maternal Diet and Tumorigenesis

Several epidemiological studies support the concept that varying the intake of one-carbon nutrients may impact on the risk for tumorigenesis in offspring. For example, a reduced incidence of acute lymphocytic leukemia (ALL) has been observed among children born to mothers who used folic acid-containing multivitamins peri-conceptionally in most [28-30] but not all case control studies [31].

A similar relationship between maternal folic acid-containing multivitamin use and risk of several other types of childhood cancers in offspring has been observed. Three such case control studies report that maternal multivitamin use was associated with a significantly reduced risk of pediatric brain tumors in offspring [32-34], while three others reported modest reductions in risk that failed to attain statistical significance [35-

37]. A decreased risk of retinoblastoma in offspring has been associated with a high maternal intakes of folate from dietary [38] as well as from supplemental sources [39]. One report also demonstrates an association between maternal folate and iron supplementation and a reduced risk of non-Hodgkin lymphoma [40]. Additionally, maternal multivitamin use has been implicated as being protective against neuroblastoma in offspring [41, 42]. Finally ecological studies indicate that, along with Wilms' tumor [43], the incidence of neuroblastoma [44] declined significantly following the introduction of mandatory folic acid fortification in Canada in the late 1990s.

As suggested by a recent meta-analysis [45], the potential protective effect bestowed upon children exposed to periconceptional multivitamins during gestation observed may be quite substantial. Among children born to mothers supplementing with multivitamins containing folic acid, the odds of having pediatric brain tumors were approximately 30% lower (OR=0.73; 95% CI=0.60, 0.88), the odds of having ALL were roughly 40% lower (OR=0.61; 95% CI=0.50, 0.74), and the odds of having neuroblastoma were nearly 50% lower (OR=0.53; 95% CI=0.42, 0.68) as compared to children born to mothers not using these vitamins. One important point that we remain cognizant of is that because many of the studies have looked at supplements in which folate is only one of many nutrients present; a role for these additional nutrients in modulating carcinogenesis in offspring cannot be excluded. However, the observation that polymorphisms in genes involved in folate metabolism such as MTHFR [46] and Methionine Synthase [47] can modulate the risk of developing ALL in adulthood, as well as the increasing appreciation for the role of DNA methylation in the pathogenesis of childhood cancers [48, 49] lend support to the

notion that alterations in one-carbon metabolism may serve as a major driver behind the apparent chemopreventive effect of maternal multivitamin intake.

It is apparent that associations between maternal one carbon intake and cancer in offspring have to date been limited to cancers that affect children, and that epidemiological evidence is not yet available to support or refute the idea that maternal one-carbon nutrient intake can impact on the risk for developing adult cancers, such as those of the colorectum or breast. This is likely because suitable databases that attempt to link maternal diet to diseases in the latter decades of the offspring's adulthood are not yet available. Nevertheless, the epidemiological observations summarized above provide support for the notion that early intervention with one-carbon nutrients in the maternal diet is associated with a decreased risk of several pediatric cancers in offspring. Given that tumorigenesis in the adult colorectum and breast has repeatedly been shown to be sensitive to one-carbon nutrient status, it is reasonable to postulate that maternal diet might impact on these cancers as well.

Animal studies on this topic to date are somewhat conflicting. Maternal folic acid supplementation has been reported to reduce offspring intestinal tumorigenesis in one preliminary report [50], while a separate group failed to observe an effect, and in fact, the latter publication suggested that maternal folate supplementation increased tumorigenesis in the offspring [51]. Likewise, maternal folate deficiency was reported to have both a beneficial effect on offspring tumorigenesis [51], while again, a separate group failed to observe an effect [52]. It is worth noting that these studies used different models of

colorectal cancer (specifically, the chemical carcinogen azoxymethane [50] and the *Apc*$^{+/min}$ mouse [51, 52]), as well as different experimental designs which, at least in part, may account for the disparate results.

C. *Maternal Diet and Epigenetics*

Landmark studies utilizing the Agouti viable yellow (A^{vy}/a) mouse are perhaps the most widely recognized demonstration of the potential for maternal diet to impact on gene-specific methylation and phenotype in offspring. In this model, feeding dams a methyl donor rich diet during gestation shifted the coat color phenotype of offspring from being predominantly yellow (agouti), to being brown in color (pseudo-agouti) [53, 54]. Importantly, this increase in the proportion of pups born with a brown coat color coincided with an elevated methylation of specific sequences within the A^{vy} promoter [53, 54]. Furthermore, it is reported that mice with the yellow coat color have an elevated propensity towards adult-onset obesity, hypertension, and insulin resistance compared to mice with brown coats [55]. More pertinent to the current topic, however, Wolff and colleagues demonstrated that following prolonged exposure to the chemical carcinogen lindane, a significant reduction in hepatic tumor multiplicity as well as tumor incidence in the lung was observed in pseudo-agouti pups relative to their agouti littermates [56]. These studies demonstrate that supplemental one-carbon nutrients in the maternal diet can shift the phenotype of the offspring to one that has elevated resistance to tumorigenesis.

Similar to the A^{vy}/a model, the potential for maternal methyl group consumption to alter the phenotype of the offspring has also been demonstrated in the *Axin Fused* (*Axin*^{Fu}), or

“kinky-tail” mouse. In this mouse, one-carbon nutrient supplementation of dams during gestation suppresses the kinky-tail phenotype which is evident in the offspring of control fed dams [57]. Furthermore, the presence of tail kinks was inversely related to the methylation density of a retrotransposon within exon 6 of the *Axin* gene.

Proof of concept that maternal one-carbon intake can impact the methylation and expression of specific genes in offspring was established in A^{vy}/a and $Axin^{Fu}$ mice; models in which an inserted retrotransposon creates a cryptic promoter that is sensitive to CpG methylation. More recently however, studies are beginning to identify genes with clear cancer relevance as being sensitive to maternal one-carbon nutrient intake.

One such gene whose methylation is modified by maternal diet is *Igf2*. Expression of *Igf2* is heavily dependent on imprinting with the maternal allele being completely silenced, leaving only the paternal allele to be expressed. However, the methylation density of imprinted maternal alleles can diminish, a process known as loss of imprinting, resulting in biallelic expression of the gene. Loss of *Igf2* imprinting has been implicated in several pathologic conditions, including cancers of the prostate and colorectum in both animal models and human subjects [58, 59]. Evidence that perturbations in the methylation of this gene may occur during development comes from a cohort of Dutch citizens who were exposed to famine *in utero* during World War II. Remarkably, those exposed to the famine during periconception had significantly lower methylation of the *Igf2* ‘differentially-methylated region’ compared to their same-sex siblings who were not exposed to famine when assessed some 60 years after the exposure [60]. In a follow-up

to this study, several additional genes in famine exposed subjects, including *Il10* and *Abca1*, were reported to have modest methylation changes relative to their unexposed siblings [61]. In a separate study, periconceptional maternal supplementation with folic acid was associated with significantly higher methylation of the same differentially-methylated region of *Igf2* in the offspring [62]. Several preclinical studies in rodents have also reported an effect of one-carbon nutrient intake on *Igf2* methylation and expression both during early post-weaning life [63] as well as during *in utero* development [64].

D. The Wnt Signaling Pathway, Colorectal Carcinogenesis, and Epigenetics

One additional pathway that may serve as a potential mediator in of the effect of maternal and individual diet on colorectal carcinogenesis is the canonical *Wnt* signaling pathway. This pathway is involved in the growth and development of several tissues, including that of the gastrointestinal mucosa [65]. This pathway is also heavily involved in colorectal carcinogenesis, as mutations in the Wnt pathway are observed in more than 80% of sporadic colorectal tumors [66]. The loss of the Wnt pathway gene *Apc*, for example, is considered to be a major initiating event in the development of colorectal tumors [66, 67]. While mutations to these genes may occur through several mechanisms, silencing of genes through promoter hypermethylation has been reported in several Wnt pathway genes such as *Apc* [68], but also *Axin2*, *Dkk1*, the *Sfrp* family (*Sfrp1*, *2, 4*, and *5*), and *Wif1*, among others [69, 70]. Epigenetic changes similar to those observed in human tumors are also seen in mouse models of colorectal cancer such as the $Apc^{+/1638N}$ mouse, namely hypermethylation of the *Sfrp1* promoter (Liu et al., unpublished data).

Several components of the *Wnt* pathway have been previously shown to be responsive to B-vitamin availability both *in vitro* and *in vivo*. The expression of both *Apc* and *β-catenin* have been shown to be responsive to folate delivery in cancer cell lines [21]. In wild type mice, a combined mild B-vitamin deficiency has been shown to result in several changes consistent with colonic *Wnt* signaling activation including diminished expression of *Apc*, increases in nuclear β-catenin localization and C*yclinD1* gene expression, and diminished apoptosis [16, 71]. More recent data from a *Wnt*-reporter mouse model crossed with the $Apc^{+/1638N}$ mouse (*BAT-LacZ* x $Apc^{+/1638N}$) has provided a clear demonstration of *in vivo* colonic *Wnt* activation as a result of mild multiple B-vitamin deficiency [72]. Therefore, when searching for potential candidate genes that mediate the effect of maternal feeding on offspring tumorigenesis, elements of the Wnt pathway are promising candidates due to their relationship with one-carbon nutrient intake, their established history of methylation aberrations in colorectal cancer, and as suggested by the $Axin^{Fu}$ model, the methylation status of Wnt pathway genes may be responsive to the presence of one-carbon nutrients in the maternal diet.

Considering these observations, it is therefore our **central hypothesis that supplemental levels of one-carbon nutrients in the maternal diet during gestation will decrease, while deficient levels will increase, dysregulation in DNA methylation patterns in Wnt pathway genes in the offspring that preserve apoptotic function leading to a reduction in tumorigenesis.**

E. Significance of Proposed Research

Colorectal cancer is a leading health concern in the United States, with over 150,000 new cases diagnosed in 2009, and as such, novel strategies for prevention of this disease are needed to ease both the financial and emotional burden of those affected. Developing a comprehensive understanding of the mechanisms through which one-carbon nutrients modulate the development of cancer in both individuals and their offspring is an essential component of developing intelligently-constructed and effective public health measures that will utilize these nutrients in the prevention of this disease.

III. Manuscript

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Maternal B-vitamin supplementation from preconception through weaning suppresses intestinal tumorigenesis in *Apc*$^{+/1638N}$ mouse offspring.

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Short title: B-vitamins suppress intestinal tumors in offspring.
Keywords: colorectal cancer, B-vitamins, folate, offspring, intestine, Wnt.

A. Abstract

Objective. Variations in the intake of folate are capable of modulating colorectal tumorigenesis; however the outcome appears to be dependent on timing. We sought to determine the effect of altering folate (and related B-vitamin) availability during in utero development and the suckling period on intestinal tumorigenesis.

Design. Female wildtype mice were fed diets either mildly deficient, replete or supplemented with vitamins B_2, B_6, B_{12} and folate for 4 weeks before mating to *Apc*$^{+/1638N}$ males. Females remained on their diet throughout pregnancy and until weaning. After weaning, all *Apc*$^{+/1638N}$ offspring were maintained on replete diets for 29 weeks.

Results. At 8 mo of age tumor incidence was markedly lower amongst offspring of supplemented mothers (21%) compared to those of replete (59%) and deficient (55%) mothers (P=0.03). Furthermore, tumors in pups born to deficient dams were most likely to be invasive (P=0.03). Expression of *Apc*, *Sfrp1*, *Wif1* and *Wnt5a* - all of which are negative regulatory elements of the Wnt signaling cascade - in the normal small intestinal mucosa of pups decreased with decreasing maternal B-vitamin intake and for *Sfrp1* this was inversely related to promoter methylation. β-catenin protein was elevated in offspring of deficient dams.

Conclusions. These changes indicate a de-repression of the Wnt pathway in pups of deficient dams and form a plausible mechanism by which maternal B-vitamin intake modulates tumorigenesis in offspring. These data indicate that maternal B-vitamin supplementation suppresses, while deficiency promotes, intestinal tumorigenesis in *Apc*$^{+/1638N}$ offspring.

Summary Box:

What is already known about this subject:

- Epidemiological data and controlled animal studies support a protective role for dietary folate and related B-vitamins against colorectal cancer.
- Maternal diet and environmental exposure are becoming increasingly recognized as important determinants of the risk for chronic disease in offspring.
- In addition to its established role in preventing birth defects, maternal folate supplementation appears to be protective against several pediatric cancers.

What are the new findings:

- Maternal supplementation with vitamins B_2, B_6, B_{12} and folate markedly suppresses intestinal tumorigenesis in mouse offspring (Odds ratio: 0.18; 95%CI = 0.0519 - 0.6308; p=0.009).
- Exceedingly mild maternal B-vitamin inadequacy increases the likelihood of tumors in offspring acquiring an invasive phenotype.
- A de-repression of the Wnt pathway characterized by the hypermethylation and suppression of *Sfrp1* and accumulation of β-catenin was observed with declining maternal B-vitamin intake.

How might it impact on clinical practice in the foreseeable future?

- Mild deficiencies of vitamins B_2, B_6 and B_{12} persist in 10-50% of the population of industrialized nations such as the US and UK. In addition, although maternal folic acid supplementation is widespread, it frequently is not initiated until after conception. These data indicate that maternal B-vitamin supplementation may not only protect offspring against birth defects but also against colorectal cancer in adulthood.

B. Introduction

Although a few exceptions exist [73], a considerable body of epidemiological evidence accumulated over the last two decades [74, 75] and scrutinized by recent meta-analyses [76], supports the notion that higher dietary folate intake is associated with a moderately reduced risk for colorectal cancer (CRC). This effect has been reproduced in rodent CRC models as well [77, 78], establishing true causality. More recently however, the potential for a paradoxical tumor-promoting effect of folate has become apparent, with our current understanding being that folate insufficiency promotes carcinogenesis by causing DNA breakage and genomic and gene-specific methylation aberrations while supplemental quantities of the vitamin, when administered in an inappropriate time frame, may paradoxically promote tumorigenesis by providing existing neoplastic lesions with an overly abundant supply of nucleotides to support DNA synthesis during proliferation [23, 79].

The critical importance of timing in determining the outcome of folate supplementation has prompted us to consider windows of exposure to folic acid other than those in adult life. Because maternal nutrition is becoming increasingly recognized as a determinant of chronic disease in offspring [80] and particularly since certain phases of *in utero* life are characterized by major transitions in DNA methylation [81], we questioned whether a mother's folate intake might impact on her offspring's risk for colorectal carcinogenesis. Epidemiological evidence is not yet available to support (or refute) this idea because suitable databases that attempt to link maternal diet to diseases in the latter decades of the offspring's adulthood are not yet available, however data does exist to support a

protective role for high maternal folate intake against certain pediatric cancers in offspring, including retinoblastoma [38, 44, 45, 82], non-Hodgkin lymphoma [40], acute lymphoblastic leukemia [83] and childhood brain tumors [45].

It is likely that the abovementioned pathways by which folate affects genetic and epigenetic stability are also operable in the developing embryo and that the embryo is just as, if not more vulnerable than adults, to nucleotide and methyl donor inadequacies as methylation patterns are rapidly changing [81] and cells are dividing rapidly. Evidence that maternal folate intake can impact on the offspring's epigenome comes from studies with 'Agouti' ($A^{vy/a}$) mice in which maternal supplementation with folate and related dietary methyl donors resulted in a shift in the offspring's coat color from yellow to brown in conjunction with *de novo* methylation of specific sequences in the agouti gene [54, 84].

In the current study we sought to determine whether maternal intake of folate and related B-vitamins impacts on intestinal cancer in offspring using the $Apc^{+/1638N}$ mouse model. Offspring from supplemented mothers had a markedly lower incidence of small intestinal tumors than those from control or deficient mothers. Furthermore, maternal deficiency resulted in a significantly greater proportion of tumors being invasive, rather than benign adenomas, compared to tumors in the control group. These data indicate that maternal supplementation with vitamins B_2, B_6, B_{12} and folate may significantly lessen the risk for intestinal tumorigenesis in offspring. The relevance of this work is underscored by the fact only around one-third of US women report taking vitamins containing folate before

conception [85] and that mild inadequacies of B_2 [86], B_6 [87] and B_{12} [88] occur in 10-50% of the US and European populations.

C. Materials and Methods

All animal procedures were approved by the institutional review board of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University. Two strains of mice were utilized for this study: C57BL6/J (Charles River, Wilmington, MA) and *Apc*$^{+/1638N}$ [89] (NCI Mouse Repository. Frederick, MD). Mice were housed on a 12hr light-dark cycle at 23°C and provided ad libitum access to water throughout the experiment.

The *Apc*$^{+/1638N}$ mouse model was utilized which has a targeted modification of exon 15 of one allele of the *Apc* gene, resulting in a chain-terminating truncation mutation of the Apc protein at codon 1638 [89]. Mice heterozygous for this particular mutation spontaneously develop between 1-5 small bowel adenomas or carcinomas.

The study design is illustrated in **Figure 1**. Six week old female wildtype C57BL6/J mice were housed individually and randomly assigned to be group pair-fed one of three amino acid-defined diets (Dyets, Bethlehem, PA) containing either mildly deficient (VBD), replete (CTRL) or supplemental (VBS) quantities of vitamins B_2, B_6, B_{12} and folate (See **Table 1**) (n=38, 26, and 22 dams for the VBD, CTRL, and VBS groups, respectively). After 4 wks, two female mice from the same group and one male *Apc*$^{+/1638N}$ were placed in a cage for 1 wk to allow mating and continued to be fed their

assigned experimental diets. Prior to this time male mice were all fed standard mouse chow (Harlan Teklad. Madison, WI) but consumed the experimental diet during the mating period. After mating, females were removed to separate cages and provided *ad libitum* access to the same diets throughout pregnancy and through weaning. Three weeks after birth, pups were genotyped immediately from tail snips. Within 3 days, *Apc*$^{+/1638N}$ offspring were removed to separate cages and all fed (*ad lib*) a standard replete diet (AIN-93G. Dyets) regardless of their mother's diet. At this time mothers and wildtype pups were all euthanized by CO_2 asphyxiation followed by cervical dislocation. Blood was collected by cardiac puncture and stored for later vitamin analyses. The abdomen was then opened and small intestine (SI) removed onto an ice-cold glass plate. The SI was flushed with ice-cold saline, opened longitudinally and washed in PBS then PBS plus protease inhibitors (Roche, Indianapolis, IN). Approximately 2cm of the normal proximal SI was fixed in formalin (<48hr) before being embedded in paraffin, sectioned and mounted on microscope slides. The remaining normal-appearing SI mucosa was scraped with microscope slides, divided into aliquots, frozen in liquid N_2 and then stored at -80°C. The liver was also removed stored and frozen (N_2, -80°C).

Apc$^{+/1638N}$ offspring were maintained on replete AIN-93 diet for 29 weeks, i.e. until 32 wk (8 mo) of age: the growth (G) formulation for the first 16 wks and maintenance (M) for the remainder. After this period, *Apc*$^{+/1638N}$ mice were euthanized as described above for the wildtype pups, with the exception that after washing the small intestine, it was systematically examined under a dissecting microscope for the presence of tumors by a blinded and experienced observer. Tumors were photographed, measured with digital

calipers then excised and stored in formalin (<48hr) before being embedding in paraffin, sectioned and mounted on microscope slides. Tumor slides were H&E stained and graded by an expert rodent pathologist (R.B.) as either anadenoma or invasive carcinoma.

Vitamin Analyses

Plasma concentrations of folate and vitamin B_{12} were quantified using a commercially available chemiluminescence assay (IMMULITE, Siemens Healthcare Diagnostics, Deerfield, IL). Hepatic and SI folate concentrations were determined by the *L. casei* microbiological assay [90]. Plasma vitamin B_6 was determined by radioenzymatic assay [91] and red cell vitamin B_2 concentrations were estimated by the erythrocyte glutathione reductase activation coefficient assay [92].

Nucleic Acid Analyses.

