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Effect Of Dietary Folate Restriction On Colon Carcinogenesis In Dna Polymerase β Haploinsufficient Mice

Lisa F. Ventrella Lucente
Wayne State University

EFFECT OF DIETARY FOLATE RESTRICTION ON COLON CARCINOGENESIS IN DNA POLYMERASE β HAPLOINSUFFICIENT MICE

by

LISA VENTRELLA LUCENTE

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DEDICATION

It was with the help and support of many people that I was able to complete this dissertation. Words cannot express my thanks to my husband, Joe and children Joey, Luka and Max for their love and support over these past few years. It has been quite the journey and each one of you was instrumental in my successes. To my mother and father, Frank and Rosemary Ventrella, my brother Michael and sister, Christina, thank you for instilling the mindset to achieve a goal and for your constant support (I know, you have no choice). Lastly, I'd like to thank my advisor and mentor, Dr Ahmad Heydari, for without his guidance and support, this dissertation would not have been possible.

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CHAPTER 1

Introduction:

Folate, a water-soluble B vitamin, has emerged as an important nutritional element in the pathogenesis of several malignancies, including cancers of the colorectum, lung, pancreas, esophagus, stomach, cervix, and breast, as well as neuroblastoma and leukemia. Studies have collectively implied an inverse association between dietary and serum folate and the risk of malignancy (1, 2). The most convincing relates to colorectal cancer (3, 4).

Folate Digestion, Absorption and Metabolism

Folate is the naturally occurring form of the vitamin found in foods, which have similar nutritional properties of folic acid (5, 6). The U.S. RDA for folate is 400 micrograms per day for nonpregnant ages 19 to 50 years. Folic acid (aka pteroylmonoglutamic acid), is rarely found in natural food items but is the source used in the fortification of foods and supplements (7). Dietary folate is only about 50% bioavailable (8), whereas folic acid is 85% bioavailable (9). Bioavailablity is dependent on the length of the polyglutamate chain to which most of the natural folate is attached. Digestion of folates begins in the small intestine. Initially, folylpoly-glutamate carboxypeptidase, a brush border enzyme, removes dietary folate polyglutamates up to their corresponding monoglutamate derivatives, 5-methyltetrahydrofolic acid and 5-formyltetrahydrofolic acid, through removal of glutamate residues. These monoglutamates, which now carry a reduced negative charge, are subsequently actively absorbed, mainly in the jejunum, via the reduced folate carrier (RFC) or the protein-coupled folate transporter and

converted to tetrahydrofolic acid (THF) by dihyrofolate reductase (DHFR), an NADPH-requiring enzyme. THF is subsequently converted to 5-methyl-THF and then transferred to the portal circulation which are taken up by the liver or converted to 5-methyl-THF, before entering the plasma. From the plasma, specific carriers (RFC) or receptors (folate receptors **α** or **β**) transport 5-methyl-THF into cells. 5-methyl-THF undergoes polyglutamation altering the properties of the molecule such that transportion through the membrane is inhibited and causing an accumulation in the liver and cells. Enzymes involved in folate metabolism have a higher affinity for polyglutamate forms of folate. Many of the inter-conversions of folates are oxidation-reduction reactions that utilize NADH, NADPH and ATP. It should be noted that folates may also be absorbed through the colon due to microbial folate production. Here the absorption rate is estimated to be ~50 times lower than in the small intestine; however, it may contribute significantly to total folate absorption (10).

Folate in one carbon metabolism

Folate has an essential role as a one carbon donor in DNA metabolism (Fig. 1) (11). It functions as a methyl donor through 5-methyltetrahydrofolate, remethylating homocysteine to methionine, which in turn is converted to *S*-adenosylmethionine (SAM), the primary intracellular methyl donor. Subsequently, SAM methylates specific cytosines in DNA and this regulates gene transcription. In addition, folate through 5,10 methylene THF, donates a methyl group to uracil (U) converting it to thymine, crucial for DNA synthesis and

repair (12). 5,10 methylene THF can be oxidized to form 10-formyl-THF for

denovo synthesis of purines (13, 14).
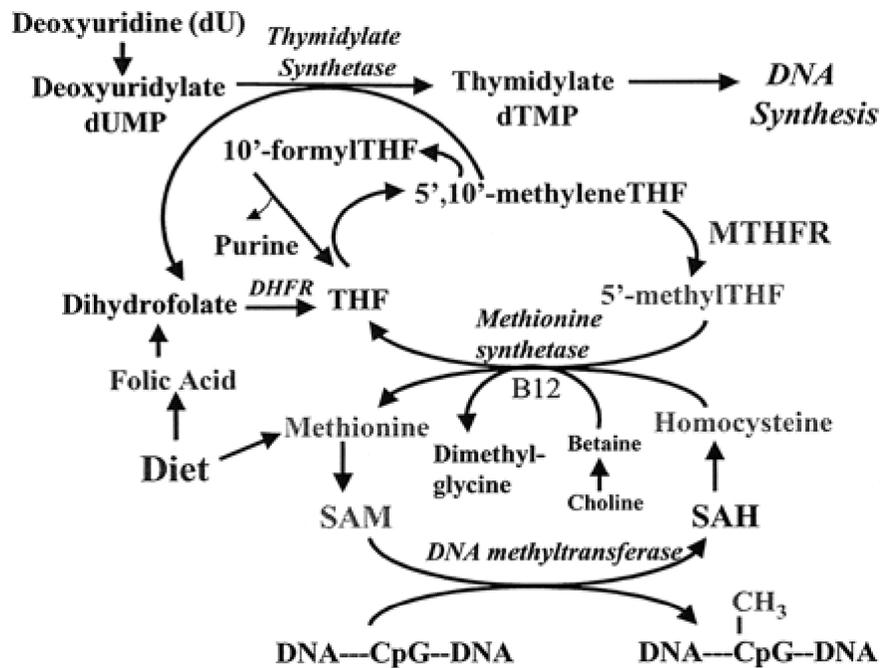
Figure 1.1

Folate and disease

Since the installation of folate fortification of cereal grains by the Food and

Drug Administration, to eradicate NTDs, median RBC folate levels of women of

childbearing age increased 65%, from 160 ng/mL to 264 ng/mL between 1988

to1994 and 1999 to 2000. The NHANES 2005 to 2006 value is 257 ng/mL. In

addition, the prevalence of low RBC folate among U.S. women of childbearing

age declined from 37.6% in 1988 to 1994 to 5.1% in 1999 to 2000. The 2005 to

2006 value was 4.5% (15). Honein et al. (2001)(16), report a 19% reduction in

NTD birth prevalence following folic acid fortification of the US food supply.

Despite this massive effort, however, there remains a high incidence of diseases

like CVD, stroke, Alzheimer's disease, depression and cancer. It is suggested that these disease states continue to prevail potentially as a result of genetic disorders of enzymes involved in folate metabolism, increasing age, and alcohol useage.

Aberrations in folate metabolism include polymorphisms in a number of essential genes related to this pathway. One example is that of the RFC, as previously described, whose variations can give rise to a variety of cancers (17, 18 and 19). As well, mutations in the Folate receptor-alpha (FR-alpha) gene which binds and transports folates, namely 5-methylTHF, into cells have been documented. Polymorphisms in this gene are said to contribute to hyperhomocysteinemia (20) increasing the risk of disease. Methylenetetrahydrofolate reductase (MTHFR) is an enzyme responsible for the conversion of 5,10-methyleneTHF to 5-methylTHF, a cosubstrate for the remethylation of homocysteine to methionine. Two well studied polymorphisms of this gene include MTHFR C677T, which is a C→T variation at nucleotide 677 (C677T), as a results of alanine to valine substitution in the MTHFR protein and MTHFR A1298C (A→C; glutamic acid substitution to alanine). Both polymorphisms lead to reduced MTHFR activity, resulting in the accumulation of 5,10-methylene THF and a decrease of 5-methyl THF (21) contributing to hyperhomocysteinemia. Other genetic variations include serine hydroxymethyltransferase (SHMT) (C→T substitution), which catalyzes the reversible conversion of serine and THF to glycine and methylene THF, thus providing one carbon units for synthesis of methionine, thymidylate and purine in

the cytoplasm, and methionine synthase (MS, A→G substitution) which catalyzes the transfer of methyl base from 5-methyl THF to homocysteine, producing methionine and tetrahydrofolate. The aforementioned is critical for maintenanace of SAM levels for DNA methylation and safeguarding of homocysteine levels. SHMT polymorphisms have been observed in ovarian (22), lung (23) and squamous cell carcinoma of the head and neck (24). Additionally polymorphisms are seen in variable number tandem repeats (VNTRs) for thymidylate synthase which catalyzes the conversion of dUMP to dTMP and thus plays a critical role in maintaining a balanced supply of deoxynucleotides required for DNA synthesis.