DNA was isolated from SI mucosal scrapings with phenol: chloroform and quantified with Pico-green reagent (Invitrogen. Carlsbad, CA). Genomic DNA methylation was measured by LC-MS after enzymatic digestion according to the method developed in our laboratory [93]. Methylation of 3 specific regions within the promoter of the *Sfrp1* gene was determined by methyl-specific PCR according to the method of Samuel et al [94]. Total RNA was extracted from SI mucosal scrapings using Trizol reagent (Invitrogen) and 2 μg subjected to reverse transcription using Superscript II enzyme and Oligo dT primers (Invitrogen). The relative expression of a panel of genes was the studied by real-time PCR using SYBR green master mix and an ABI7300 thermocyler (Applied Biosystems). Genes selected for analysis included tumor suppressor genes, genes

commonly methylated in CRC and/or known to be sensitive to B-vitamin status including: *Apc*, *Gsk3β*, β-*catenin*, *Sfrp1*, *Wif1*, *Wnt5a*, *Tgf-β1*, *Smad4*, *Smad7*, *p53*, *p21*, *Cdx2* and *MMP9*. *GAPDH* was used as control gene. Primer sequences specific to each of these genes of interest were retrieved from the qPrimer database (http://mouseprimerdepot.nci.nih.gov).

Apoptosis & Proliferation.

Apoptosis was assessed in SI mucosal scraping using the CaspACE colorimetric assay according to manufacturer's instructions (Promega. Madison, WI). Results were confirmed by western blotting with rabbit anti-cleaved caspase-3 (Cell signaling, Danvers, MA) and goat α-rabbit (Santa Cruz) antibodies. Staining was normalized to GAPDH using mouse 1° antibody (Millipore. Billerica, MA) and goat α-mouse 2° antibody (Santa Cruz). Cell proliferation was assessed by immunohistochemistry for Ki-67 using rabbit α-Ki-67 1° (Abcam. Cambridge, MA) followed by detection with the 'ABC' peroxidation system (Vector Labs. Burlingame, CA). The number of Ki-67 positive cells in the crypt was counted. Villi were scored separately and assigned the number 0 - 4 based on whether 0, 25, 50, 75 or 100% cells in the lower third of the villus were positively stained. At least 20 crypts and villi were scored per sample.

Statistical Analyses

Comparisons of the incidence of tumors in pups between maternal dietary groups were done using a Poisson regression using the mother as the offset variable (PROC GENMOD). Comparisons of tumor grade between maternal dietary groups were

performed using Chi-square analysis. Comparisons of numeric endpoints between maternal dietary groups (i.e., vitamin analyses, gene expression, etc) were made using one-way analysis of variance (ANOVA). Testing for significant trends between diet groups was done using linear regression. Statistical analyses were performed using SAS v9.2 (SAS. Cary, NC) and SYSTAT v11 (Systat Software. Chicago, IL). Significance was accepted when $P \leq 0.05$. All data are reported as mean ± SEM.

D. Results

Vitamin Analyses

Blood and tissue vitamin concentrations are reported in **Table 2** for mothers (wildtype), weanling pups (wildtype) and 8 month old pups (*Apc*$^{+/1638N}$). Incremental increases in plasma vitamin B_6 and B_{12} concentrations were observed for both mothers and weanling wildtype offspring going from deficient to supplemented maternal diets. Interestingly, these differences were also present, albeit to a lesser degree, in the 8 month old *Apc*$^{+/1638N}$ offspring for plasma B_6 and B_{12}.

No significant differences were detected in plasma and hepatic folate concentrations between mothers of different dietary groups although significant incremental increases in hepatic folate concentration were detected among weanling offspring with increasing maternal intake. An effect of maternal supplementation, but not depletion, was also seen on plasma folate in weanling offspring. No differences in folate concentration were detected in small intestinal scrapings of weanling or adult offspring between maternal groups.

No differences in red cell vitamin B_2 status could be detected between mothers from different groups. Compared to offspring of control mothers, weanling offspring of deficient mothers did have a significantly reduced B_2 status while offspring of supplemented mothers did not display any increase. Although B_2 status was slightly reduced in the deficient pups at 8 months, this difference failed to reach statistical significance (P=0.10).

Tumor Incidence

No intestinal tumors were detected in wildtype offspring which were euthanized at weaning. Across all 96 $Apc^{+/1638N}$ pups, 58 small intestinal tumors were distributed amongst 46 mice; 33 of these tumors were adenomas, 16 were invasive cancers while 9 could not be classified due to tissue autolysis secondary to premature death. The incidence of small intestinal tumors in 8 month old $Apc^{+/1638N}$ offspring was markedly lower amongst offspring of supplemented mothers compared to those of both control and deficient-fed mothers (P< 0.025). Small intestinal tumors were observed in 25 of 45 (55.6%), 16 of 27 (59.3%) and 5 of 24 (20.8%) of offspring from deficient, control and supplemented mothers respectively (**Figure 1**). The Odds Ratio for offspring of supplemented dams displaying tumors was 0.18 (95%CI = 0.0519 - 0.6308. p=0.009). Among the mice that developed tumors, no difference in tumor multiplicity (tumors/mouse) was observed (P= 0.89). Although there was no difference in tumor incidence between offspring of deficient and control mothers, a significant difference in the likelihood of tumors being invasive was observed between these two groups. After excluding tumors that could not be histologically-confirmed, tumors in the offspring of deficient dams were significantly more likely to be invasive than those in offspring of control dams (14 of 26 [53.85%] vs. 3 of 17 [17.65%] respectively. P< 0.03). None of the classifiable tumors (n=2) in offspring of supplemented dams were invasive. The average maximum diameter of all tumors from all groups was 3.27 ± 0.17 mm (range 1.82 – 7.78 mm) with no significant differences between groups (P> 0.05).

<u>Molecular Analyses of Small Intestinal Mucosa</u>

Genomic DNA methylation was measured in small intestinal scrapings from 3 week old wildtype and 8 month old *Apc*$^{+/1638N}$ mice. In the former group, maternal diet did not impact on genomic methylation (P= 0.28. **Table 3**), however, in *Apc*$^{+/1638N}$ offspring there was a trend for offspring of both deficient and supplemented fed mothers to display a mild, although significant, degree of genomic hypomethylation (P= 0.003 and 0.07 for VBD and VBS vs. CTRL respectively). Global methylation was not associated with tumor incidence number (P= 0.90).

Of the genes for which expression was measured in the normal SI mucosa, 8 genes were responsive to maternal diet in WT and/or *Apc*$^{+/1638N}$ offspring, returning an ANOVA P for diet of ≤ 0.05 or an ANOVA P of ≤ 0.1 and a P_{trend} of <0.05 (**Figure 3**). In general, expression changes were of a smaller magnitude in the *Apc*$^{+/1638N}$ pups than their WT littermates. The genes *Apc*, *Sfrp1*, *Wif1* and *Wnt5a*, the products of which negatively regulate the Wnt pathway, displayed significant stepwise reductions in expression in both sets of pups with reducing maternal vitamin intake. Within the Tgf-β pathway, there was a tendency for the expression for *Tgfβ1 and Smad4* to be reduced with decreasing maternal B-vitamin intake in either WT or *Apc*$^{+/1638N}$ offspring. The cell cycle inhibitor p21 was also suppressed in WT pups of deficient dams (**Figure 3**). Upon application of the Bonferroni correction for multiple comparisons (P= 0.05/13 genes = 0.003), only differences in *Sfrp1* expression in *Apc*$^{+/1638N}$ mice remained significant (P=0.002).

Because we observed a significant repression of *Sfrp1* expression with declining maternal B-vitamin consumption, we measured the methylation of 3 specific regions in and around the promoter of this gene in both *Apc*$^{+/1638N}$ and WT pups. For each of the two strains of animals, two of the three amplicons that were examined were significantly, and inversely, correlated with expression (**Table 4**), underscoring a potential functional relationship between methylation and expression. In order to determine whether these changes could result in an increased activation of the Wnt pathway, we measured cellular levels of total β-catenin, the effector molecule of this pathway. No differences in nuclear (IHC) or total β-catenin (western blotting) were detected in the small intestine of the *Apc*$^{+/1638N}$ offspring, but this is not unexpected because this animal already has a 40-fold up-regulation of the Wnt pathway (Liu et al, submitted), so modest differences due to diet are difficult to detect on this high background of activation. Therefore we measured total β-catenin in wildtype offspring and observed a ~45% elevation in pups of deficient dams compare to those of replete and supplemented dams (P=0.04, **Figure 4**).

Estimates of apoptosis obtained using a colorimetric assay for Caspase-3 activity indicated that in both WT and *Apc*$^{+/1638N}$ pups, offspring of deficient dams had a significantly higher Caspase-3 activity compared to both replete and supplemented dams (**Figure 5A**). Because we expected to see the highest apoptotic activity in the offspring of supplemented dams and the lowest in those of deficient dams we sought to verify these results by western blotting. Protein extracts from *Apc*$^{+/1638N}$ pups were analyzed in this fashion, and the results concurred with those of the activity assay; caspase-3 expression was significantly higher in offspring of deficient compared to control and supplemented

dams (**Figure 5B**). These endpoints were significantly and positively associated with each other (R^2=0.21, P=0.006).

In order to gain an estimate of cellular proliferation we stained for Ki-67 in sections of the small intestine. Because crypts displayed an almost ubiquitous degree of Ki-67 staining we focused on the lower third of the villi in an attempt to detect a possible expansion of the proliferative zone on the high tumor groups. No effect of maternal diet was seen on the abundance of positively stained cells in the lower third of the villi in either WT 3 wk (P=0.28) or 8 mo $Apc^{+/1638N}$ or (P=0.93) offspring (**Table 3**).

E. Discussion

Our data clearly indicates that peri-conceptional maternal supplementation with four-times the basal requirement of vitamins B_2, B_6, B_{12} and folate markedly reduces small intestinal tumor incidence in *Apc*$^{+/1638N}$ offspring (OR= 0.18). Furthermore, while a maternal diet mildly deficient in these B-vitamins did not elevate tumor incidence above control levels, tumors in the offspring of deficient-fed mothers were significantly more likely be invasive, rather than benign, compared to tumors in offspring of control-fed mothers (**Figure 2**).

The data of the current study are in good agreement with two other studies investigating the effect of maternal B-vitamin intake on intestinal tumorigenesis in offspring. Firstly, similar to the outcome of our depletion arm, McKay *et al* [95] reported that mild maternal folate depletion (0.4 mg/kg), compared to adequacy (2mg/kg), did not alter the intestinal tumor incidence amongst replete fed *Apc*$^{+/min}$ offspring, however tumor grade was not reported. Data from Sie *et al* [50] agrees with findings from our supplemental arm: peri-conceptional maternal folate supplementation (5 mg/kg), compared to adequacy (2mg/kg), significantly reduced azoxymethane-induced colorectal tumor incidence in offspring, irrespective of which diet offspring were fed.

In the current study we were solely concerned with the effect of maternal diet on offspring tumorigenesis, and therefore assigned the offspring of all three maternal groups to consume the same replete AIN-93 diet after weaning. In contrast, Lawrance *et a*l [51], fed mouse dams either folate deficient, replete or supplemented diets (0.3, 2.0 and 20

mg/kg) peri-conceptionally and through weaning but assigned the *Apc*$^{+/min}$ offspring to continue on the same diets their mother received. Significantly fewer intestinal tumors were observed in deplete offspring than in those fed control and supplemented diets. We suggest that these results highlight the dual role of folate in cancer, whereby folate supplementation before the appearance of neoplastic lesions may be protective while supplementation afterwards may fuel the growth of tumors, with the opposite being true for folate depletion. In the *Apc*$^{+/min}$ model, because the tumorigenic phenotype is so severe (15-45 tumors/mouse) and begins so early, the ability of folate to act in its preventive capacity may be overwhelmed, thus biasing the overall results towards the effect folate has in promoting tumor growth and evolution. Although the design of this study precludes dissecting out the effects of maternal versus offspring diet, the findings nevertheless may still have important public health relevance because it is known that a child's dietary habits are modeled largely upon those of their parents [96].

Although the mechanistic importance of genomic DNA methylation in tumorigenesis is unclear, a graded decrease in whole-genome methylation going from normal to malignant tissue has been repeatedly observed in CRC [97]. Because folate status [14] and variants in folate-metabolizing enzymes [98] have been reported to impact on genomic methylation we also studied this endpoint. No significant differences in genomic methylation were observed between weanling wildtype pups of different maternal dietary groups, however a small but significant reduction in genomic methylation was seen amongst adult *Apc*$^{+/1638N}$ pups of VBD dams, (i.e.: those possessing the highest likelihood of developing invasive cancers, compared to CTRL dams) (**Table 3**). We speculate that

maternal deficiency may initiate a specific metabolic program that, over time, results in genomic hypomethylation which might subsequently promote tumorigenesis. Mechanistically, genomic DNA hypomethylation may cause chromosomal instability [99] and loss [100], both recognized risk factors for cancer [101, 102].

Our previous work showing that multiple B-vitamin depletion can cause aberrant activation of the Wnt pathway [16] prompted us to again consider a role for this pathway in the current study. Taken together, the reduced expression of Wnt pathway negative regulators (**Figure 3**) with the elevated level of total β-catenin protein observed in WT VBD pups (**Figure 4**), points towards a de-repression of this pathway in offspring with decreasing maternal B-vitamin intake. In the case of *Sfrp1*, we observed a significant inverse relationship between expression and methylation (**Table 4**), which is consistent with observations of prior investigators who have demonstrated a functional relationship between these two phenomena in human colorectal carcinogenesis [70, 103]. This is of particular relevance as previous work indicates that loss of *Sfrp1* function is an early event in human colorectal tumorigenesis that results in constitutive *Wnt* signaling [70]. Furthermore expression of exogenous *Sfrp1* inhibits the growth of established tumors by promoting apoptosis and suppressing vascularization [104], and this latter observation may have relevance to the increased risk of invasiveness that was observed with depletion in our study.

In an effort to explain the kinetic mechanisms underlying observed differences in tumor incidence we evaluated apoptosis and proliferation in the SI of offspring. We observed a

significantly elevated caspase-3 activity in VBD pups compared to pups of both CTRL and VBS dams, a finding subsequently confirmed by western blotting in the *Apc*$^{+/1638N}$ pups (**Figure 5**). Since apoptosis is highest in the group with an elevated tumor incidence and the highest proportion of invasive tumors, apoptosis is unlikely a key factor explaining the differences in tumor burden seen here. Rather, we suggest that the elevated apoptosis amongst the pups of deficient dams may be explained by an elevated frequency of cells harboring deleterious genetic and epigenetic aberrations and is an effort to remove these cells for the benefit of the organism.

Proliferation was also measured in the small intestine of offspring. Among both WT and *Apc*$^{+/1638N}$ pups there were no differences in Ki-67 staining between pups of different dietary groups (**Table 3**). When comparing proliferation scores between the two offspring genotypes it was clear that *Apc*$^{+/1638N}$ mice had a greater expansion of the proliferative cells into the villi than WT mice ($P< 0.0001$). Although caution should be used when comparing these two genotypes, others have previously shown that SI proliferation is not different between WT and *Apc*$^{+/1638N}$ mice of the same age [105]. Therefore we speculate that, although we did not observe differences in proliferation between groups at either time point, it is plausible that pups of deficient and CTRL dams attained a hyper-proliferative state significantly earlier than pups of supplemented dams. In this case we may have missed the critical window of time when differences in proliferation were apparent. Sampling of *Apc*$^{+/1638N}$ offspring at various ages would have been required to detect such a difference.

It is well-recognized that all non-human models of colorectal cancer have limitations, and this includes the *Apc*$^{+/1638N}$ mouse. Virtually all genetic models of colorectal carcinogenesis in the rodent (including the widely used *Apc*$^{+/min}$ mouse) have a strong predilection for forming small intestinal, rather than colonic, neoplasms and we have found this to be true with the *Apc*$^{+/1638N}$ mouse model as well. Although the original characterization of this model [89, 106] indicated that it develops colorectal tumors as well, we have found this to be a relatively rare event. Nevertheless, genetically-induced models of colorectal carcinogenesis have been used with considerable effectiveness to study the effects of diet on cancer development since the small intestinal tumorigenesis generally responds in a fashion that mimics the effects of diet on the colon, and this has certainly been true of the *Apc*$^{+/1638N}$ mouse [107, 108]. Although tumor location differs from the human situation, where the vast majority of tumors appear in the colorectum as opposed to the small intestine, there are aspects of tumorigenesis in *Apc*$^{+/1638N}$ mice that closely adhere to molecular carcinogenesis in the human. For instance, Apc protein is reported to be absent in 71% of human colorectal adenocarcinomas (and only 31% of SI adenocarcinomas)[109]. Similarly, inactivation of *Apc* is critical in *Apc*$^{+/1638N}$ tumorigenesis where 81% of tumors display a loss of the wild type *Apc* allele [106].

In conclusion, our data clearly demonstrate that maternal B-vitamin intake can modulate both the incidence of small intestinal neoplasms in mouse offspring as well as the likelihood of progressing to an invasive phenotype. Our data are supportive of a relative de-repression of the Wnt pathway in pups of CTRL, but especially of deficient dams. Quite aside from the well-documented benefits in the prevention of birth defects, our

observations indicate that, at least within the framework of this animal model, mothers who initiate B-vitamin supplementation before conception may also be protecting their offspring against colorectal cancer in adulthood.

Acknowledgements

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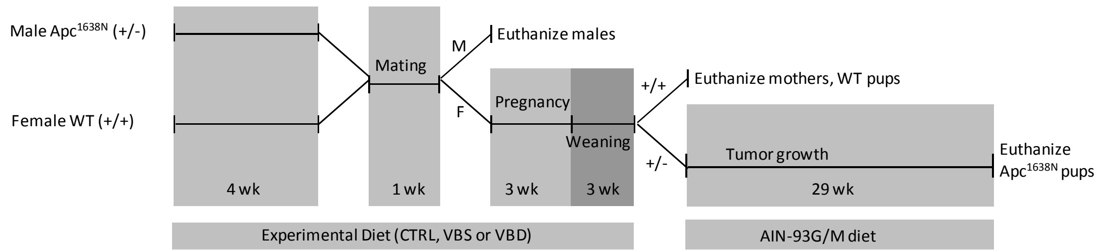

Figure 1. Design of animal experiment.
M, male. F, female. CTRL, control diet. VBD, vitamin B deficient diet. VBS, vitamin B supplemented diet. WT (+/+), C57BL6/J mice wildtype for Apc gene. Apc$^{+/1638N}$ (+/-), mice heterozygous for truncation mutation in the Apc gene.

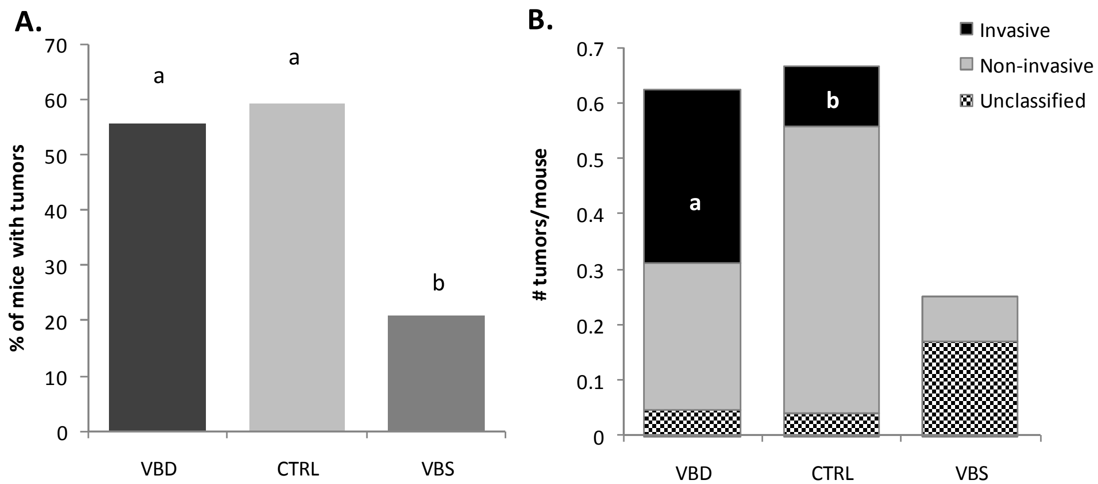

Figure 2. Peri-conceptional maternal B-vitamin supplementation suppresses tumor occurrence, while depletion promotes tumor invasiveness in the small intestine of *Apc$^{+/1638N}$* offspring.
A. *Tumor incidence (percentage of mice with tumors) and* ***B.*** *tumor grade (# of tumors per mouse) in 8 month old Apc$^{+/1638N}$ mice. Groups with different letters are significantly different (P< 0.05). Maternal diets = VBD, vitamin B deficient; CTRL, control; VBS, vitamin B supplemented. N= 45, 27 and 24 mice/group respectively (from 9 – 15 litters).*

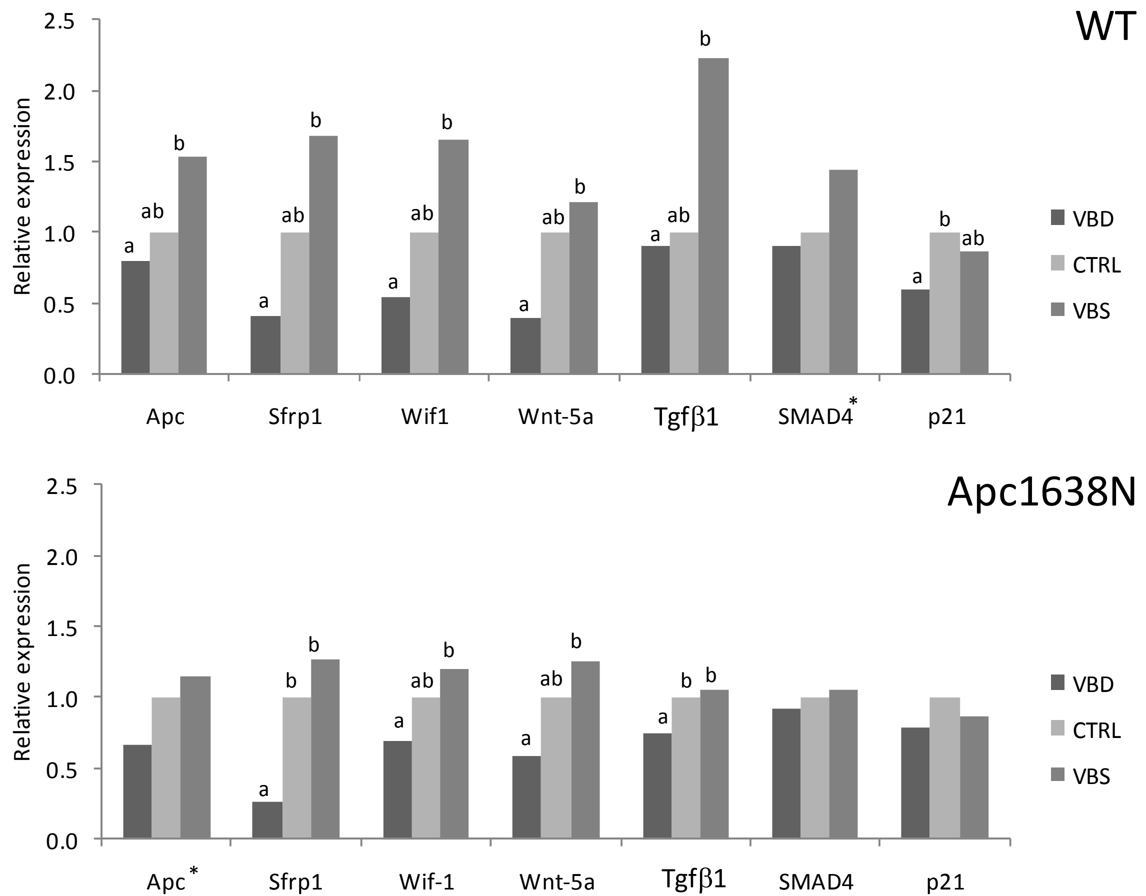

Figure 3. Effect of maternal B-vitamin intake on the expression of select genes in the small intestine of weanling wildtype and adult *Apc*$^{+/1638N}$ offspring.
Gene expression in the small intestinal mucosa of 3 week old wildtype (upper panel) and 8 month old Apc$^{+/1638N}$ *(lower panel) offspring. Mothers were fed diets either mildly deficient (VBD), replete (CTRL) or supplemented (VBS) with vitamins* B_2, B_6, B_{12} *and folate. Data expressed as Relative Expression* ($2^{-\Delta\Delta Ct}$)*. Statistical analyses performed on* ΔCt *values. Different letters denote significant difference (P<0.05). * indicates* P_{trend} *<0.05.N= 10-18/gp (from 6-12 litters).*

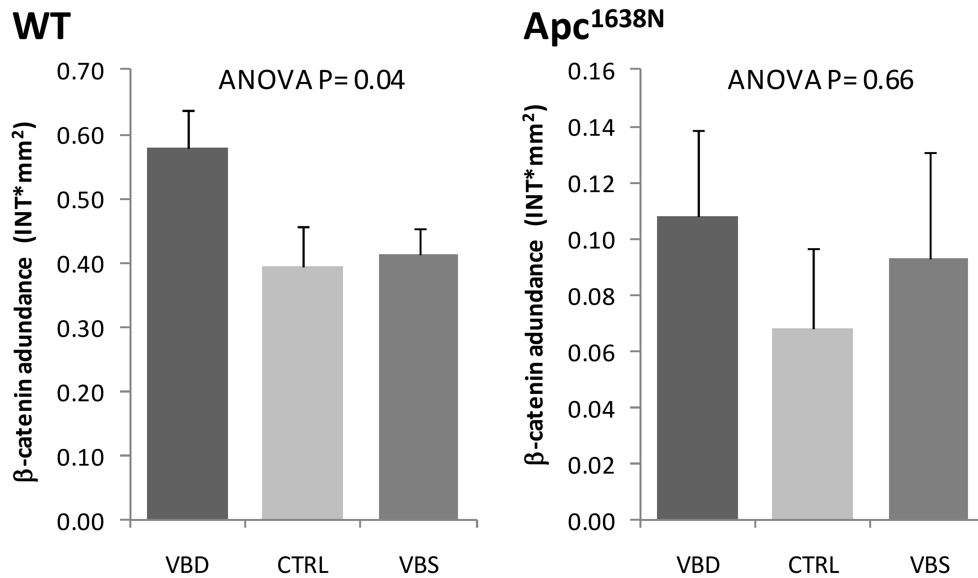

Figure 4. Effect of maternal B-vitamin intake on the abundance of total β-catenin protein in the SI mucosa of offspring.
Total β-catenin protein abundance in small intestinal mucosa scrapings of 3 week old wildtype (left panel) and 8 month old $Apc^{+/1638N}$ *(right panel) offspring of mothers fed diets either mildly deficient (VBD), replete (CTRL) or supplemented (VBS) in vitamins* B_2, B_6, B_{12} *and folate. Protein levels were evaluated by western blotting and band volumes for β-catenin (intensity x* mm^2*) were corrected for those of GAPDH. Data = mean ± SEM. N= 7 - 13/group (from 6-12 litters).*

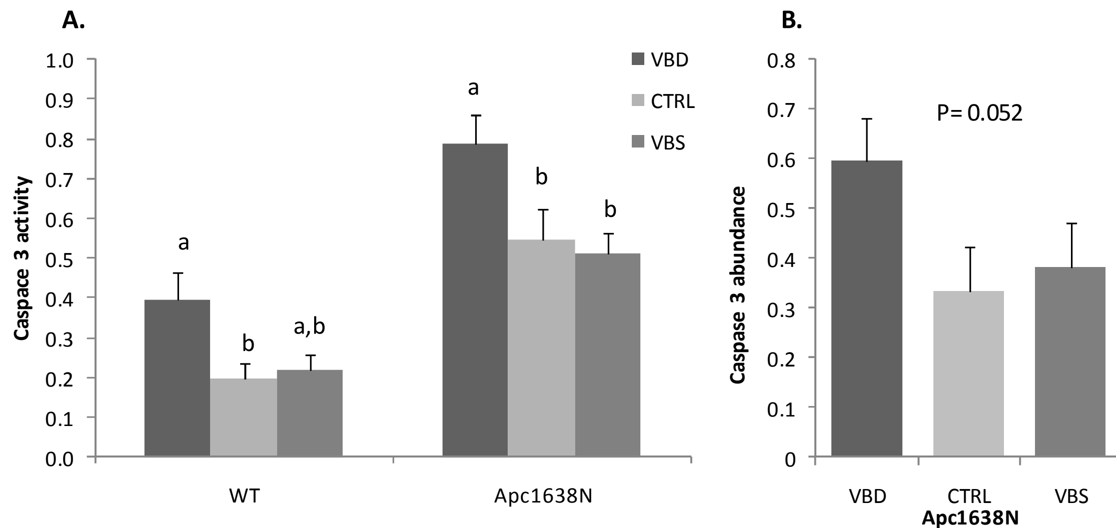

Figure 5. Maternal B-vitamin deficiency elevates apoptosis in the small intestine of offspring.
Estimation of apoptotic activity in small intestinal mucosal scrapings by ***A)*** *colorimetric assay for caspase-3 activity in 3 week old WT and 8 month old* $Apc^{+/1638N}$ *and* ***B)*** *western blotting for cleaved caspase-3 protein in 8 mo* $Apc^{+/1638N}$ *mice. Groups with different letters are significantly different. Data for caspase 3 activity and abundance were significantly correlated (*R^2*= 0.21, P=0.007). Data = mean ± SEM. N= 9 - 18/group (from 6 – 12 litters).*

Mice	Diet type	Folic Acid (mg/kg diet)	Vitamin B_2 (mg/kg diet)	Vitamin B_6 (mg/kg diet)	Vitamin B_{12} (μg/kg diet)
Dams[1]	Vit. B Deficient (VBD)	0.5	2.0	2.0	10.0
	Control (CTRL)	2.0	6.0	7.0	50.0
	Vit. B Supplemented (VBS)	8.0	24.0	28.0	200.0
$Apc^{+/1638N}$ Offspring[2]	AIN-93G then M	2.0	6.0	7.0	25

Table 1. Vitamin composition of maternal and offspring diets.
VBD, vitamin B deficient diet. CTRL, control diet. VBS, vitamin B supplemented diet. [1]*Maternal diet consumption began 4 wk prior to mating and lasted through weaning.* [2] $Apc^{+/1638N}$ *offspring diet consumption began at weaning and lasted until animals were euthanized (29 wk).*

	Generation	Maternal Diet Vit B Deficient			CTRL		Vit B Supplemented			ANOVA P
Plasma folate	*Mothers*	81.4 ± 7.3		*(23)*	84.7 ± 7.8	*(7)*	104.4 ± 26.3		*(7)*	0.95
(ng/ml)	*3 Wk offspring (WT)*	76.1 ± 4.1		*(54)*	63.3 ± 6.1	*(17)*	107.8 ± 10.3	a,b	*(16)*	< 0.01
	8 Mo offspring (Apc)	59.3 ± 2.0		*(40)*	52.5 ± 3.4	*(26)*	50.2 ± 3.2		*(21)*	0.12
Hepatic folate	*Mothers*	11.12 ± 0.70		*(14)*	13.20 ± 0.88	*(9)*	13.29 ± 1.43		*(8)*	0.17
(μg/g)	*3 Wk offspring (WT)*	8.38 ± 0.62	a	*(9)*	13.37 ± 1.19	*(10)*	14.31 ± 1.45	b	*(10)*	< 0.01
	8 Mo offspring (Apc)	12.90 ± 0.65		*(9)*	14.14 ± 1.24	*(10)*	13.34 ± 1.05		*(10)*	0.69
Small intestine folate	*3 Wk offspring (WT)*	924.9 ± 89.0		*(8)*	1358.2 ± 155.0	*(10)*	1295.0 ± 205.3		*(9)*	0.16
(ng/g)	*8 Mo offspring (Apc)*	1264.7 ± 126.1		*(10)*	1205.9 ± 136.7	*(9)*	1172.4 ± 146.8		*(9)*	0.89
Plasma vitamin B_{12}	*Mothers*	3.56 ± 0.14	a	*(23)*	10.96 ± 0.72	*(7)*	37.59 ± 4.91	a,b	*(8)*	< 0.01
(ng/ml)	*3 Wk offspring (WT)*	3.31 ± 0.08	a	*(54)*	8.26 ± 0.80	*(17)*	24.48 ± 1.59	a,b	*(16)*	< 0.01
	8 Mo offspring (Apc)	27.21 ± 0.86		*(41)*	24.94 ± 0.84	*(26)*	32.87 ± 1.45	a,b	*(20)*	< 0.01
Plasma vitamin B_6	*Mothers*	151.2 ± 7.2	a	*(7)*	262.9 ± 12.6	*(6)*	310.6 ± 29.7	b	*(6)*	< 0.01
(ng/ml)	*3 Wk offspring (WT)*	150.5 ± 13.1	a	*(22)*	281.5 ± 17.3	*(13)*	349.7 ± 28.8	b	*(12)*	< 0.01
	8 Mo offspring (Apc)	255.9 ± 13.4		*(38)*	294.0 ± 18.8	*(25)*	319.9 ± 17.2	b	*(20)*	0.02
Red cell vitamin B_2	*Mothers*	1.43 ± 0.01		*(21)*	1.36	*(1)*	1.40		*(1)*	0.50
(activity coeff)	*3 Wk offspring (WT)*	1.27 ± 0.01	a	*(50)*	1.20 ± 0.01	*(10)*	1.22 ± 0.03		*(3)*	< 0.01
	8 Mo offspring (Apc)	1.32 ± 0.01		*(41)*	1.28 ± 0.01	*(26)*	1.29 ± 0.01		*(20)*	0.10

Table 2. Blood and tissue B-vitamin concentrations in dams consuming different B-vitamin intakes and their offspring. *Data = mean ± SEM. Sample size in parentheses . [a] Significantly different from CTRL. [b] Significantly different from VBD. Note: Red cell B_2 activity coefficients are inversely related to status, hence a higher value means a lower B_2 status.*

		Maternal Diet						
		Vit B Deficient		CTRL		Vit B Supplemented		ANOVA P
Genomic DNA methylation	*3 Wk offspring (WT)*	4.73 ± 0.02	*(49)*	4.74 ± 0.03	*(18)*	4.68 ± 0.03	*(16)*	0.28
(% cytosine methylated)	*8 Mo offspring (Apc)*	4.73 ± 0.03 [a]	*(28)*	4.92 ± 0.05	*(16)*	4.79 ± 0.05	*(18)*	< 0.01
Ki-67 staining score	*3 Wk offspring (WT)*	0.45 ± 0.16	*(10)*	0.81 ± 0.21	*(10)*	0.87 ± 0.22	*(10)*	0.28
(Rating for lower 3rd of villus) (0-5)	*8 Mo offspring (Apc)*	1.83 ± 0.18	*(17)*	1.78 ± 0.16	*(15)*	1.72 ± 0.21	*(8)*	0.93

Table 3. Effect of maternal diet on genomic methylation and proliferation in the small intestine of offspring.
Data = mean ± SEM. Sample size in parentheses (Pups from 7-19 litters/gp and 6-12 litters/gp for methylation and Ki-67 respectively). [a] *Significantly different from CTRL.*

	3 Wk offspring (WT)			*8 Mo Offspring ($Apc^{+/1638N}$)*		
	P	R		P	R	
Amplicon 1	NS	-	*(30)*	0.04	0.37	*(31)*
Amplicon 2	0.02	0.43	*(28)*	NS	-	*(30)*
Amplicon 3	0.02	0.37	*(37)*	0.02	0.40	*(35)*

Table 4. Relationship between *Sfrp1* expression and methylation in weanling wildtype and adult *$Apc^{+/1638N}$* offspring.
Correlation analyses performed on ΔCt values vs. methylation (intensity methyl/unmethyl band) of each methyl-specific PCR amplicon. Location of methyl (M) and unmethyl (UM) amplicons in relation to start of gene (NM_013834): Amplicon 1 (M: 23 - 136, UM: 19 - 139); Amplicon 2 (M:-140 - 5, UM: -140 - 5); Amplicon 3(M: 246 – 372, UM: 243 – 375). Note that because ΔCt values are inversely related to gene expression, the positive R values reported here reflect an inverse association. Sample size in parentheses. NS, not significant.

IV. Summary and Discussion

A. *Summary of Research Findings*

The studies described herein provide the most comprehensive data set available to date regarding intestinal cancer-relevant molecular changes induced by maternal one-carbon nutrient intake in offspring. In addition, our observation of reduced offspring tumorigenesis following maternal B-vitamin supplementation is among the first published, and our observation of an increased likelihood of invasion secondary to maternal B-vitamin deficiency is the first to ever be published.

1. Discussion of Specific Aim 1

The studies in Specific Aim 1 were designed to examine the impact of periconceptional maternal B-vitamin intake on nutritional status and tumorigenesis in offspring. Initially, a more severe multiple B-vitamin deficient diet (used previously by Liu et al [16]) was fed to dams, however, this diet proved to be too harsh for the developing *Apc*$^{+/1638N}$ pups. As *Apc*$^{+/1638N}$ males were mated with wild type females, approximately half of the offspring were expected to be heterozygous for the *Apc* mutation. Among dams fed this deficient diet, however, only 21.4% of pups born harbored the *Apc*$^{+/1638N}$ mutation, a proportion significantly lower (p=0.035) than what was observed in the control and supplemented groups (**Appendix A**). Previous work [16], as well as our subsequent work in Specific Aim 2 (**Chapter IV.A.2**), demonstrates that a state of B-vitamin depletion results in significantly lower expression of *Apc*. Furthermore, the phenotype of mice homozygous for the *Apc*$^{+/1638N}$ allele is embryonic lethality [89]. Thus, we suspect that the reason for this altered distribution is that the combination of the already defective *Apc* allele coupled

with diminished *Apc* expression secondary to the deficient diet resulted in embryonic lethality due to a functional loss of heterozygosity in the *Apc* gene.

Regardless of the mechanism, the fact that the proportion of $Apc^{+/1638N}$ pups born to deficient dams was so low prompted the decision to change the diet to one that induced a milder degree of depletion. On this new diet, the distribution of $Apc^{+/1638N}$ and wild type pups was no longer skewed, and overall 48% of pups born bore the defective *Apc* allele. No effect of maternal diet on distribution of the genotypes (p=0.59). (Note: hereafter, discussions regarding the deficient maternal diet will refer to the 'second generation' deficient diet.)

The rate of mating resulting in live births was 63.2, 46.1 and 70% for dams fed B-vitamin deficient, control and supplemented diets, however these differences were not statistically significant (p= 0.23). Litter size was also not affected by maternal diet, with mean litter sizes of 5.3, 5.9 and 6.4 pups/litter for the above groups (p= 0.30).

In order to minimize stress to the animals, birth weights were not obtained. Wild type pups were euthanized after weaning (3 weeks of age). At this time body weight of the wild type pups was significantly different between groups, with deficient pups being heavier than both control and supplemented pups among both males and females (males: Deficient = 14.3 ± 0.45g, Control = 12.2 ± 0.51g, Supplemented = 11.4 ± 0.85g, $p<0.001$; females: Deficient = 12.4 ± 0.24g, Control=11.2 ± 0.59g, Supplemented=10.4 ± 0.82g,

p=0.01). Among the *Apc*$^{+/1638N}$ pups, however, no significant differences in body weight between maternal diet groups were observed over time (see **Appendix B**).