Aging has profound effects on 1-carbon metabolism, discernible by hyperhomocysteinemia, genomic DNA hypomethylation, and impact on DNA repair systems. Achlorhydria, which is a lack of hydrochloric acid in digestive juices, is a common physiological change that occurs with aging (25). A pH < 7 is required for optimal absorption of folate monoglutamate (26), as such, achlorhydria can result in reduced folic acid absorption and folate depletion (25, 27). Ethanol associated folate deficiency can develop because of poor dietary practices, intestinal malabsorption, altered hepatobiliary metabolism, enhanced colonic metabolism, and increased renal excretion of folate secondary to alcohol consumption. Ethanol reduces the intestinal and renal uptake of folate by changing the binding and transport kinetics of folate transport systems. Also, ethanol may lead to a folate deficiency as a result of reduced expression of folate transporters in both intestine and kidney contributing to folate malabsorption (28).

Folate Deficiency and Cancer

Over the last several decades' research studies have indicated an inverse association of low dietary folate intake and the development of certain cancers such as those of the esophagus, stomach, breast, leukemia, pancreas and others with the evidence most compelling for colon cancer. Larsson et al.(29), conducted a meta-analysis for studies published through March 2006 which supported low dietary folate intake with increased risk of esophageal squamous cell carcinoma, esophageal adenocarcinoma and pancreas, cancers that were 40%–50% less likely to develop when dietary folate intake was high. Most, although not all, showed an increased risk of these cancers with the MTHFR 677TT genotype, which disrupts folate metabolism. Others have shown, in mucosal tissue samples from patients with gastric cancer, a lower folate concentration, detected by the FOL ACS:180 automated chemiluminescence system, then in controls (30). Likewise, Gao et al. (31) observed a significant inverse relationship in 669 breast cancer cases between folate intake and breast cancer risk. Of interest, Giovanucci et al. (32), in their study of 88 756 women from the Nurses' Health Study showed a reduced risk of development of colon cancer with long term high folate intake. In addition, animal studies have demonstrated time constraint for folate supplementation with initiation prior to the establishment of neoplastic foci essential for suppression of development and progression of cancer. If supplementation coincides with preexisting foci formation, this B-vitamin can enhance the growth of these preneoplastic lesions

(33, 34 and 35). Lindzon et al. (2009)(36) in their male Sprague-Dawley rats showed an increase in progression of colonic preneoplastic lesions to colon tumors when folic acid was supplemented six weeks post-ACF induction.

It is believed that the mechanism of folates protection may be a result of the maintenance of normal DNA synthesis and DNA methylation (37). If folate is deficient, 5-methylTHF will not be sufficient to provide the methyl group to methylate homocysteine to methionine, SAM will become depleted. This would give rise to abnormal metabolism of methyl groups which impacts DNA methylation, a cellular process that may be detrimental in neoplastic transformation (38). Atypical methylation of cytosine residues of cytosine-guanine dinucleotide pairs impacts epigenetic gene expression and also has a role in maintaining DNA stability. Hypermethylation of promoter regions has the ability to silence tumor suppressor genes, which inhibit uncontrolled cell growth that could lead to the formation of tumors. In contrast, global hypomethylation of protoncogenes may cause their overexpression, a root for the transformation of normal cells into cancer cells via chromosomal instability and increase mutational frequency (39). As well, if cellular folate becomes deficient DNA stability may be reduced by a disruption in the nucleotide pool, negatively altering DNA synthesis and repair. Reduced synthesis of thymidylate from the imbalances in the deoxyribonucleotide pool results in massive uracil misincorporation into the DNA, leading to "a catastrophic repair cycle". This may lead to DNA strand breaks, deletions, chromosomal breaks, micronucleus formation and loss of heterozygosity (40, 41, 41). In contrast, folate supplementation after

development of preneoplastic lesions maybe attributed to folate's tumor promoting effect (43). Cancer cells are cells that are categorized as having rapid levels of DNA replication and cell division occurring. Folate once again is the cofactor in the synthesis of nucleic acids and is required for DNA repair, which would only facilitate the growth of preneoplastic cells. If one considers the basis of use of antifolates in the treatment of certain cancers, it would only seem practical to recognize that a reduction of folate would cause inhibition of tumor growth.