We intentionally sought to induce a very mild degree of vitamin depletion in mice in order to model the situation that exists in industrialized nations such as the US, where frank deficiencies are rare but mild inadequacies (so-called 'subclinical deficiencies') of vitamins B_2 [86], B_6 [87] and B_{12} [88] occur in 10-50% of the population. Analyses of liver and blood confirmed that we were successful in achieving mild, not severe, deficiencies. It is notable that the depletion of folate was so modest that no significant reductions in plasma or hepatic folate were observed in deficient mothers, although a 40% reduction in hepatic folate was observed in wild type weanling offspring. Plasma vitamin B_6 and B_{12} concentrations were 46% and 60% lower, respectively, in offspring of deficient compared to control dams while vitamin B_2 status (activity coefficient) was reduced by 6%. Combining these deficiencies is relevant since the abovementioned nutrition surveys demonstrate that subclinical deficiencies of each of these nutrients remain prevalent even in industrialized nations. Moreover, we have previously shown that the combined deficiencies amplify the molecular aberrations in the Wnt pathway induced by folate depletion alone [16]. Although the food supply of the United States and several other nations is fortified with folate, maternal folate intakes above those considered adequate appear to be protective against several pediatric cancers [38, 40, 44, 45, 82, 83] and, in rats, against chemically-induced intestinal tumors [50]. Our supplemented diet (VBS) contained four-times the amount of vitamins B_2, B_6, B_{12} and folate found in the basal or control diet. This level of supplementation induced 24% and 70% increases in vitamin B_6

and folate concentrations, respectively, in the plasma of weanling pups. A two-fold elevation in B_{12}, but no significant elevation of B_2 status was observed in weanling pups.

An interesting finding in the study was that significant differences in systemic vitamin status were present for vitamins B_2, B_6, and B_{12} in the 32 week old *Apc*$^{+/1638N}$ pups whose mothers were in different dietary groups despite the fact that, by the time of measurement, all pups had been fed identical diets for roughly 29 weeks. If one assumes that the defective *Apc* allele present in these pups does not contribute in any way to this phenomenon, then this is of particular interest since as these three nutrients are water soluble vitamins, they typically do not possess long biological half lives, and are excreted from the body rapidly [110]. The reason that the impact of the maternal diet is still evident in these pups is not known, especially when one considers that most reactive mechanisms to fluctuations in nutrient intake are intended to restore homeostasis (i.e., excretion/catabolism is directly correlated to the degree of intake). Although speculative, perhaps the degree of intrauterine exposure to these nutrients may have lasting effects on the pups due to a "homeostatic resetting" of sorts, akin to what is observed in the relationship between excessive maternal caloric intake and offspring adiposity. In this scenario, excess caloric intake by mothers during gestation creates an *in utero* environment which alters the adipocyte metabolism of the developing fetus in a manner in which it predisposes the offspring to an obesigenic phenotype [111]. Future work should be done regarding the impact of different levels of intrauterine exposure to these B-vitamin nutrients, and the compensatory changes in the absorption, storage, and excretion of these vitamins in the offspring.

The primary endpoint of the study was the incidence of tumors among offspring born to dams fed different diets. After 32 weeks, we observed that offspring born to supplemented mothers had a roughly 40% reduction in small intestine tumor incidence relative to control (Odds Ratio = 0.18, 95%CI: 0.05, 0.63). Small intestinal tumors were observed in 25 of 45 (55.6%), 16 of 27 (59.3%) and 5 of 24 (20.8%) of offspring from deficient, control and supplemented mothers respectively. Even though our hypothesis was that tumor incidence would be higher in pups born to deficient dams, the incidence of small intestinal tumors was the not greater among pups born to deficient dams compared to those born to controls. However, histological examination revealed that there were two tumor phenotypes present, standard adenomas and invasive carcinomas, the latter displaying downward invasion into the muscularis mucosa and submucosa. There was a significantly ($p=0.03$) higher proportion of tumors that displayed invasive characteristics among pups born to deficient mothers (54% of tumors) relative to control (18% of tumors).

The data from this experiment are in agreement with some of the currently available literature, although this is a field for which there exist some inconsistencies between different studies. One preliminary report states that maternal folic acid supplementation shown suppressed intestinal tumorigenesis in offspring [50], while others [51] observed no such a protective effect. Rather, the latter group reports that maternal folic acid deficiency, not supplementation, conferred a relative protection against intestinal tumorigenesis in offspring [51]. In contrast, our own studies show that combined maternal B-vitamin deficiency - compared to provision of the basal requirement - does not increase

tumor incidence in offspring. This agrees with the maternal folic acid depletion studies of McKay et al [52], although we also showed that despite an unchanged tumor incidence, a significantly higher proportion of tumors that developed in pups of deficient dams were invasive relative to tumors from pups born to replete dams.

Given the purported dual role of folate in carcinogenesis, we suggest that important determinants of the outcome of such maternal supplementation or depletion studies are 1) the severity of the tumor phenotype in the animal model used, and 2) the timing of the folate intervention. When we consider studies with dietary interventions limited to the periconceptional and suckling period, it is apparent that when a model with a mild tumor burden and long latency is used, such as the azoxymethane (AOM) [50] and $Apc^{+/1638N}$ models, folate with or without additional B-vitamins acts in a protective fashion. If dietary interventions continue into the offspring's adolescence (thereby exposing neoplastic lesions to varied folate concentrations) then there is an opportunity for the dual role to be expressed. Such is the case in the studies of Lawrance et al, who observed a suppression of tumorigenesis in offspring with maternal folate deficiency carried through to the pup's adolescence [51]. Additionally, when an animal with a severe tumor phenotype is used such as the $Apc^{+/min}$ mouse [51], the ability of folate to act in its protective capacity may be overwhelmed, thus biasing the outcome towards the promoting effect of folate by supplying nucleotides for proliferation.

However, it is apparent that the relative proclivity of the model towards tumorigenesis is an important determinant of outcome, and therefore must be taken into consideration.

Therefore, an interpretation of this body of literature is that, especially for folate, when a model with a relatively weak tumorigenic phenotype and long latency period is used, the potential chemoprotective potential of one-carbon nutrients is observed, and may even be enhanced in the presence of other related nutrients such as vitamins B_2, B_6, and B_{12}. In contrast, in models where tumor incidence is high and tumor growth is fast, this chemoprotective potential of folate and other one-carbon nutrients is overwhelmed, leaving only their capacity to fuel cell proliferation. In this latter case, and especially in situations where supplementation is continued into the pup's adolescence, the resulting exposure of neoplastic lesions to an abundance of one-carbon nutrients would be expected to fuel tumorigenesis.

Overall, the studies in Specific Aim 1 clearly indicate that supplemental maternal B-vitamin intake during periconception significantly reduces intestinal tumor incidence among offspring in the $Apc^{+/1638N}$ mouse model. Furthermore, although the deficient maternal diet did not impact tumor incidence in offspring relative to control, it did significantly increase the likelihood that offspring tumors would be invasive carcinomas. Furthermore, the maternal diet led to alterations in systemic markers of nutrient status in weanling wild type offspring consistent with that of the maternal diet, and curiously, remained apparent even in the 32 week old $Apc^{+/1638N}$ pups.

2. Discussion of Specific Aim 2

Specific Aim 2 was designed to elucidate the potential epigenetic effects that mediate the relationship between maternal diet and offspring tumorigenesis. It is worth noting here

that these studies were not designed to provide a definitive proof of the underlying molecular mechanisms. Rather, they were intended to identify likely candidate mechanisms that can then be verified in a more decisive manner by subsequent investigations. After identifiying a portfolio of candidate genes through a systematic evaluation of the literature, the expression of these genes was examined using real time PCR. These genes were chosen based upon 1) a known relevance to colorectal carcinogenesis, 2) a known history of being aberrantly methylated in carcinogenesis, and 3) being part of pathways known to be sensitive to B-vitamin status. Primarily, the genes chosen for examination were in the Wnt, p53, and Tgf-β and signaling pathways.

As described in **Chapter II.D**, there is increasing evidence that Wnt signaling throughput is increased in a state of mild multiple B-vitamin deficiency, and aberrations leading to elevated Wnt signaling throughput are believed to play an early mechanistic role in more than 80% of colorectal cancers [66]. Given the prominent role of the Wnt pathway in colorectal carcinogenesis, as well as our previous results demonstrating Wnt activation in multiple B-vitamin deficiency [16, 72], this pathway was the primary focus of our investigation. We had hypothesized that differences in expression of Wnt pathway genes would occur secondary to alterations in gene-specific methylation, and indeed this is what we had observed.

Several Wnt pathway genes were found to be responsive to the maternal diet in the small intestine of both our 3 week old wild type and 32 week old *Apc*$^{+/1638N}$ pups. Among these were the Wnt-pathway genes *Apc*, *Sfrp1*, *Wif1*, and *Wnt5a*. These genes are widely

known for their roles as early repressors of Wnt-signaling activity, which is therefore consistent with our hypothesis that maternal B-vitamin intake mediates offspring tumorigenesis through an interaction in Wnt signaling.

The methylation of the gene *Sfrp1* was chosen for examination through methyl-specific PCR as a proof of principle experiment since *Sfrp1* is well documented in the literature as being commonly hypermethylated early in colorectal carcinogenesis and previous unpublished data from our laboratory has shown that *Sfrp1* hypermethylation is seen in the tumors of *Apc*$^{+/1638N}$ mice (Liu et al., unpublished). Indeed, we observed a significant correlation between the methylation of the promoter region of *Sfrp1* and the expression of the gene (as ΔCt, hence expression is interpreted as inverse) in both the 3 week wild type and 32 week *Apc*$^{+/1638N}$ pups. Although the nature of the study design precludes us from making conclusions regarding the causality of the methylation changes on expression, the well documented inverse relationship between methylation and *Sfrp1* expression provides strong support for our hypothesis. The observation that *Sfrp1* was relatively hypermethylated in normal, non-tumor tissue is also consistent with the known biology of the gene, as hypermethylation of *Sfrp* genes are believed to be one of the earliest events in colorectal carcinogenesis [112]. It also worth noting that the four Wnt pathway genes in which significant changes were observed due to the maternal diet in both WT and *Apc*$^{+/1638N}$ pups – *Apc*, *Sfrp1*, *Wif1*, and *Wnt5a* – have been repeatedly identified as being silenced by hypermethylation during colorectal carcinogenesis [68-70, 94].

To gain an understanding of the functional ramifications of the alterations in gene expression, we opted to probe for Sfrp1 protein expression using a sandwich based ELISA method (USCN Life Sciences). We found a significant elevation of Sfrp1 protein in the small intestine of 3 week old supplemented wild type pups, but no difference among maternal diet groups in our 32 week $Apc^{+/1638N}$ mice. Upon careful examination of the data, however, we lost faith in this assay due to poor reproducibility, and ultimately chose to exclude these data from our final data set. For the sake of full disclosure, these data are provided in **Appendix F**.

The expression of select genes within the p53 pathway has been repeatedly shown to be susceptible to alterations induced from B-vitamin status, possibly related to changes in both methylation and DNA damage (i.e., strand breaks). Folate depletion has been shown to induce strand breaks in the so-called "hypermutable region" (corresponding to exons 5-8) in colonic tissue, where approximately 90% of *p53* mutations in human cancer occur. Although it is not definitively proven that strand breaks in this gene invariably lead to reductions in expression [113], reductions in *p53* expression are observed in folate depletion [20, 114].

Additionally, although there are no CpG islands within the *p53* promoter region, the coding region of *p53* is rich with CpG islands that are typically hypermethylated under normal physiological conditions. In the setting of folate deficiency, either alone [114] or alongside deficiencies of other nutrients (i.e., vitamins B_2, B_6, and B_{12}) [20], hypomethylation within the *p53* hypermutable region has been observed. Furthermore,

colonic expression of *p53* is diminished further in a state of mild multiple B-vitamin deficiency [20] relative to replete animals. The diminished expression of other p53 pathway genes, such as *Mdm2* and *p21* [20], has also been reported in folate deficiency.

In the small intestinal scrapings of 3 week old wild type pups in this study, a significant reduction in *p21* expression was observed in pups born to deficient dams, but no effect was seen on the expression of *p53* ($P_{trend} = 0.14$). In the 32 week old $Apc^{+/1638N}$ pups, no effect of maternal diet was seen with respect to *p21* expression, although the expression of *p53* responded in a dose-dependent manner with maternal dietary B-vitamin concentration, the trend narrowly missed statistical significance ($P_{trend} = 0.06$). Given that p53 is largely regulated post-translationally, it is difficult to make definitive conclusions regarding the role of the p53 pathway in our observed maternal diet induced protection against tumorigenesis since we did not quantify p53 protein level. The fact that our observations are somewhat consistent with what we have observed in the past, however, does provide a degree of confidence in our current findings.

Relative to our understanding of the response to the Wnt and p53 pathways in the presence of one-carbon nutrient insufficiency and supplementation, little is known about the Tgf-β pathway. The Tgf-β signaling pathway is becoming recognized for its mechanistic importance in colorectal carcinogenesis and is thought to have a dual role in carcinogenesis [115, 116], not unlike the relationship seen between folate and cancer [23]. There is a significant degree of cross-talk between the Tgf-β and Wnt signaling pathways [115], and the loss of Tgf-β signaling has been shown to accelerate both the incidence and

invasiveness of tumors in the *Apc*$^{+/1638N}$ mouse [117]. Furthermore, there has been an increased degree of appreciation for the role of epigenetics in Tgf-β signaling in carcinogenesis, as the expression of several genes within this signaling pathway such as *Tsp1* [118], *Smad4* [119], *TgfβR1* and *TgfβR2* [120], and *Tgf-β1* [121] have been shown to be silenced through methylation in various tissues. Previous data generated in our laboratory indicates that folate depletion led to a significant reduction in *Smad4* expression *in vitro* [21], and microarray data also showed a significant decrease in the expression of the Tgf-β pathway intermediate *Bmp3* [122].

In this study, the expression of *Tgf-β1* was elevated in supplemented 3 week old wild type pups, and this was accompanied by a significant trend for elevated expression of the downstream gene *Smad4*. In the 32 week *Apc*$^{+/1638N}$ pups, however, a significant decrease in *Tgf-β1* expression was observed in the deficient pups, while no effect of maternal diet was observed for *Smad4* expression. Given that the loss of Tgf-β signaling has been shown to accelerate invasiveness in the *Apc*$^{+/1638N}$ mouse, it would be worth investigating through future experiments to determine the importance of Tgf-β signaling in the changes in tumorigenesis observed in this study.

The difference in *Tgf-β1* expression observed in the supplemented wild type pups was the greatest change in terms of magnitude observed in our dataset. Although the data are limited regarding the role of *Tgf-β1* methylation in colorectal cancer at present, there nevertheless remains a large CpG island immediately upstream of the *Tgf-β1* promoter and this gene has been shown to be hypermethylated in tumors of both the lung and prostate

[121]. At this stage, this relationship is strictly speculative, but nevertheless remains an interesting area for future research.

In addition to gene-specific hypermethylation, the second major epigenetic event that has been shown to occur in some settings of B-vitamin depletion is that of genomic hypomethylation. This global reduction in methyl-cytosine levels is an early and consistently observed event in colorectal carcinogenesis [123, 124], and is considered to be a procarcinogenic event as it can lead to chromosomal instability and therefore facilitate chromosome breaks, translocations, and allelic loss [125, 126]. Several labs have observed genomic hypomethylation in several tissues in response to B-vitamin depletion, although the data are hardly unanimous (reviewed in [127]). In the context of maternal diet and offspring methylation, pilot work from our laboratory that, in rats, pups born to dams fed a diet mildly deficient in multiple B-vitamins displayed significant reductions in global methylation in both the colon (17%) and liver (11%) relative to control [128].

No significant differences in genomic methylation were observed between weanling wild type pups of different maternal dietary groups, however a small but significant reduction in genomic methylation was seen amongst adult $Apc^{+/1638N}$ pups of deficient dams, (i.e.: those possessing the highest likelihood of developing invasive cancers), compared to control dams. Although caution should be used when comparing between $Apc^{+/1638N}$ and wild type animals, if we assume that the defective *Apc* allele does not affect DNA methylation, then one might argue that because genomic hypomethylation was not present

in weanling mice and only appeared in adult mice that it is unlikely to be mechanistically involved in tumorigenesis. Alternatively, maternal deficiency may initiate a specific metabolic program that, over time, results in hypomethylation which might subsequently promote tumorigenesis. The answer to this question will only be attained through time course experiments where offspring are sampled periodically through life to study genomic methylation.

In summary, the studies in Specific Aim 2 indicate that the expression of several tumor suppressor genes in the small intestine, particularly within the Wnt pathway, was proportional to the quantity of B-vitamins in the maternal diet. Furthermore, the expression of one of these genes, *Sfrp1*, was significantly and inversely correlated to the degree of promoter methylation at two specific amplicons corresponding to a region within the first exon in the 32 week old $Apc^{+/1638N}$ pups. Genomic hypomethylation was not observed among 3 week old wild type pups, but was apparent in the small intestinal mucosa of $Apc^{+/1638N}$ pups born to deficient dams. Overall, the data from Specific Aim 2 suggest that the maternal diet can induce changes in methylation at the gene-specific level in the offspring that persist throughout life in a manner that functionally affects gene expression.

3. Discussion of Specific Aim 3

Specific Aim 3 was to assess the effect of the maternal diet on downstream Wnt signaling events, namely the accumulation of β-catenin, the expression of oncogenes such as *cMyc*

and *CyclinD1*, and ultimately the resulting effects on the cytokinetics of the small intestinal epithelium of the offspring.

The accumulation of β-catenin in the cell nucleus is a major hallmark of Wnt signaling activation, as once inside β-catenin (upon binding with LEF and TCF) forms a transcriptionally active complex that stimulates the expression of downstream target genes, namely *CyclinD1* and *c-Myc*, among others [65]. The elevated expression of these downstream oncogenes then stimulates proliferation and inhibits apoptosis, thus creating an environment conducive to tumorigenesis [65]. Indeed, previous work from our laboratory demonstrated that several of these molecular events associated with Wnt signaling occurred in colonic tissue of mice fed a diet mildly deficient in multiple B-vitamins, namely, elevated nuclear β-catenin, elevated transcription of *CyclinD1*, and a suppression of apoptosis [16]. Definitive proof of Wnt activation in vivo comes from the *BAT-LacZxApc*$^{+/1638N}$ reporter mouse, wherein the expression of the β-galactosidase reporter construct was elevated 4-fold in the colons mice fed a multiple B-vitamin deficient diet [72].

In our study, a significant elevation of β-catenin was observed in the small intestine of 3 week old wild type pups (via total β-catenin western blot) as well as in *Apc*$^{+/1638N}$ pups (via immunohistochemistry) born to deficient dams. No effect on β-catenin concentration in either genotype was observed among pups born to supplemented dams relative to pups born to control fed dams. This is curious considering the relative suppression of tumorigenesis observed in pups born to supplemented dams, however it may simply be

that neither immunohistochemistry nor western blots were sensitive enough to detect the subtle differences between these pups and those born to control fed dams. A large proportion of total cellular β-catenin is present in cytoskeletal elements, obscuring changes that might be occurring in other pools of β-catenin. Furthermore, the antibody used for both experiments was directed at total β-catenin, which is the sum of both the phosphorylated (i.e., inactive) and non-phosphorylated (i.e., active) forms of the protein. It may be that there were differences in the quantity of the active form between the groups, but the use of this antibody precluded the observation of this difference. Our lab has previously used an antibody specific to the active form of β-catenin (Millipore, #05-665) successfully in tissues such as the colon and liver, however, protein extracts from the small intestine were largely non-responsive to the antibody.

We had hypothesized that the elevation in β-catenin in the small intestine of offspring would lead to an increased expression of downstream Wnt targets, namely *c-Myc*, *CyclinD1*, *Cdx2*, *Cd44*, as well as the oncogene *K-ras*. Contrary to our expectations, however, the expression of these genes were unaffected by the maternal diet. We believe that the disconnect between β-catenin concentration and the expression of downstream targets in the small intestinal mucosa highlights the complexity of the Wnt signaling network. Clearly β-catenin is not the only factor controlling the expression these genes and studying the nuclear abundance of related Wnt regulators, such as TCF, LEF, and Groucho may shed further light onto the precise mechanisms operable here. Alternatively, the use of a Wnt reporter animal, such as the $BAT\text{-}LacZxApc^{+/1638N}$ mouse,

would provide a definitive demonstration of elevated gene transcription secondary to β-catenin entry to the nucleus.

In an effort to explain the cytokinetic mechanisms underlying observed differences in tumor incidence, we evaluated proliferation and apoptosis in the small intestine of offspring. In this study, we did not observe significant differences between maternal diet groups in cellular proliferation in the crypt nor in the expansion of the proliferative zone into the lower 1/3rd of the villus in pups of either genotype. The small intestinal crypts are highly proliferative, and this was evident in the immunohistochemistry slides from pups of both genotypes. Virtually 100% of crypt cells in the small intestine were positive for Ki-67. Since the detection of any modification of small intestinal proliferation by diet was obscured by this high degree of proliferation, we chose to examine the colons of 3 week wild type mice for proliferation via Ki-67 immunohistochemistry. Again, however, we were unable to find significant differences between maternal diet groups. The lack of an effect of the maternal diet on cellular proliferation was entirely consistent with our *a priori* hypothesis. Previously, we had not observed a significant effect of multiple B-vitamin deficiency on the proliferative status of the colon of wild type mice [72], hence we expected a similar finding in this transgenerational setting.

When comparing proliferation scores between the 3 week old wild type and 32 week old $Apc^{+/1638N}$ pups, it was apparent that the level of expansion into the proliferative zone was significantly higher in the adult $Apc^{+/1638N}$ pups ($p<0.0001$). While it is plausible that this difference in proliferation can be attributed to the effect of the defective *Apc* allele, at least

one other report demonstrated that small intestinal proliferation is not different between wild type and *Apc*$^{+/1638N}$ mice of the same age [105], suggesting that perhaps the age of the pups is responsible for this difference rather than genotype. Age, as a drive of increased proliferation in the intestinal epithelium has previously been observed in rodent models [129].

To coincide with the reduced tumor incidence in pups born to supplemented dams and increased tumor invasiveness in pups born to deficient dams, we anticipated a reduction in apoptosis with decreasing maternal B-vitamin intake. On the contrary, we observed a significantly elevated caspase-3 activity in pups born to deficient fed dams as compared to pups of both replete and supplemented dams. In order to confirm this finding, we performed a western blot directed against cleaved caspase-3 in *Apc*$^{+/1638N}$ pups, and the abundance of cleaved caspase-3 was also highest among pups born to deficient dams. The data from both the caspase activity assay as well as the western blots for caspase abundance were significantly and positively correlated (R^2=0.21, p=0.006).

Thus, because apoptosis is highest in the group with an elevated tumor incidence and the highest proportion of invasive tumors, it appears that apoptosis – at least in the fashion in which we assessed it – may not be a key factor explaining the differences in tumor incidence seen here. Rather, we suggest that the elevated apoptosis amongst the pups of deficient dams may be explained by an elevated frequency of cells harboring deleterious molecular characteristics, such as elevated β-catenin accumulation, methylation aberrations, or DNA damage, and this finding represents an effort clear these cells for the

benefit of the organism. To this end, other groups have shown data indicating that β-catenin overexpression *in vitro* led to increases in apoptosis, supporting this hypothesis [130]. While somewhat counterintuitive, perhaps this elevation in apoptosis induced by the maternal diet may predispose the animals to tumorigenesis by creating selective pressure which favors those cells that harbor inappropriate methylation changes, such as those that result in silencing of pro-differentiation/anti-proliferation genes (i.e., *Sfrp1*). This may then later facilitate the expansion and progression of those cells which harbor these epigenetic mutations into neoplasms once the appropriate genetic mutation develops (i.e., loss of the wild type *Apc* allele through methylation or genetic mutation, activating genetic mutations in β-catenin, etc). Given the lack of an observed effect on proliferation in the small intestine and colon, however, perhaps the examination of differentiation of the cells in the small intestine would be a viable alternative endpoint to investigate in the future.

In conclusion, the studies in Specific Aim 3 suggest that the epigenetic changes in Wnt repressor genes among offspring that occur secondary to the maternal diet lead to a de-repression of the Wnt pathway, culminating in an increase in apoptosis among healthy cells in the intestine.

B. Limitations

Our study design has a few major limitations, although in each instance our limitations serve as extremely important guideposts pointing us to subsequent experiments that need to be done in the future. The first being that, as the experimental maternal diets are carried from preconception through weaning, we are unable to determine if and when a so-called

"critical window" of feeding exists. Therefore, we will be unable to tell whether the observed alterations in tumorigenesis, methylation, and gene expression in offspring that occured secondary to the maternal diet were a consequence of exposure *in utero*, early in the life of the offspring, or a combination of these exposures. In this initial stage of investigation, however, we believed it to be appropriate to alter the diet throughout periconception in order to verify the presence of an effect, while future experiments can parse out these details.

Second, conclusions from comparisons of endpoints such as gene expression, proliferation, etc between genotypes are confounded by the age of the pups. All wild type pups were 3 weeks old, whereas all $Apc^{+/1638N}$ were 8 months old, thus limiting any conclusions of the effect of genotype on individual endpoints. Logistical considerations prevented us from collecting samples from offspring of both genotypes at both time points, but this is something that should addressed through future study.

Third, the changes we observed regarding gene expression, methylation, β-catenin accumulation, etc; although consistent with the hypothesis that the mechanism of suppression as due to elevated Wnt signaling throughput in the pups born to control and deficient fed dams, the lack of a true reporter assay precludes us from making definitive conclusions in this regard. Our laboratory has access to a Wnt-reporter animal, namely the *BAT-LacZ* mouse, and subsequent experiments could easily be performed utilizing this mouse to definitively demonstrate alterations Wnt signaling in the offspring in response to the maternal diet. Additionally, maternal feeding studies using knockout or transgenic

mice with alterations in Wnt inhibitor genes (i.e., *Sfrp1*, *Wif1*, etc) could be used to determine whether the mechanism of tumor suppression/invasiveness are dependent on the expression of these genes.

Finally, the *Apc*$^{+/1638N}$ mouse, while it does have desirable characteristics that are similar to human sporadic colorectal cancer (i.e., low tumor burden, similar genetic and epigenetic alterations that occur in human colorectal tumors, etc.), the extent to which this mouse can be used as a model of human sporadic colorectal cancer is unknown. This mouse harbors a germline truncating mutation in the *Apc* gene unlike most human sporadic cases of colorectal cancer, and primarily develops tumors in the small intestine rather than the colon.

In addition, the fact that this animal harbors a mutation within the Wnt pathway (namely the *Apc* gene) may have potentially biased our examination of Wnt pathway gene expression. Therefore, it would be worthwhile to determine whether the observed effects of the maternal diet on the expression of Wnt inhibitors such as *Apc* and *Sfrp1* in models of colorectal cancer that do not harbor mutations within this pathway, such as the AOM or dextran sodium sulfate (DSS) models. The observations that other groups have documented a reduction in tumorigenesis in offspring in the AOM model secondary to maternal folic acid supplementation [50], and the fact that similar gene expression patterns were observed in the 3 week wild-type offspring indicate that the presence of the *Apc* mutation did not bias our results. Despite these limitations, this animal model serves as a

worthwhile and commonly used tool for studying dietary factors that influence the risk of colorectal cancer, given our present knowledge of the disease.

C. Conclusions

Taken together, the work presented in this dissertation demonstrates that supplemental intakes of folate, vitamins B2, B6, and B12 from preconception through weaning suppress tumor incidence among offspring in the $Apc^{+/1638N}$ mouse model, while deficient maternal diets enhance the likelihood that offspring tumors will be invasive. Furthermore, the degree of Wnt pathway activation (as evidenced by β-catenin accumulation) was inversely related to maternal B-vitamin intake. We believe this phenomenon to be related to the expression of soluble Wnt pathway inhibitors such as *Sfrp1*, which was shown to be inversely and significantly associated with the methylation of the promoter region.

This work represents one of the first observations that maternal B-vitamin supplementation can significantly reduce the degree of offspring tumorigenesis in the intestine of offspring, and furthermore, for the first time demonstrates that deficient maternal intakes lead to a higher likelihood that offspring tumors are invasive. In addition, this work is the first to demonstrate significant differences in gene expression in the offspring secondary to methylation changes related to the maternal diet in cancer relevant signaling pathways such as Wnt, p53, and Tgf-β. This work is significant as it provides an important base of preclinical data to guide subsequent investigations regarding cancer-relevant epigenetic alterations induced by maternal B-vitamin intake in offspring.

V. Detailed Methods

A. Genotyping Protocol for Apc$^{+/1638N}$ Mice

Materials – Tail DNA Extraction

- Tail buffer:
  - Tris HCl: 6.05g
  - EDTA: 37.22 g
  - NaCl 5.84 g
  - SDS: 10 g
- Use 10M NaOH adjust pH until close to 7, then use 1M NaOH adjust pH till 7.5,
- Add dH_2O to 1L.
- Proteinase K (Sigma, p2308) (Proteinase K (10 mg/ml)
- NaCl (6 M)
- 2-Propanol
- Ethanol

Protcol – DNA Extraction from Tail Samples

1. Cut tail; add 750 ul Tail buffer and 37.5 ul Proteinase K (10 mg/ml).
2. Place in 55 °C heat block overnight.
3. Vortex vigorously, add 250 ul of 6M NaCl, and vortex again.
4. Spin for 10 min at 14,000 rpm at 4 °C.
5. Transfer 750ul of the upper layer to a new eppendorf tube.
6. Add 500ul of 2-Propanol, and vortex well.
7. Spin for 15 min at 14,000 rpm at 4°C.
8. Aspirate the propanol, and add 1ml 70% ethanol to wash away the rest propanol.
9. Spin for 5 min at 14,000 rpm at 4°C.
10. Aspirate ethanol, and air dry at room temperature.
11. Add 500 ul H_2O and mix gently. Store at -80 °C until PCR.

Materials – PCR

Primer Sequences:
A001: 5'-TGC CAG CAC AGA ATA GGC TG-3'
A002: 5'-GTT GTC ATC CAG GTC TGG TG-3'
A003: 5'-TGG AAG GAT TGG AGC TAC GG-3'

Master Mix Preparation:

Reagent	Volume (x1 sample)
H2O	20.4 ul
10x PCR buffer	3 ul
dNTP mix	3 ul
Primer A001	0.2 ul
Primer A002	0.2 ul
Primer A003	0.2 ul
Taq	1 ul

Protocol – PCR for Apc1638N allele

1. Multiply volume of each reagent by the number of samples (adding a few extras to account for pipetting error).
2. Combine in a separate eppendorf tube labeled “master mix” and store on ice until ready.
3. Add 28 ul of the master mix to each PCR tube. Add 2 ul of tail DNA template to each reaction tube. Keep on ice during this step.
4. Place into PCR machine using the following conditions (Listed as “Liu APC” on PCR machine):

Temperature	Time	# Cycles
94°C	3 min	1
94°C	1 min	35
60°C	2 min	
72°C	3 min	
72°C	3 min	1
4°C	Hold	1

5. Keep product at 4°C until ready for electrophoresis.

Materials – Agarose Gel Electrophoresis

- 50 X TAE Buffer (Tris Acetic EDTA):
  - 242 g Tris
  - 57.1 ml Acetic Acid Glacial
  - 100 ml of 0.5M EDTA, pH 8.0
  - Bring to 1 liter with ddH2O. Store in a glass container. Discard any buffers that develop a precipitate. Dilute to 1X for use in agarose gels.
- Ethidium Bromide (EBr) Staining Solution (Mix 5 ul EBr stock [10 mg/ml] + 100 ml ddH2O)

Protocol:

1. Prepare the gel tray by securing both ends. Insert comb with desired number of wells.
2. Prepare 100 ml of a 1.8% agarose solution:
3. 1.8g Agarose (UltraPure Agarose, Invitrogen)+100 ml of 1X TAE
4. Mix sample with loading buffer at appropriate ratio, and load into gel.
5. Load your samples and the marker in separate wells of the gel.
6. Turn on the power and run the gel at 95-105 V for 30-45 min or until the tracking dye is 2/3 to 3/4 of the distance to the end of the gel.
7. Turn off power, remove gel, and photograph under UV light.
8. Animals with 1638N allele will have two bands corresponding to 300 and 400bp upon visualization in the agarose gel.

Primer Combinations:

A001 + A003 = 400bp product = 1638N allele
A002 + A003 = 300bp product = WT allele

B. RNA Extractions Using Trizol

Materials

- Trizol Reagent (Gibco/Life #15596-026)
- Chloroform.
- Isopropyl alcohol (aka isopropanol, 2-propanol).
- Ethanol.
- RNase free water.
- Optional: RNase away to swab pipettes and workspace.

Protocol

1. Place tissue biopsy into 1.5 mL eppendorf tube, add 500 ul Trizol. Homogenize with tissue grinder.
2. Incubate at room temp for 10 minutes.
3. Transfer lysate to a conical based tube. At this stage lysates may be stored at -20ºC overnight (-70ºC for longer periods) before continuing.
4. Add 0.2 volumes chloroform, shake vigorously and sit at room temp for 5-10 min. Spin at 3270g for 30 min at 4ºC*.
5. Remove upper aqueous layer containing RNA into a new conical tube. Interface contains DNA while lower layer contains protein. These can be extracted from the same preparation (see product insert).
6. Add 0.5 volumes isopropanol and mix by inversion to precipitate RNA. Sit at room temp for 10 min.
7. Spin at 3270g for 30 min at 4ºC*.
8. Decant supernatant and wash RNA pellet with 1 volume 75% ethanol.
9. Spin at 3270 g for 30 min at 4ºC*.
10. Decant supernatant and stand tubes upside down on tissue paper to drain for 10 min.
11. Stand tubes to air dry at room temp or use speed-vac.
12. Resuspend RNA in 50 -100 µl RNase free water.
13. Incubate at 60ºC for 10 min to dissolve.

* If using microfuge tubes spin at 12,000 g for 10 min at 4ºC.

C. Reverse Transcription

Materials

- Superscript II Reverse Transcriptase (Invitrogen #18064-014)
- 0.1M DTT & 5x first strand buffer (supplied with Superscript II)
- Oligo (dT)12-18 primer (Invitrogen #18418-012)
- 10 mM dNTPs (Invitrogen #18427-013 (or -088))
- RNaseOUT (Invitrogen #10777-019)
- RNase free/sterile water

Protocol

1. Add 1 ng – 5 µg total RNA to a sterile RNase-free 0.2 ml reaction (PCR) tube and make up volume to 10 µl with water.
2. Add 1 µl dNTPs and 1 µl Oligo(dT)12-18.
3. Incubate at 65ºC for 5 min and then chill on ice.
4. Make a mixture of 4 µl 5x buffer, 2 µl 0.1 M DTT and 1 µl RNaseOUT per reaction. Add 7 µl of this mixture to each reaction.
5. Mix gently, spin and incubate at 42ºC for 2 min.
6. Add 1 µl (200 Units) of Superscript II enzyme to each tube and incubate at 42ºC for 50 min. Final volume = 20 µl.
7. Inactivate the reaction by incubating at 70ºC for 15 min.
8. Store at -70ºC.

Notes:

- Use 2 µl of the single-stranded cDNA product for real time PCR. 1 µl is sufficient for GAPDH.
- May scale reaction up to 50 µl: Add 2.5 µl each of dNTPs and Oligo(dT) 12-18, then 10 µl buffer, 5.0 µl DTT and 2.5µl RNase OUT. Add 1 µl SSII enzyme.

D. DNA Extraction Using Phenol:Chloroform

Materials
• TE (pH 8.0).
• 10% SDS.
• Phenol (Tris-saturated).
• Phenol:Chloroform:Isoamylalcohol (25:24:1).
• Chloroform:Isoamylalcohol (24:1).
• 3M Sodium Acetate.
• 100% Ethanol.
• 70% Ethanol.
• RNase (Roche, 1119915 or similar).
• Proteinase K (Sigma, P6556 or similar).
• Polypropylene tubes- DO NOT use polystyrene.

Protocol
1. Suspend cells in TE buffer and add 10% SDS to a final concentration of 0.5%. Mix by inversion and add 2-5 µg/ml RNase. As a guide, suspend 50x 106 cells in 2mls TE and add 100 µl 10%SDS.
2. Incubate at 37ºC for 1- 2 hours.
3. Add Proteinase K to a final concentration of 0.1 mg/ml and incubate overnight at 37ºC.
4. Add an equal volume of Phenol and mix vigorously.
5. Spin tubes at maximum speed for 10 min.
6. Remove the upper layer (DNA, TE) into a clean tube. Solution may be highly viscose and pipette tips may need to be cut to increase the bore-size.
7. Add an equal volume of Phenol:Chloroform:Isoamylalcohol (25:24:1) and mix vigorously. Spin at maximum speed for 10 min and remove upper layer into a new tube.
8. Add an equal volume of Chloroform:Isoamylalcohol (24:1) and mix vigorously. Spin at maximum speed for 10 min and remove upper layer into a new tube.
9. Add 2 volumes of cold ethanol and 1/10 volume 3M NaOAc. Mix by inversion to precipitate DNA.
10. Spin tube at maximum speed for 10 min and decant off supernatant. Wash with 2 volumes of 70% ethanol.
11. Spin tube at maximum speed for 10 min and decant off supernatant.
12. Allow DNA to air dry (do not over-dry) and dissolve DNA in sterile water (pH 8.0) or TE buffer (pH 8.0).

E. DNA Quantification Using Picogreen

Materials

- Quant-iT Picogreen ® ds DNA Assay kit.
- Picogreen dye P7581 (desiccate store 4°C protect from light) thaw before opening
- 20x TE buffer P7589 (store at RT)
- DNA standard P (store at 4°C)
- DNA (ase)-free water.
- Black-sided Microplate (VWR cat. #BD353293) (well volume 100-250µL)

Protocol

1. Dilute 20X TE (2.0mL 20x in 38mL DNA water) in 50.0mL conical vial.
2. Make DNA dilutions for standard curve: 2µL of standard DNA (100ng/µL) in 198µL of 1X TE (tube 1). Add 100µL of 1X TE to 5 labeled tubes (2-6). Vortex and centrifuge tube 1 remove 100µL and add to tube 2. Continue to tube 6. DNA concentrations will be 1, 0.5, 0.25, 0.125, .0625, 0.3125ng/µL.
3. Dilute DNA samples to be quantified as necessary to fall into this curve. May need to do a cursory quantification on the spectrophotometer. Total well volume of DNA and 1X TE cannot exceed 100µL.
4. Make dye solution 1:200 in TE in plastic as glass may absorb dye. Cover vial in foil--light sensitive. Each sample (including STD (n=6) and blank (n=1) require 100µL.
5. Add experimental DNA to black walled 96 well plate + 1X TE buffer make-up volume to 100µL.
6. To each well add 100µL of 1:200 Picogreen dye-- Total well volume=200.uL
7. Cover plate in foil and shake gently on plate shaker for 10mins.
8. Read plate using CytoFlour multi-well plate reader series 4000 or similar (Lab 717).
9. Open software, turn on lamp (must warm up for at least 10mins). *Excitation 485/20, Emission 530/20, Gain 60*
10. Open door put plate on press START
11. Must have floppy to save data. When finished reading go to File→ Export→a\: drive→ name file→export dialog box will open→ export current.
12. Make a STD curve, get the equation of the line and apply it to fluorescence readings multiply by DNA dilution factor, do not need to subtract blank.