The Base Excision Repair Pathway (BER)

BER is the predominant pathway in mammalian cells for repair of small DNA base lesions and abasic sites which arise spontaneously as a result of alkylation, oxidation and deamination events (44, 45, 46). It is estimated that BER is responsible for the repair of as many as one million nucleotides per cell per day (47). In general, the BER pathway involves the following steps: i) DNA glycosylase enzymes remove damaged nucleic acid bases (purines or pyrimidines), ii) Endonuclease enzyme cleaves the phosphodiester backbone, iii) synthesis of new DNA by a DNA polymerase using the complementary strand as a template, iv) excision of the 5'-deoxyribose phosphate (dRp) terminus, v) recreation of the phosphodiester bond by a ligase (48). Two subpathways of BER have been recognized: 'short patch' which is responsible for the replacement of a damaged base with a single new nucleotide, and the 'long patch' which results in the replacement of approximately 2-10 nucleotides

Mechanisms of Base Excision Repair Pathway
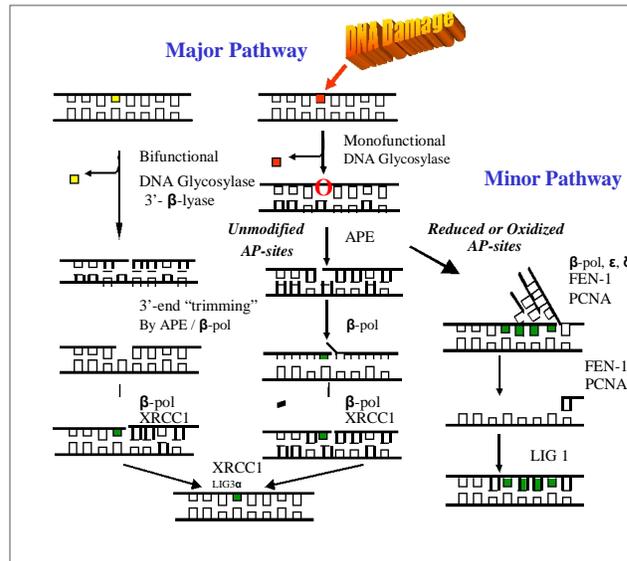
Figure 1.2

including the damaged base (49). The 'short patch repair pathway is the dominant pathway representing approximately 75-90% of all BER (50).

'Short-patch' base excision repair: The short-patch repair pathway is a DNA polymerase ß-dependent pathway (51). There are two types of glycosylases, monofunctional and bifunctional. In monofunctional glycosylase-initiated BER, a damaged or improper base is recognized and removed by enzymatic hydrolysis of the *N*-glycosyl bond creating an AP site. This AP site serves as a substrate for the endonuclease APE, which nicks the backbone directly 5' of the AP site, creating a single strand break with a normal 3'-hydroxyl group and an abnormal 5'dRP terminus. **β** -pol then inserts a new base followed by the excision of the of the abnormal 5'dRP (52). This latter step has proven to

be rate limiting (53). In bifunctional glycosylase initiated BER, a DNA specific glycosylase recognizes the damaged base, excises it and makes an incision in the backbone with its associated AP lyase activity, yielding a normal 5'-terminal deoxynucleoside-5'-phosphate residue and an abnormal 3'-terminal **α,β**-unsaturated aldehyde residue that must be processed prior to completion (53). By virtue of its 3'phosphodiesterase activity, Ape 1 performs this rate-limiting step that excises the deoxyribose fragments and phosphate groups at the 3' terminus of DNA (54). Completion of BER requires seal of the nicked phosphodiester bond, the majority of which is completed by a complex of XRCC1 and DNA ligase III (55).

'Long-patch' base excision repair: If damage is refractory to dRP moiety excision, then 'long patch' BER completes the repair. In this pathway, DNA polymerase **β** or e adds a few more nucleotides to the 3'-end of the nicked AP site (56) creating a flap that is removed by a flap endonuclease (FEN1) (57). The DNA ends are subsequently sealed by DNA ligase I (58). It has been shown that cells insufficient in **β** -pol were deficient in 'short-patch' BER, but proficient in 'long-patch' BER. This suggests that**β** -pol is the polymerase in the 'short-patch' BER, whereas DNA polymerase **β** or **ε** are the main polymerases of the 'long-patch' BER pathway (59).

The BER deficient mouse model

Based on **β**-pol role in the BER pathway on removal of uracil, alkylated and oxidized bases we decided to utilize the **β**-pol haploinsufficient mouse model to study the effects of folate deficiency in colon carcinogenesis. While

homozygous deletion of the **β**-pol gene in the mouse germ line results in lethality,

β-pol$^{+/-}$ heterozygote transgenic mice appears normal and fertile. We have

previously characterized the **β**-pol$^{+/-}$ mice as having a 40 to 50% reduction in

mRNA **β**-pol levels and a 50 to 60% decrease in **β**-pol protein levels.

Additionally, we have shown an increased accumulation of SSBs and

chromosomal aberrations that occur spontaneously in this animal model relative

to their WT littermates. These incidences are exacerbated with the inclusion of

oxidizing and alkylating agents and in addition demonstrate an increase in

mutational frequency (60). These happenings proved similar to the DNA damage

that is induced in folate deficiency making it plausible to further study the

connection between this repair pathway and folate.

Polymerase **β** is indispensible in 'short patch' repair for both its lyase and

gap filling. It appears that **β**-pol also has a role in 'long patch' repair by the

insertion of several nucleotides (61). Examples of base lesions repaired by BER

pathway include oxidized bases such as 8-oxoguanine, 2,6-diamino-4-hydroxy-5-

formamidopyrimidine, alkylated bases like 3-methyladenine, 7-methylguanine,

deaminated bases like hypoxanthine that arise from the deamination of adenine or

uracil formed by deamination of 5-methylcytosine or its misincorporated in DNA.

Although the 'short patch' repair pathway is the dominant pathway representing

approximately 75-90% of all BER (50), **β**-pol has an important role in both BER

pathways. Accordingly, removal of **β**-pol would be reasonable to study the BER

deficient model. We have previously (62) shown that WT animals exposed to

oxidative damage had increased BER and **β**-pol levels, whereas, **β**-

pol haploinsufficient mice, as previously mentioned, exhibited a greater occurrence of SSB than WT (60).

$\beta\beta$-pol$^{+/-}$ Mice as a Model for BER Polymorphisms

β-pol is a monomeric polypeptide of 335 amino acids consisting of two domains: an 8-kDa amino-terminal lyase domain and a 31-kDa active site carboxy-terminal polymerase domain (63, 64). The amino-terminal domain is involved in binding to single stranded DNA, recognition of the 5'-phosphate in gapped DNA, removal of the 5'-dRP group from AP endonuclease-incised DNA, and AP-site strand incision (65). The carboxy-terminal domain binds weakly to double stranded DNA and possess nucleotidyltransferase activity (63, 66). **β**-pol is located on the proximal region of the short arm of chromosome 8 (p12-p11) (67).

The Heydari lab has previously shown that **β**-pol$^{+/-}$ results in genomic instability (68). The **β**-pol haploinsufficient mouse has a reduced ability to tolerate carcinogen exposure, demonstrating reduced BER capacity and this translates into concern regarding the development of certain cancers. In fact, mutations in the gene encoding **β**-pol have been previously identified in human colorectal, prostate, lung and breast carcinomas (69, 70, 71, 72). These mutations are found only in the tumor tissue and not in the adjacent normal tissue, thus corresponding to sporadic mutations (73).