F. Sample Preparation for Genomic DNA Methylation Analysis

Materials
- Ammonium Acetate (Sigma A7262)
- Ammonium Bicarbonate (Sigma A6141)
- Methanol (Sigma 494437)
- Nuclease p1 200u/vial (Fisher(MP Biomedicals) ICN1953520)
- Phosphodiesterase I from Crotalus adamanteus venom 0.43u/vial (Sigma P-3243)
- Phosphatase, Alkaline Escherichia coli 100u/vial (Sigma P 4252)
- [15N3]2'-deoxycytidine (uM Cyt-internal std 3.5ng/µL) Cambridge Isotope Laboratories.
- (methyl-d3, ring-6-d1)5-methyl-2'-deoxycytidine (M Cyt-internal std 1.75ng/µL) Cambridge Isotope Laboratories.
- HPLC grade H2O.

Solutions
- 0.1M ammonium acetate pH 5.3: Dissolve 1.93g of ammonium acetate (FW=77.08) in 200mL HPLC grade H2O. Bring pH to 5.3 with glacial acetic acid. Add HPLC grade H2O to bring final volume to 250 mL. Store at 4°C.
- 1.0M ammonium bicarbonate pH 7.8: Dissolve 19.8 g ammonium bicarbonate (FW=79.06) in 250 mL of HPLC grade H2O. Store at 4°C
- 0.5M ammonium acetate pH 6.7 (Mobile Phase Constituent): Dissolve 19.3 g of ammonium acetate (FW=77.08) in 500 mL HPLC grade H2O. Store at 4°C
- Mobile Phase: Combine 14.0 mL Mobile Phase Constituent and 50.0 mL MeOH. Bring volume to 1000 mL with HPLC grade H2O. Sterile filter through 0.2µm nylon membrane filter. Store @ 4°C.
- Nuclease p1: Add 100µL 0.05M ammonium acetate pH 5.3 to vial (200u/mg-1mg/vial). Vortex- aliquot and store at -20°C.
- Phosphodiesterase I: Add 430 µL HPLC grade H2O to vial (0.43u/vial) to make .001u/µL. Vortex- aliquot and store at -20°C.
- Phosphatase, Alkaline: Transfer total volume into an 1.5 ml tube. Centrifuge 12,000 rpm at 4°C for 1.0 min. Discard supernatant, add 200 µL HPLC grade H2O to make final concentration 0.5u/µL. Store aliquots at 4°C.

Protocol
1. Put 1µg of DNA in a lock-top 1.5 ml microfuge tube.
2. Denature the DNA by heating 100°C for 3.0 min then immediately chill on ice for > 1 min
3. Briefly centrifuge and then add 0.5µL of 0.1M ammonium acetate pH 5.3.
4. Add 1.0µL (2u) of Nuclease p1 and incubate at 45°C for 2.0 hrs.
5. Briefly centrifuge and add 1.0µL 1.0M ammonium bicarbonate pH 7.8.
6. Add 2.0uL (.001u) Phosphodiesterase I and incubate at 37°C for 2.0 hrs.
7. Briefly centrifuge, add 1.0µL Alkaline Phosphatase and incubate at 37°C for 1.0 hrs.
8. Add 10µL uM Cyt-internal std 3.5ng/µL
9. Add 10µL M Cyt-internal std 1.75ng/µL
10. Bring total volume to 35µL w/ HPLC grade H2O.

G. *Promoter Methylation of* Sfrp1 *in Mouse DNA*

Materials

A. Bisulfite treated DNA (Note: EZ Methylation Gold Kit from Zymo used – cat# D5006)
B. Qiagen Taq PCR master mix (cat # 201443)
C. Primers

Name	Forward Primer Sequence	Reverse Primer Sequence
Amplicon A – Methyl	5'-TAG GTG TAG TAG TTC GTA GTT CGT C-3'	5'-AAT CCT CCG CTA CAA CAA ATC GCC G-3'
Amplicon A – Unmethyl	5'-TAA GTA GGT GTA GTA GTT TGT AGT TTG TT-3'	5'-TAA AAT CCT CCA CTA CAA CAA ATC ACC A-3'
Amplicon B – Methyl	5'-GTA AAT CGA TTT TTT TGG TCG GCG C-3'	5'-CGA ATA CGC GAT ATA CGA ATA ACC G-3'
Amplicon B – Unmethyl	5'-TTT AGT AAA TTG ATT TTT TTG GTT GGT GT-3'	5'-AAC TCA AAT ACA CAA TAT ACA AAT AAC CA-3'
Amplicon C – Methyl	5'-TGA AGG TAG CGT GGG TAG TTT CGA C-3'	5'-GAA CCC GCG ACC AAC AAA ACG ACG-3'
Amplicon C – Unmethyl	5'-GTG TGA AGG TAG TGT GGG TAG TTT TGA T-3'	5'-ACC AAA CCC ACA ACC AAC AAA ACA ACA-3'

Protocol

1. Dilute primers to 100 uM on arrival with PCR grade H2O.
2. Create forward and reverse primer mixes for the methyl and unmethyl sets of each amplicon by adding 50 ul of the forward primer and 50 ul of the reverse with 900 ul PCR grade H2O. (new concentration = 5 uM).
3. For each PCR reaction, use 10 ul Taq master mix, 7.4 ul H2O, and 1.6 ul primer mix (primer concentration in reaction = 400 nM). Set up reactions for both the methyl and unmethyl primer sets for each amplicon.
4. Load 1 ul of bisulfate treated DNA (total reaction volume = 20 ul).
5. Run PCR according to the following conditions:

Amplicons A & B			***Amplicon C***		
Temp.	**Time**	**Cycles**	**Temp.**	**Time**	**Cycles**
94^oC	3 min	1	94^oC	3 min	1
95^oC	30 sec	35	95^oC	30 sec	35
60^oC	30 sec		62^oC	10 sec	
72^oC	30 sec		72^oC	30 sec	
4^oC	Hold	1	4^oC	Hold	1

Note: Conditions for Amplicons A and B listed on BioRad PCR machine as "Sfrp1 msp ampA", and Amplicon C is listed as "Sfrp1 msp ampC".

6. Mix loading buffer in with PCR product.
7. Load into a 2% agarose gel. Run at 90 volts for 25-40 min.
8. Quantify bands using BioRad Image Quantifier.

H. Folate Extraction Procedure

Materials

• Extraction buffer:

1 g Na Ascorbate (2%)

1 g Bis-Tris (2%)

50 ml dd H_2O

35.5 ul 2-mercaptoethanol

K_2HPO4

Na Ascorbate

Chicken pancreas conjugase

Protocol

- Extraction buffer is 20 ml/g sample. Put a fraction of the volume you will probably be using into tube.
- Weigh the biopsy sample quickly, remove from foil and put it into the buffer fraction.
- Weigh the foil alone, subtract to obtain the weight of the sample and multiply by 20. Example:

Sample ID	Total Wt (g)	Foil Wt (g)	Sample Wt (g)	Buffer (ul)
1	0.4610	0.4540	0.0070	140
2	0.4781	0.4729	0.0052	104

- Add remaining buffer volume. (Ex: for sample 1, add 50 ul extraction buffer at first, then add the remaining 90 ul extraction buffer at this time).
- Homogenize with motor-driven homogenizer
- Cap tubes with aluminum foil. And place tubes in boiling water bath for 15 minutes.
- Remove from water bath, cool on ice for 5 minutes.
- Centrifuge at 36000 g, 4 °C for 20 minutes. (*17,500 rpm for rotor code: SS34, Sorvall Intrument RC%C centrifuge in Jacob's Lab is equal to 36,600 g. the adaptor for 11*75 tubes (cat. 00381), and the 4 ml flanged (with lip) 11*75 tubes (cat. 03015) can be obtained from www.kendro.com (bought out Sorvall and Revco)*),
- Transfer the supernatant into red top vacutainer using LP needle (to prevent air from entering which causes oxidation) and keep at -80 °C until ready for conjugase treatment.
- Conjugase Treatment: Make 0.1 M K_2HPO4 (pH 8.6, note: it is already 8.6, should not need to adjust pH) containing 10 mg/ml NaAscorbate
- In an 1.5 ml eppendorf tube mix 20ul folate extract, 10ul purified chicken pancreas conjugase (rat plasma conjugase will work too), 70ul 0.1 M K_2HPO4/Na Ascorbate mixture
- Incubate at 37°C for 2 h, store in -80°C until ready for quantification.

I. Folate Quantification Using L. Casei

Materials

- Cryoprotected *L. casei* :(ATCC 7469)
- *L casei* folic acid media: (Disco, Ref.282210/4114584, BD)
- Folic acid: Sigma F-7876
- Ascorbic acid
- Na Ascorbate
- Sodium chloride (NaCl)
- Sodium hydroxide (NaOH)
- Potassium phosphate, mono- and dibasic

Protocol

Solutions:

1) **0.9% physiological saline (optimal):**
   a. dissolve 4.5 g NaCl in 500 ml H2O.
   b. autoclave or sterile filter solution.
2) **0.1 M Potassium phosphate buffer:** (pH 6.3, and containing 1 mg/ml Na Ascorbate).
   a. Dissolve 5.25 g KH2PO4, 2.0 g K2HPO4, and 0.5 g Na Ascorbate in 500 ml H2O.
   b. Sterile filter solution (The pH usually is 6.3, it is not needed to adjust again).
3) **Folic acid standards:** (Prepare and dispense into cryotubes and store -70°C).
   a. 10 mg folic acid in 10 ml water, add 4 drops 5 N NaOH to dissolve.
   b. Verify the concentration using a spectrophotometer at 282 nm using 0.1 M K PO4 buffer, pH 7 as a blanking solution. (Ex. Coeff.=27600, MW=441.4).
   c. Dilute stock 1:20 in MeOH, store aliquots at -70°C.
   d. On day of use, dilute MeOH solution to 2 ng/ml in 0.1 M KPO4 buffer.
4) **0.5 M Manganous sulfate:** (MnSO4·H2O, MW=169.01).
   a. Dissolve 8.45 g MnSO4·H2O in 100 ml ddH2O.
   b. Sterile filter and store 4°C.
5) **L. casei media:** (dissolve following the instructions on the bottle)
   a. Weigh 47 (9.4 g/100ml *5) g media, and put it into a 1000 ml flask.
   b. Add 500 ml ddH2O.
   c. boil it, and then cool it.
   d. After completely cooled, add 250 mg (50 mg/100 ml*5) ascorbic acid free acid
   f. Filter solution.

Procedure:

- Mix 50 ul cryoprotected L. casei with 1.25 mL physiological saline.
- Mix 10 ml 0.1 M KPO4 buffer with 15 ml L. casei folic acid media per plate
- Add 0.5 ml above saline L. Casei inoculum and 0.27 ml 0.5 M MnSO4 (final concentration = 5.2 mmol/L).
- Pipette 50 ul 0.1 M potassium phosphate buffer into all wells of a 96-well plate.
- In columns 1 and 2:
    - Pipette 50 ul of 6 ng/ml folic acid standard (in KPO4 buffer) into well H and pipette mix.

  - Transfer 50 ul from well H to well G.
  - Repeat mix and transfer steps until well A and discard 50 ul.
  - Final standard is 0.15 ng FA in well H with serial dilution through well A.
- In columns 3 and 4:
  - Pipette 50 ul of internal control plasma folate (1:1 dilution) into well H and pipette mix.
  - Transfer 50 ul from well H to well G.
  - Repeat mix and transfer steps until well A and discard 50 ul.
- In columns 5 through 12: (as an example when using 5 ul sample)
  - Pipette 40 ul 0.1 M potassium phosphate buffer into wells D and H.
  - Pipette 10 ul of a sample into well D and pipette mix.
  - Transfer 50 ul from well D to well C.
  - Repeat mix and transfer steps until well A. After transferring from well B to well A, pipette mix well A and then discard 50 ul.
- Repeat steps b, c, and d with a different sample going from well H to well E.
- Add 250 ul of L. casei suspension (see media preparation) to each well.
- Cover plate with a mylar seal instead of the plate lid, incubate the plate overnight at 37 °C in a dry incubator. Or incubate the plate in a plastic bag overnight in a humid incubator.
- Determine total folate concentration for each sample using a 96-well plate reader.
- Gently place plate in shaker for 5-10 min, remove mylar seal and allow bubbles to burst before reading.
- Read plate at 630 nm.

Solution Preparation:

Plasma Standard – 55ul + 55 ul ddH2O

Folic Acid Standard – Mix 1: 5 ul stock + 995 ul ddH2O – use 23.55 ul of mix 1 and add to 976.45 ul ddH2O for final working solution (conc: 254.75 ng/mL)

VI. Appendices

A. Breeding Statistics

Diet Group	Dams (n)	Matings Resulting in Live Births (%)	Litters (n)	Mean Litter Size
Deficient 2	38	63.2%	24	5.3
Control	26	46.1%	12	5.9
Supplemented	20	70.0%	14	6.4
Chi Square P value	-	0.23	-	0.30

Table 5. Mating and breeding statistics for each maternal diet group.

Diet Group	$Apc^{+/1638N}$ pups born (n)	Wild type pups born (n)	%$Apc^{+/1638N}$
Deficient 1	3	11	21%*
Deficient 2	56	65	46%
Control	29	24	55%
Supplemented	26	29	47%

Table 6. Proportion of pups born of both genotypes stratified by maternal diet. **Significantly different than control (by chi-square), p=0.036. Note: 10 $Apc^{+/1638N}$ mice were sacrificed at weaning in order to reduce the number of mice in the study. 5 additional $Apc^{+/1638N}$ mice died over the course of the study without tumors. None of these mice were included in the tumor data.*

B. Offspring Weight Data and Growth Charts

Maternal Diet	Weight at meaning, males (g)	Weight at weaning, females (g)
Deficient 2	14.3 ± 0.45	12.4 ± 0.24
Control	12.2 ± 0.51	11.2 ± 0.59
Supplemented	11.4 ± 0.85	10.4 ± 0.82
ANOVA p-value	<0.001	0.01

Table 7. Weights at weaning for wild type offspring.
Mean ± SD.

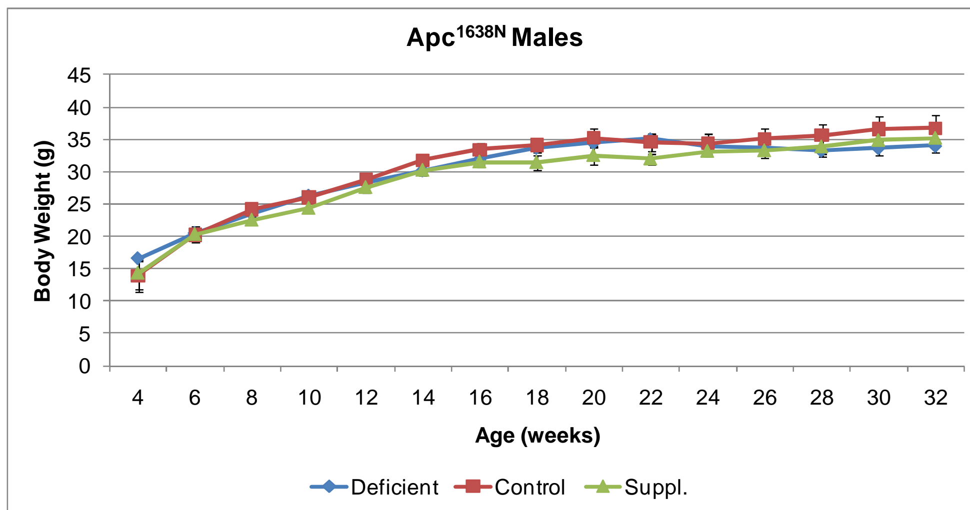

Figure 6. Growth curve for male *Apc*$^{+/1638N}$ offspring from weaning until sacrifice.
Repeated measures ANOVA for diet effect, p=0.43. Mean ± SEM.

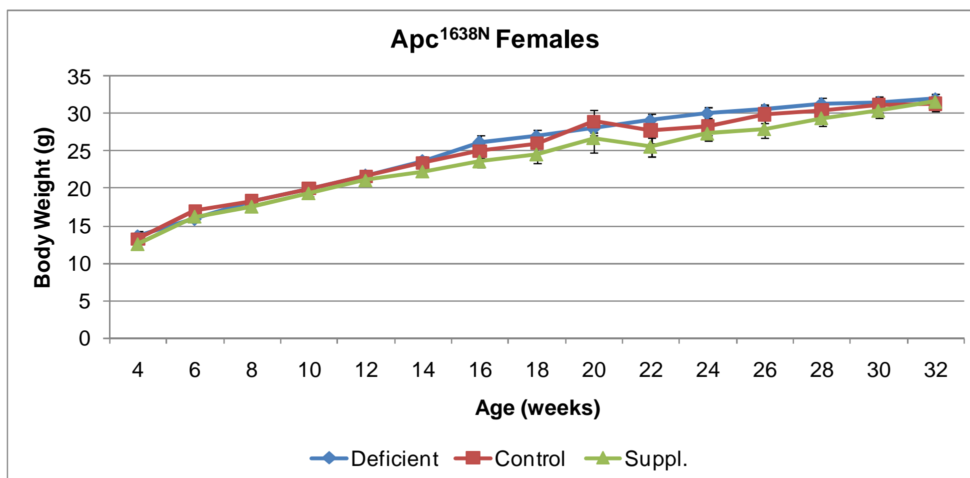

Figure 7. Growth curve for female *Apc*$^{+/1638N}$ offspring from weaning until sacrifice.
Repeated measures ANOVA for diet effect, p=0.37. Mean ± SEM.

C. Aberrant Crypt Foci (ACF) Analysis

Maternal Diet	**ACF Incidence Proportion**	**Mean # ACF**
Deficient 2	30.7%	1.5 ± 0.8
Control	23.1%	1.2 ± 0.4
Supplemented	13.6%	1.0 ± 0.0

Table 8. Aberrant Crypt Foci data in the cecum of *Apc*$^{+/1638N}$ pups.
Mean ± SD

D. Primer Sequences

Gene	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
Apc	GCCAGGATCCAGCAAATAGA	CATGCCTGCTCTGAGATGAC
Axin2	ACTGACCGACGATTCCATGT	TGCATCTCTCTCTGGAGCTG
Cdc42	GAGACTGCTGAAAAGCTGGC	CAGCCAATATTGCTTCATCAAA
Cd44	CAAGTTTTGGTGGCACACAG	AGCGGCAGGTTACATTCAAA
Cdh1	GAGGTCTACACCTTCCCGGT	AAAAGAAGGCTGTCCTTGGC
Cdx2	GAAACCTGTGCGAGTGGATG	TCTGTGTACACCACCCGGTA
c-Myc	AGAGCTCCTCGAGCTGTTTG	TGAAGTTCACGTTGAGGGG
CyclinD1	TCCTCTCCAAAATGCCAGAG	GGGTGGGTTGGAAATGAAC
GSK3β	GACCGAGAACCACCTCCTTT	GTGGTTACCTTGCTGCCATC
K-Ras	CTTTGTGGATGAGTACGACCC	TTGACCTGCTGTGTCGAGAA
mTOR	CTCAGGCTGCTGGAGCTTAT	GCCAAAGCACTGCACTACAA
MMP9	AGACGACATAGACGGCATCC	CTGTCGGCTGTGGTTCAGT
p21	CGGTGTCAGAGTCTAGGGGA	ATCACCAGGATTGGACATGG
p53	CTAGCATTCAGGCCCTCATC	TCCGACTGTGACTCCTCCAT
Sfrp1	CATCTCTGTGCAAGCGAGTT	GGGTTTCTTCTTCTTGGGGA
Smad4	GGTTGTCTCACCTGGAATTGA	GGCTGTCCTTCAAAGTCGTG
Smad7	GAACGAATTATCTGGCCCCT	CTTCTCCTCCCAGTATGCCA
Tgf-β1	GGAGAGCCCTGGATACCAAC	CAACCCAGGTCCTTCCTAAA
uPA	GTGTAGACACCGGGCTTGTT	GGACCCAGAGTGGAAAACAG
Wif1	CCATCAGGCTAGAGTGCTCA	GCATTCTTTGTTGGGCTTTC
Wnt5a	AACTTGGAAGACATGGCACC	ACGCTTCGCTTGAATTCCT
β-catenin	GAGCCGTCAGTGCAGGAG	CAGCTTGAGTAGCCATTGTCC
GAPDH	TTGATGGCAACAATCTCCAC	CGTCCCGTAGACAAAATGGT

Table 9. Primer sequences for all real time PCR targets examined.
Note: All primers used at a 300 nM concentration in the PCR reaction.

E. Full Real Time PCR mRNA Expression Data

Gene	**Relative Expression ($2^{-\Delta\Delta Ct}$)**			**ANOVA P**	**P_{trend}**
	Deficient	**Control**	**Suppl.**		
Extracellular Wnt Inhibitors					
Sfrp1	0.41[a]	1[ab]	1.69[b]	**0.04**	**0.01**
Wif1	0.54[a]	1[ab]	1.66[b]	**0.01**	**0.003**
Wnt5a	0.50	1	1.21	0.09	**0.04**
Wnt Pathway Mediators					
Apc	0.80[a]	1[ab]	1.53[b]	**0.03**	**0.01**
Axin2	0.77	1	1.05	0.58	0.33
β-catenin	0.98	1	1.13	0.86	0.61
Cdh1	0.99	1	1.30	0.43	0.25
GSK3β	0.77	1	1.11	0.16	0.06
Oncogenes					
CyclinD1	0.70	1	1.12	0.58	0.31
c-Myc	0.61	1	1.37	0.14	**0.047**
K-Ras	0.81	1	1.22	0.14	**0.047**
Tgf-β1 Pathway					
Tgf-β1	0.90[a]	1[a]	2.24[b]	**0.008**	**0.005**
Smad4	0.91	1	1.44	0.08	**0.03**
Smad7	0.82	1	0.84	0.58	0.91
p53 Pathway					
p21	0.59[a]	1[b]	0.86[ab]	**0.03**	0.07
p53	0.85	1	1.19	0.38	0.16
Other					
Cdx2	0.75	1	0.84	0.55	0.70
Cdc42	0.81	1	1.17	0.26	0.10
MMP9	0.75	1	0.87	0.73	0.70
uPA	1.09	1	1.85	0.26	0.19

Table 10. Real time PCR data (as relative expression) in small intestinal scrapings of 3-week old wild type pups.

Letters with different superscripts are significantly different with Tukey's HSD, $p<0.05$

Gene	Relative Expression ($2^{-\Delta\Delta Ct}$)			ANOVA P_{diet}	P_{trend}
	Deficient	Control	Suppl.		
Extracellular Wnt Inhibitors					
Sfrp1	0.27[a]	1[b]	1.27[b]	**0.002**	**0.001**
Wif1	0.69[a]	1[ab]	1.20[b]	**0.02**	**0.005**
Wnt5a	0.59[a]	1[ab]	1.26[b]	**0.01**	**0.004**
Wnt Pathway Mediators					
Apc	0.73[a]	1[a]	1.14[a]	**0.04**	**0.02**
Axin2	0.75	1	1.23	0.16	0.06
β-catenin	0.73	1	0.79	0.31	0.55
Cdh1	0.78	1	1.16	0.25	0.10
GSK3β	0.75	1	0.98	0.09	0.06
Oncogenes					
Cd44	0.66[a]	1[a]	1.18[a]	**0.04**	**0.01**
CyclinD1	0.79	1	0.98	0.66	0.44
c-Myc	0.55	1	0.96	0.06	**0.048**
K-Ras	0.87	1	1.05	0.25	0.11
Tgf-β1 Pathway					
Tgf-β1	0.74[a]	1[b]	1.05[ab]	**0.02**	**0.01**
Smad4	0.93	1	1.05	0.57	0.29
Smad7	0.89	1	1.11	0.43	0.19
p53 Pathway					
p21	0.83	1	0.95	0.54	0.41
p53	0.77	1	1.07	0.14	0.06
Other					
Cdx2	0.91	1	0.87	0.73	0.92
mTOR	0.88	1	1.20	0.23	0.09
MMP9	0.82	1	1.07	0.54	0.28
uPA	0.43[a]	1[b]	0.85[ab]	**0.006**	**0.01**

Table 11. Real time PCR data (as relative expression) in small intestinal scrapings of 32-week old *Apc*$^{+/1638N}$ pups.

Letters with different superscripts are significantly different with Tukey's HSD, p<0.05

F. Sfrp1 Protein Quantification in Small Intestine (ELISA)

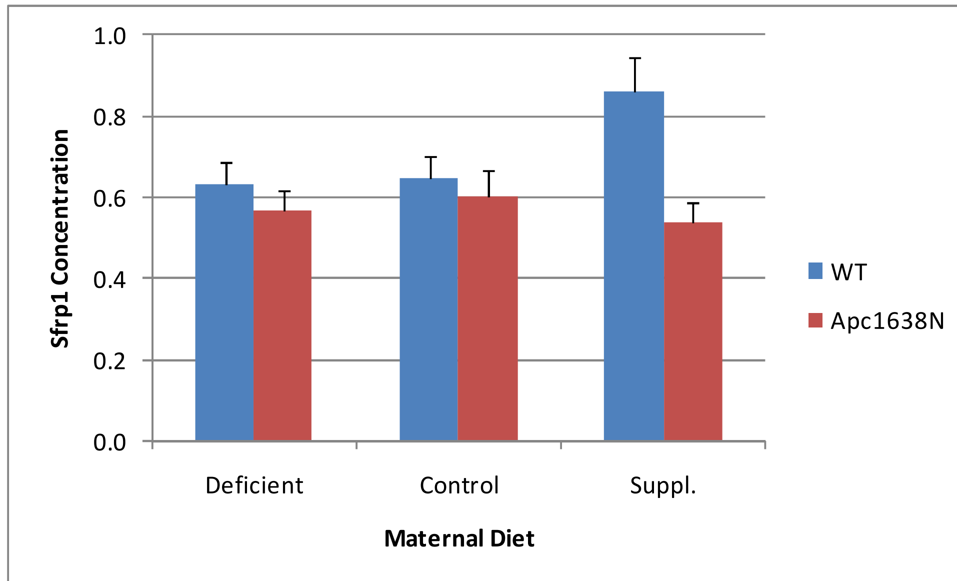

Figure 8. Effect of maternal diet on Sfrp1 protein level measured by ELISA in small intestinal scrapings from wild type and *Apc*$^{+/1638N}$ pups.
Wild type (WT) p=0.03; Apc$^{+/1638N}$ *p=0.71. Mean ± SEM.*

G. Nuclear β-catenin Immunohistochemistry Data

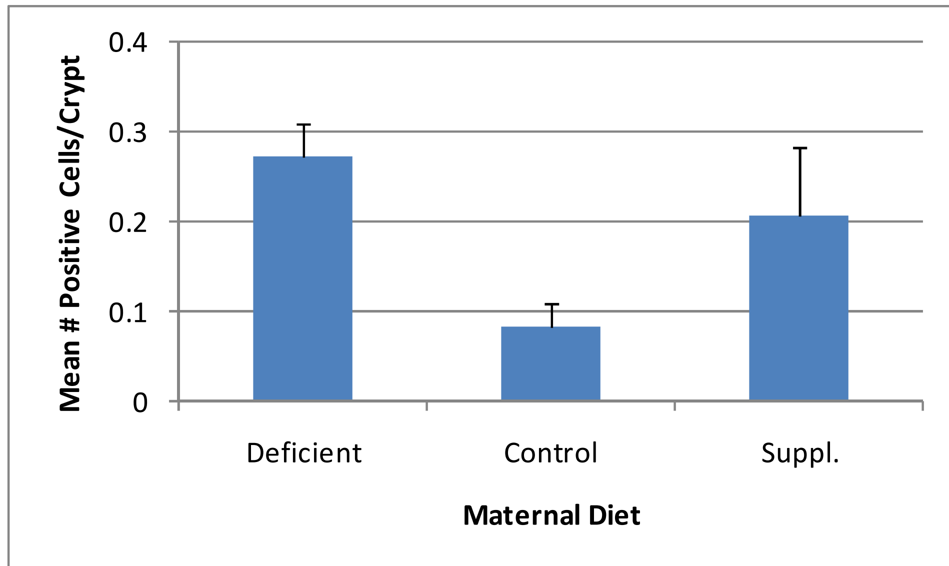

Figure 9. Effect of maternal diet on nuclear β-catenin accumulation in the small intestine of 32-week old *Apc*$^{+/1638N}$ pups.
ANOVA P_{diet}*=0.006. Mean ± SEM.*

H. Effect of Maternal Diet on Cytokinetics in the Colon of Offspring

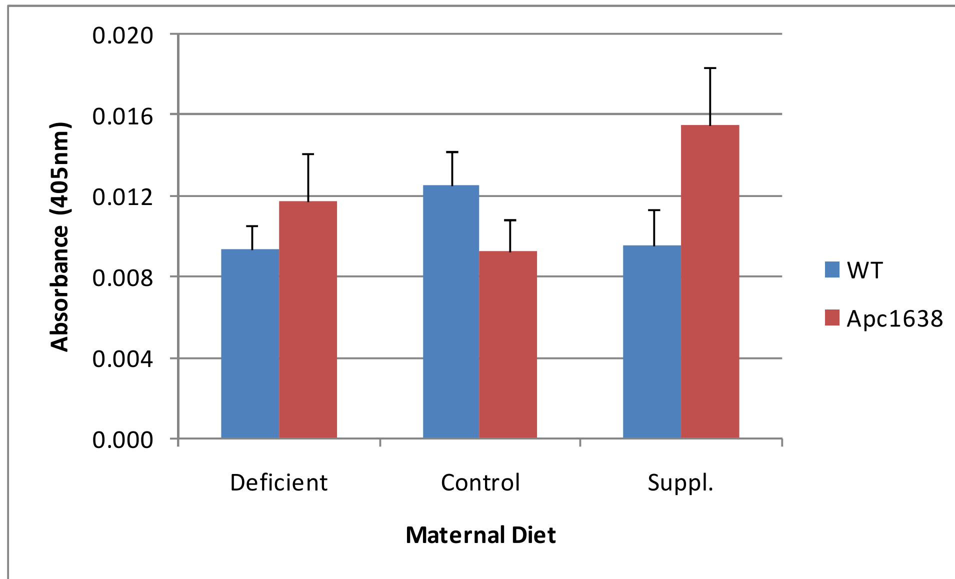

Figure 10. Effect of maternal diet on apoptosis measured by caspase-3 activity in colonic scrapings from wild type and *Apc*$^{+/1638N}$ pups.
Wild type (WT) p=0.33; Apc$^{+/1638N}$ p=0.23. Mean ± SEM.

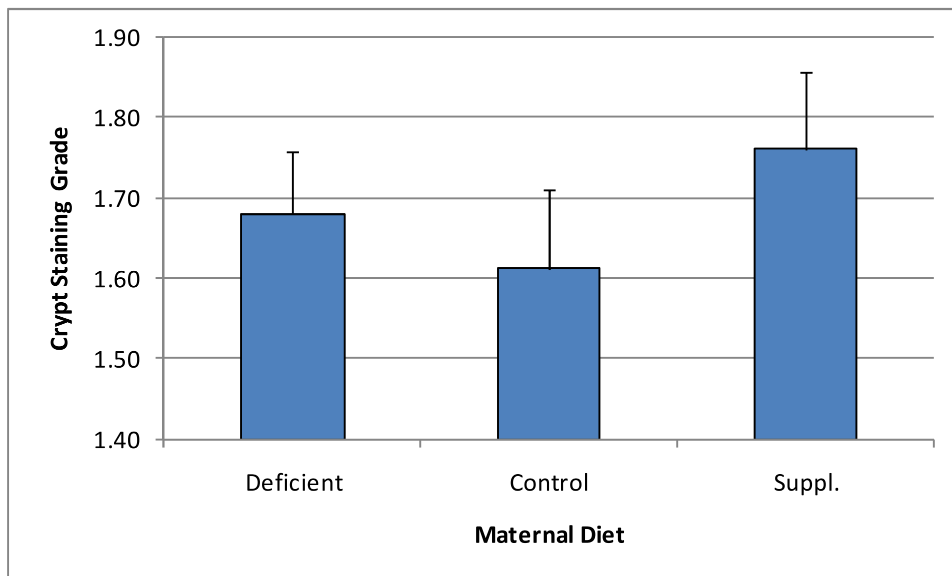

Figure 11. Effect of maternal diet on proliferation in colonic crypts in 3-week old wild type offspring.
ANOVA P_{diet}=0.52. Mean ± SEM.

VII. Bibliography

1. Wei EK, Giovannucci E, Selhub J, Fuchs CS, Hankinson SE, Ma J. Plasma vitamin B6 and the risk of colorectal cancer and adenoma in women. *J Natl Cancer Inst.* 2005; 97(9): 684-692.
2. Larsson SC, Giovannucci E, Wolk A. Vitamin B6 intake, alcohol consumption, and colorectal cancer: a longitudinal population-based cohort of women. *Gastroenterology.* 2005; 128(7): 1830-1837.
3. Zhang SM, Moore SC, Lin J, et al. Folate, vitamin B6, multivitamin supplements, and colorectal cancer risk in women. *Am J Epidemiol.* 2006; 163(2): 108-115.
4. Lee JE, Li H, Giovannucci E, et al. Prospective study of plasma vitamin B6 and risk of colorectal cancer in men. *Cancer Epidemiol Biomarkers Prev.* 2009; 18(4): 1197-1202.
5. James SJ, Basnakian AG, Miller BJ. In vitro folate deficiency induces deoxynucleotide pool imbalance, apoptosis, and mutagenesis in Chinese hamster ovary cells. *Cancer Res.* 1994; 54(19): 5075-5080.
6. Melnyk S, Pogribna M, Miller BJ, Basnakian AG, Pogribny IP, James SJ. Uracil misincorporation, DNA strand breaks, and gene amplification are associated with tumorigenic cell transformation in folate deficient/repleted Chinese hamster ovary cells. *Cancer Lett.* 1999; 146(1): 35-44.
7. Crott JW, Mashiyama ST, Ames BN, Fenech MF. Methylenetetrahydrofolate reductase C677T polymorphism does not alter folic acid deficiency-induced uracil incorporation into primary human lymphocyte DNA in vitro. *Carcinogenesis.* 2001; 22(7): 1019-1025.
8. Duthie SJ, Hawdon A. DNA instability (strand breakage, uracil misincorporation, and defective repair) is increased by folic acid depletion in human lymphocytes in vitro. *FASEB J.* 1998; 12(14): 1491-1497.
9. Koury MJ, Horne DW, Brown ZA, et al. Apoptosis of late-stage erythroblasts in megaloblastic anemia: association with DNA damage and macrocyte production. *Blood.* 1997; 89(12): 4617-4623.
10. Blount BC, Mack MM, Wehr CM, et al. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. *Proc Natl Acad Sci U S A.* 1997; 94(7): 3290-3295.
11. Pogribny IP, Miller BJ, James SJ. Alterations in hepatic p53 gene methylation patterns during tumor progression with folate/methyl deficiency in the rat. *Cancer Lett.* 1997; 115(1): 31-38.
12. Reidy JA. Deoxyuridine increases folate-sensitive fragile site expression in human lymphocytes. *Am J Med Genet.* 1987; 26(1): 1-5.
13. Dianov GL, Timchenko TV, Sinitsina OI, Kuzminov AV, Medvedev OA, Salganik RI. Repair of uracil residues closely spaced on the opposite strands of plasmid DNA results in double-strand break and deletion formation. *Mol Gen Genet.* 1991; 225(3): 448-452.
14. Jacob RA, Gretz DM, Taylor PC, et al. Moderate folate depletion increases plasma homocysteine and decreases lymphocyte DNA methylation in postmenopausal women. *J Nutr.* 1998; 128(7): 1204-1212.

15. Pogribny IP, Basnakian AG, Miller BJ, Lopatina NG, Poirier LA, James SJ. Breaks in genomic DNA and within the p53 gene are associated with hypomethylation in livers of folate/methyl-deficient rats. *Cancer Res.* 1995; 55(9): 1894-1901.
16. Liu Z, Choi SW, Crott JW, et al. Mild depletion of dietary folate combined with other B vitamins alters multiple components of the Wnt pathway in mouse colon. *J Nutr.* 2007; 137(12): 2701-2708.
17. Jhaveri MS, Wagner C, Trepel JB. Impact of extracellular folate levels on global gene expression. *Mol Pharmacol.* 2001; 60(6): 1288-1295.
18. Pogribny IP, James SJ. De novo methylation of the p16INK4A gene in early preneoplastic liver and tumors induced by folate/methyl deficiency in rats. *Cancer Lett.* 2002; 187(1-2): 69-75.
19. Motiwala T, Ghoshal K, Das A, et al. Suppression of the protein tyrosine phosphatase receptor type O gene (PTPRO) by methylation in hepatocellular carcinomas. *Oncogene.* 2003; 22(41): 6319-6331.
20. Liu Z, Choi SW, Crott JW, Smith DE, Mason JB. Multiple B-vitamin inadequacy amplifies alterations induced by folate depletion in p53 expression and its downstream effector MDM2. *Int J Cancer.* 2008; 123(3): 519-525.
21. Crott JW, Liu Z, Keyes MK, et al. Moderate folate depletion modulates the expression of selected genes involved in cell cycle, intracellular signaling and folate uptake in human colonic epithelial cell lines. *J Nutr Biochem.* 2008; 19(5): 328-335.
22. Mason JB, Choi SW, Liu Z. Other one-carbon micronutrients and age modulate the effects of folate on colorectal carcinogenesis. *Nutr Rev.* 2008; 66(s1): S15-S17.
23. Mason JB. Folate, cancer risk, and the Greek god, Proteus: a tale of two chameleons. *Nutr Rev.* 2009; 67(4): 206-212.
24. Song J, Sohn KJ, Medline A, Ash C, Gallinger S, Kim YI. Chemopreventive effects of dietary folate on intestinal polyps in Apc+/- Msh2-/- mice. *Cancer Res.* 2000; 60(12): 3191-3199.
25. Cole BF, Baron JA, Sandler RS, et al. Folic acid for the prevention of colorectal adenomas: a randomized clinical trial. *JAMA.* 2007; 297(21): 2351-2359.
26. Wu K, Platz EA, Willett WC, et al. A randomized trial on folic acid supplementation and risk of recurrent colorectal adenoma. *Am J Clin Nutr.* 2009; 90(6): 1623-1631.
27. Kim YI. Folate: a magic bullet or a double edged sword for colorectal cancer prevention? *Gut.* 2006; 55(10): 1387-1389.
28. Wen W, Shu XO, Potter JD, et al. Parental medication use and risk of childhood acute lymphoblastic leukemia. *Cancer.* 2002; 95(8): 1786-1794.
29. Thompson JR, Gerald PF, Willoughby MLN, Armstrong BK. Maternal folate supplementation in pregnancy and protection against acute lymphoblastic leukaemia in childhood: a case-control study. *Lancet.* 2001; 358(9297): 1935-1940.
30. Ross JA, Blair CK, Olshan AF, et al. Periconceptional vitamin useand leukemia risk in children with Down syndrome: a Children's Oncology Group study. *Cancer.* 2005; 104(2): 405-410.

31. Milne E, Royle JA, Miller M, et al. Maternal folate and other vitamin supplementation during pregnancy and risk of acute lymphoblastic leukemia in the offspring. *Int J Cancer.* 2010; 126(11): 2690-2699.
32. Preston-Martin S, Pogoda JM, Mueller BA, Holly EA, Lijinsky W, Davis RL. Maternal consumption of cured meats and vitamins in relation to pediatric brain tumors. *Cancer Epidemiol Biomarkers Prev.* 1996; 5(8): 599-605.
33. Preston-Martin S, Pogoda JM, Mueller BA, et al. Prenatal vitamin supplementation and risk of childhood brain tumors. *Int J Cancer.* 1998; 11 Suppl.: 17-22.
34. Bunin GR, Kuijten RR, Buckley JD, Rorke LB, Meadows AT. Relation between maternal diet and subsequent primitive neuroectodermal brain tumors in young children. *N Engl J Med.* 1993; 329(8): 536-541.
35. Bunin GR, Kuijten RR, Boesel CP, Buckley JD, Meadows AT. Maternal diet and risk of astrocytic glioma in children: a report from the Childrens Cancer Group (United States and Canada). *Cancer Causes Control.* 1994; 5(2): 177-187.
36. Lubin F, Farbstein H, Chetrit A, et al. The role of nutritional habits during gestation and child life in pediatric brain tumor etiology. *Int J Cancer.* 2000; 86(1): 139-143.
37. Sarasua S, Savitz DA. Cured and broiled meat consumption in relation to childhood cancer: Denver, Colorado (United States). *Cancer Causes Control.* 1994; 5(2): 141-148.
38. Orjuela MA, Titievsky L, Liu X, et al. Fruit and vegetable intake during pregnancy and risk for development of sporadic retinoblastoma. *Cancer Epidemiol Biomarkers Prev.* 2005; 14(6): 1433-1440.
39. Bunin GR, Meadows AT, Emanuel BS, Buckley JD, Woods WG, Hammond GD. Pre- and postconception factors associated with sporadic heritable and nonheritable retinoblastoma. *Cancer Res.* 1989; 49(20): 5730-5735.
40. Schuz J, Weihkopf T, Kaatsch P. Medication use during pregnancy and the risk of childhood cancer in the offspring. *Eur J Pediatr.* 2007; 166(5): 433-441.
41. Michalek AM, Buck GM, Nasca PC, Freedman AN, Baptiste MS, Mahoney MC. Gravid health status, medication use, and risk of neuroblastoma. *Am J Epidemiol.* 1996; 143(10): 996-1001.
42. Olshan AF, Smith JC, Bondy ML, Neglia JP, Pollock BH. Maternal vitamin use and reduced risk of neuroblastoma. *Epidemiology.* 2002; 13(5): 575-580.
43. Grupp SG, Greenberg ML, Ray JG, et al. Pediatric cancer rates after universal folic acid flour fortification in Ontario. *J Clin Pharmacol.* 2011; 51(1): 60-65.
44. French AE, Grant R, Weitzman S, et al. Folic acid food fortification is associated with a decline in neuroblastoma. *Clin Pharmacol Ther.* 2003; 74(3): 288-294.
45. Goh YI, Bollano E, Einarson TR, Koren G. Prenatal multivitamin supplementation and rates of pediatric cancers: a meta-analysis. *Clin Pharmacol Ther.* 2007; 81(5): 685-691.
46. Skibola CF, Smith MT, Kane E, et al. Polymorphisms in the methylenetetrahydrofolate reductase gene are associated with susceptibility to acute leukemia in adults. *Proc Natl Acad Sci U S A.* 1999; 96(22): 12810-12815.
47. Lightfoot TJ, Johnston WT, Painter D, et al. Genetic variation in the folate metabolic pathway and risk of childhood leukemia. *Blood.* 2010; 115(19): 3923-3929.

48. Banelli B, Di Vinci A, Gelvi I, et al. DNA methylation in neuroblastic tumors. *Cancer Lett.* 2005; 228(1-2): 37-41.
49. Garcia-Manero G, Yang H, Kuang SQ, O'Brien S, Thomas D, Kantarjian H. Epigenetics of acute lymphocytic leukemia. *Semin Hematol.* 2009; 46(1): 24-32.
50. Sie K, Medline,A., Sohn,K-J., Croxford,R., Kim, Y-I. *The effect of in utero folic acid supplementation on colorectal cancer risk in the offspring in a chemical carcinogen model.* in *American Association for Cancer Research Annual Meeting*. 2008. San Diego, CA.
51. Lawrance AK, Deng L, Rozen R. Methylenetetrahydrofolate reductase deficiency and low dietary folate reduce tumorigenesis in Apc min/+ mice. *Gut.* 2009; 58(6): 805-811.
52. McKay JA, Williams EA, Mathers JC. Gender-specific modulation of tumorigenesis by folic acid supply in the Apc mouse during early neonatal life. *Br J Nutr.* 2008; 99(3): 550-558.
53. Wolff GL, Kodell RL, Moore SR, Cooney CA. Maternal epigenetics and methyl supplements affect agouti gene expression in Avy/a mice. *FASEB J.* 1998; 12(11): 949-957.
54. Waterland RA, Jirtle RL. Transposable elements: targets for early nutritional effects on epigenetic gene regulation. *Mol Cell Biol.* 2003; 23(15): 5293-5300.
55. Yen T, Gill A, Frigeri L, Barsh G, Wolff G. Obesity, diabetes, and neoplasia in yellow A(vy)/- mice: ectopic expression of the agouti gene. *FASEB J.* 1994; 8(8): 479-488.
56. Wolff GL, Roberts DW, Morrissey RL, et al. Tumorigenic responses to lindane in mice: potentiation by a dominant mutation. *Carcinogenesis.* 1987; 8(12): 1889-1897.
57. Waterland RA, Dolinoy DC, Lin JR, Smith CA, Shi X, Tahiliani KG. Maternal methyl supplements increase offspring DNA methylation at Axin Fused. *Genesis.* 2006; 44(9): 401-406.
58. Sakatani T, Kaneda A, Iacobuzio-Donahue CA, et al. Loss of imprinting of Igf2 alters intestinal maturation and tumorigenesis in mice. *Science.* 2005; 307(5717): 1976-1978.
59. Cheng YW, Idrees K, Shattock R, et al. Loss of imprinting and marked gene elevation are 2 forms of aberrant IGF2 expression in colorectal cancer. *Int J Cancer.* 2010; 127(3): 568-577.
60. Heijmans BT, Tobi EW, Stein AD, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci U S A.* 2008; 105(44): 17046-17049.
61. Tobi EW, Lumey LH, Talens RP, et al. DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. *Hum Mol Genet.* 2009; 18(21): 4046-4053.
62. Steegers-Theunissen RP, Obermann-Borst SA, Kremer D, et al. Periconceptional maternal folic acid use of 400 microg per day is related to increased methylation of the IGF2 gene in the very young child. *PLoS One.* 2009; 4(11): e7845.
63. Waterland RA, Lin JR, Smith CA, Jirtle RL. Post-weaning diet affects genomic imprinting at the insulin-like growth factor 2 (Igf2) locus. *Hum Mol Genet.* 2006; 15(5): 705-716.

64. Kovacheva VP, Mellott TJ, Davison JM, et al. Gestational choline deficiency causes global and Igf2 gene DNA hypermethylation by up-regulation of Dnmt1 expression. *J Biol Chem.* 2007; 282(43): 31777-31788.
65. Klaus A, Birchmeier W. Wnt signalling and its impact on development and cancer. *Nat Rev Cancer.* 2008; 8(5): 387-398.
66. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell.* 1996; 87(2): 159-170.
67. Fearon E, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell.* 1990; 61(5): 759-767.
68. Arnold CN, Goel A, Niedzwiecki D, et al. APC promoter hypermethylation contributes to the loss of APC expression in colorectal cancers with allelic loss on 5q. *Cancer Biol Ther.* 2004; 3(10): 960-964.
69. Ying Y, Tao Q. Epigenetic disruption of the WNT/beta-catenin signaling pathway in human cancers. *Epigenetics.* 2009; 4(5): 307-312.
70. Suzuki H, Watkins DN, Jair K-W, et al. Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. *Nat Genet.* 2004; 36(4): 417-422.
71. Kim YI, Shirwadkar S, Choi SW, Puchyr M, Wang Y, Mason JB. Effects of dietary folate on DNA strand breaks within mutation-prone exons of the p53 gene in rat colon. *Gastroenterology.* 2000; 119(1): 151-161.
72. Liu Z, Ciappio ED, Crott JW, Brooks RS, Mason JB. Mild inadequacy in multiple one-carbon vitamins elevates Wnt-signaling and promotes intestinal tumorigenesis in the BAT-LacZxApc1638N mouse model. *FASEB J.* 2010; 24(1_MeetingAbstracts): lb382-.
73. Eussen SJ, Vollset SE, Igland J, et al. Plasma folate, related genetic variants, and colorectal cancer risk in EPIC. *Cancer Epidemiol Biomarkers Prev.* 2010; 19(5): 1328-1340.
74. Giovannucci E, Rimm EB, Ascherio A, Stampfer MJ, Colditz GA, Willett WC. Alcohol, low-methionine--low-folate diets, and risk of colon cancer in men [see comments]. *J Natl Cancer Inst.* 1995; 87(4): 265-273.
75. Baron JA, Sandler RS, Haile RW, Mandel JS, Mott LA, Greenberg ER. Folate intake, alcohol consumption, cigarette smoking, and risk of colorectal adenomas. *J Natl Cancer Inst.* 1998; 90(1): 57-62.
76. Kim DH, Smith-Warner SA, Spiegelman D, et al. Pooled analyses of 13 prospective cohort studies on folate intake and colon cancer. *Cancer Causes Control.* 2010.
77. Kim YI, Salomon RN, Graeme-Cook F, et al. Dietary folate protects against the development of macroscopic colonic neoplasia in a dose responsive manner in rats. *Gut.* 1996; 39(5): 732-740.
78. Cravo ML, Mason JB, Dayal Y, et al. Folate deficiency enhances the development of colonic neoplasia in dimethylhydrazine-treated rats. *Cancer Res.* 1992; 52(18): 5002-5006.
79. Arasaradnam RP, Commane DM, Bradburn D, Mathers JC. A review of dietary factors and its influence on DNA methylation in colorectal carcinogenesis. *Epigenetics.* 2008; 3(4): 193-198.

80. Barker DJ. The developmental origins of adult disease. *J Am Coll Nutr.* 2004; 23(6 Suppl): 588S-595S.
81. Li E. Chromatin modification and epigenetic reprogramming in mammalian development. *Nat Rev Genet.* 2002; 3(9): 662-673.
82. Baethmann M, Wendel U, Hoffmann GF, et al. Hydrocephalus internus in two patients with 5,10- methylenetetrahydrofolate reductase deficiency. *Neuropediatrics.* 2000; 31(6): 314-317.
83. Thompson JR, Gerald PF, Willoughby ML, Armstrong BK. Maternal folate supplementation in pregnancy and protection against acute lymphoblastic leukaemia in childhood: a case-control study. *Lancet.* 2001; 358(9297): 1935-1940.
84. Cooney CA, Dave AA, Wolff GL. Maternal methyl supplements in mice affect epigenetic variation and DNA methylation of offspring. *J Nutr.* 2002; 132(8 Suppl): 2393S-2400S.
85. March-of-Dimes, *Improving Preconception Health: Women's knowledge and Use of Folic Acid*. 2008, March of Dimes Foundation.: White Plains, NY.
86. Henderson L IK, Gregory J. National Diet and Nutrition Survey: adults aged 19-64. Volume 4. http://www.food.gov.uk/multimedia/pdfs/ndnsfour.pdf. 2004.
87. Planells E, Sanchez C, Montellano MA, Mataix J, Llopis J. Vitamins B6 and B12 and folate status in an adult Mediterranean population. *Eur J Clin Nutr.* 2003; 57(6): 777-785.
88. Lindenbaum J, Rosenberg IH, Wilson PW, Stabler SP, Allen RH. Prevalence of cobalamin deficiency in the Framingham elderly population. *Am J Clin Nutr.* 1994; 60(1): 2-11.
89. Fodde R, Edelmann W, Yang K, et al. A targeted chain-termination mutation in the mouse Apc gene results in multiple intestinal tumors. *Proc Natl Acad Sci U S A.* 1994; 91(19): 8969-8973.
90. O'Broin S, Kelleher B. Microbiological assay on microtitre plates of folate in serum and red cells. *J Clin Pathol.* 1992; 45(4): 344-347.
91. Camp VM, Chipponi J, Faraj BA. Radioenzymatic assay for direct measurement of plasma pyridoxal 5'- phosphate. *Clin Chem.* 1983; 29(4): 642-644.
92. Nichoalds GE. Assessment of status riboflavin nutriture by assay of erythrocyte glutathione reductase activity. *Clin Chem.* 1974; 20(5): 624-628.
93. Friso S, Choi SW, Dolnikowski GG, Selhub J. A method to assess genomic DNA methylation using high-performance liquid chromatography/electrospray ionization mass spectrometry. *Anal Chem.* 2002; 74(17): 4526-4531.
94. Samuel MS, Suzuki H, Buchert M, et al. Elevated Dnmt3a activity promotes polyposis in Apc(Min) mice by relaxing extracellular restraints on Wnt signaling. *Gastroenterology.* 2009; 137(3): 902-913, 913 e901-911.
95. McKay JA, Williams EA, Mathers JC. Gender-specific modulation of tumorigenesis by folic acid supply in the Apc mouse during early neonatal life. *Br J Nutr.* 2007: 1-9.
96. Anzman SL, Rollins BY, Birch LL. Parental influence on children's early eating environments and obesity risk: implications for prevention. *Int J Obes (Lond).* 2010; 34(7): 1116-1124.

97. Cravo M, Fidalgo P, Pereira AD, et al. DNA methylation as an intermediate biomarker in colorectal cancer: modulation by folic acid supplementation. *Eur J Cancer Prev*. 1994; 3(6): 473-479.
98. Friso S, Choi SW, Girelli D, et al. A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. *Proc Natl Acad Sci U S A*. 2002; 99(8): 5606-5611.
99. Rodriguez J, Frigola J, Vendrell E, et al. Chromosomal instability correlates with genome-wide DNA demethylation in human primary colorectal cancers. *Cancer Res*. 2006; 66(17): 8462-9468.
100. Guttenbach M, Schmid M. Exclusion of specific human chromosomes into micronuclei by 5-azacytidine treatment of lymphocyte cultures. *Exp Cell Res*. 1994; 211(1): 127-132.
101. Duesberg P, Rasnick D, Li R, Winters L, Rausch C, Hehlmann R. How aneuploidy may cause cancer and genetic instability. *Anticancer Res*. 1999; 19(6A): 4887-4906.
102. Pino MS, Chung DC. The chromosomal instability pathway in colon cancer. *Gastroenterology*. 2010; 138(6): 2059-2072.
103. Caldwell GM, Jones CE, Ashley AM, et al. Wnt signalling in adenomas of familial adenomatous polyposis patients. *Br J Cancer*. 2010; 103(6): 910-917.
104. Hu J, Dong A, Fernandez-Ruiz V, et al. Blockade of Wnt signaling inhibits angiogenesis and tumor growth in hepatocellular carcinoma. *Cancer Res*. 2009; 69(17): 6951-6959.
105. Mahmoud NN, Bilinski RT, Churchill MR, Edelmann W, Kucherlapati R, Bertagnolli MM. Genotype-phenotype correlation in murine Apc mutation: differences in enterocyte migration and response to sulindac. *Cancer Res*. 1999; 59(2): 353-359.
106. Smits R, Kartheuser A, Jagmohan-Changur S, et al. Loss of Apc and the entire chromosome 18 but absence of mutations at the Ras and Tp53 genes in intestinal tumors from Apc1638N, a mouse model for Apc-driven carcinogenesis. *Carcinogenesis*. 1997; 18(2): 321-327.
107. Yang WC, Mathew J, Velcich A, et al. Targeted inactivation of the p21(WAF1/cip1) gene enhances Apc-initiated tumor formation and the tumor-promoting activity of a Western-style high-risk diet by altering cell maturation in the intestinal mucosal. *Cancer Res*. 2001; 61(2): 565-569.
108. Yang K, Edelmann W, Fan K, et al. Dietary modulation of carcinoma development in a mouse model for human familial adenomatous polyposis. *Cancer Res*. 1998; 58(24): 5713-5717.
109. Zhang MQ, Chen ZM, Wang HL. Immunohistochemical investigation of tumorigenic pathways in small intestinal adenocarcinoma: a comparison with colorectal adenocarcinoma. *Mod Pathol*. 2006; 19(4): 573-580.
110. *Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline*. 2000, Washington, DC: National Academy Press.
111. Mcmillen IC, Robinson JS. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. *Physiol Rev*. 2005; 85(2): 571-633.

112. Jones PA, Baylin SB. The Epigenomics of Cancer. *Cell.* 2007; 128(4): 683-692.
113. Crott JW, Liu Z, Choi SW, Mason JB. Folate depletion in human lymphocytes up-regulates p53 expression despite marked induction of strand breaks in exons 5-8 of the gene. *Mutat Res.* 2007; 626(1-2): 171-179.
114. Kim YI, Pogribny IP, Basnakian AG, et al. Folate deficiency in rats induces DNA strand breaks and hypomethylation within the p53 tumor suppressor gene. *Am J Clin Nutr.* 1997; 65(1): 46-52.
115. Xu Y, Pasche B. TGF-Beta signaling alterations and susceptibility to colorectal cancer. *Hum Mol Genet.* 2007; 16(R1): R14-R20.
116. Bierie B, Moses HL. Transforming growth factor beta (TGF-β) and inflammation in cancer. *Cytokine Growth Factor Rev.* 2010; 21(1): 49-59.
117. Munoz NM, Upton M, Rojas A, et al. Transforming Growth Factor Beta Receptor Type II inactivation induces the malignant transformation of intestinal neoplasms initiated by Apc mutation. *Cancer Res.* 2006; 66(20): 9837-9844.
118. Rojas A, Meherem S, Kim Y-H, et al. The aberrant methylation of TSP1 suppresses TGF-β1 activation in colorectal cancer. *Int J Cancer.* 2008; 123(1): 14-21.
119. Aitchison AA, Veerakumarasivam A, Vias M, et al. Promoter methylation correlates with reduced Smad4 expression in advanced prostate cancer. *Prostate.* 2008; 68(6): 661-674.
120. Hinshelwood RA, Huschtscha LI, Melki J, et al. Concordant epigenetic silencing of transforming growth factor-β signaling pathway genes cccurs early in breast carcinogenesis. *Cancer Res.* 2007; 67(24): 11517-11527.
121. Shah J, Shao G, Hei T, Zhao Y. Methylation screening of the TGFBI promoter in human lung and prostate cancer by methylation-specific PCR. *BMC Cancer.* 2008; 8(1): 284.
122. Crott JW, Choi SW, Ordovas JM, Ditelberg JS, Mason JB. Effects of dietary folate and aging on gene expression in the colonic mucosa of rats: implications for carcinogenesis. *Carcinogenesis.* 2004; 25(1): 69-76.
123. Feinberg AP, Gehrke CW, Kuo KC, Ehrlich M. Reduced genomic 5-methylcytosine content in human colonic neoplasia. *Cancer Res.* 1988; 48(5): 1159-1161.
124. Goelz SE, Vogelstein B, Hamilton SR, Feinberg AP. Hypomethylation of DNA from benign and malignant human colon neoplasms. *Science.* 1985; 228(4696): 187-190.
125. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R. DNA hypomethylation leads to elevated mutation rates. *Nature.* 1998; 395(6697): 89-93.
126. Hoffmann MJ, Schulz WA. Causes and consequences of DNA hypomethylation in human cancer. *Biochem Cell Biol.* 2005; 83(3): 296-321.
127. Kim YI. Folate, colorectal carcinogenesis, and DNA methylation: Lessons from animal studies. *Environ Mol Mutagen.* 2004; 44(1): 10-25.
128. Lai C-H, Liu Z, Chen C-Y, Crott J. Induction of genomic DNA hypomethylation in the offspring of rats consuming diets mildly deficient in vitamin B2, B6, B12 and folate. *AACR Meeting Abstracts.* 2008; 2008(1_Annual_Meeting): 3061-.
129. Holt PR, Yeh KY. Small intestinal crypt cell proliferation rates are increased in senescent rats. *J Gerontol.* 1989; 44(1): B9-14.

130. Kim K, Pang KM, Evans M, Hay ED. Overexpression of beta-catenin Induces apoptosis independent of its transactivation function with LEF-1 or the involvement of major G1 cell cycle regulators. *Mol Biol Cell.* 2000; 11(10): 3509-3523.