Studies have shown that approximately 30%of human tumors are characterized by mutations in **β**-pol including those of the colon, prostate, lung, breast and bladder (Starcevic et al, 2004). In particular those of the colon include

a **β**-pol single nucleotide polymorphism (SNP) where positively charged Lys-289 was substituted for a neutral Met (K289M). This variant leads to altered positioning of DNA within the K289M enzyme leading to misincorporation of nucleotides (74) during BER, lowering its fidelity. In Lang's study they utilized *cII* mutants from cells expressing K289M showing an increased frequency of C to G base substitutions within the AACAAA sequence. This polymorphism observed resembles the mutational hotspot within the APC gene, a gene that is frequently mutated in colon carcinoma. Wang et al. (75), observed this alteration in human colorectal carcinomas, a mutation found only in the tumor tissue and not in normal, surrounding mucosa (74). These abovementioned findings indicate that this variant could impact the cell through haploinsufficiency and modify risk for colon cancer. This SNP, along with others found in various tissues (I260M prostate carcinoma and E295K gastric carcinoma, (76)), confer that the **β**-pol haploinsufficient animal can serve as a model mimicking human polymorphisms.

We have previously shown that **β**-pol haploinsufficiency results in genomic instability (60). Furthermore, **β**-pol haploinsufficient mice display an acceleration of normal, age-related tumors, e.g., lymphomas, as well as increased susceptibility to epithelial tumors, such as adenocarcinomas, phenomenon that do not typically occur to a high degree in C57BL/6 mice (77). Consequently, previous experimental work done in our lab and others strengthens the cause for using a **β**- pol haploinsufficient mouse as a model to study CRC.

Folate deficiency and Base Excision Repair

While uracil is not a normal constituent of DNA it can arise in DNA during replication when dUMP:dTMP ratios are imbalanced. It may also arise as a result of the deamination of cytosine which is the hydrolysis reaction of cytosine into uracil. The frequency of cytosine deamination is elevated when SAM levels are reduced (78), as seen in folate deficiency. The DNA repair pathway primarily responsible for removal of uracil is the base excision repair (BER) pathway. Misincorporation of uracil, as seen in folate deficiency (79), constitutes a nucleotide that is normally repaired by the 'short-patch' BER pathway. Briefly, the removal of uracil by BER involves i) uracil-DNA glycosylase (UDG) binding specifically to the affected base and hydrolyzing the N-glycosidic bond, releasing the damaged base while keeping the DNA backbone intact. ii) The abasic site is subsequently recognized by an endonuclease, notably Ape 1, which nicks the phosphodiester backbone immediately 5' to the lesion leaving a strand break with a normal 3'-hydroxyl group and an abnormal 5'dRp residue. iii) synthesis of new DNA by incorporation of the correct nucleotide by DNA polymerase, iv) excision of the 5'dRp moiety, and v) ligation by a DNA ligase (Ligase 1 or Ligase 3/XCRR1 (1).

Evidence from our lab, as well as others, demonstrate a reduced ability to process DNA damage when folate deficiency is present. We have shown that folate deficient mice display an initiation of BER, as observed by an increase in uracil DNA glycosylase; however there is a stalling at the rate-limiting enzyme DNA polymerase **β**. Consequently, we observed an accumulation of DNA single strand breaks (80), which if left unrepaired could emerge as a DNA double strand

break. Similarly, Blount et al.(81) using folate deficient human erythrocytes, showed increased levels of uracil in DNA as well as, an increase in micronuclei formation. In addition, Branda et al. (82) have demonstrated that folate deficient Weanling Fischer 344 rats are less able to repair damages induced by alkylating agents. Although they show that folate deficiency on its own was mutagenic, in conjunction with ENU, an alkylating agent, it was found to be even more mutagenic. Branda has also shown (83) that folate deficiency acts synergistically with EMS in CHO cells to increase DNA strand breaks and mutant frequency. Duthie and Hawdon (84) have shown that folate depletion in human lymphocytes makes the cells more sensitive to oxidative damage induced by hydrogen peroxide. Duthie et al. (85) have further shown that human colon epithelial cells grown in the absence of folate are poorly able to repair damages induced by MMS (an alkylating agent) and hydrogen peroxide (an oxidizing agent). Growth in a low folate medium has also been shown to impair excision repair capacity of colonocytes (86). More oxidative damage, which is repaired by BER, accumulates in response to amyloid **β**-peptide in neuronal cells depleted of folate

(87). These data indicate that the pathway responsible for repairing these damages may be ineffective when folate is limiting. These findings are rather compelling for the effects of folate on cellular DNA appear to mimic those that are seen when **β**-pol is deficient. We would suggest that this phenomenon occurs conceivably by overwhelming the capacity of BER through inhibition of its rate determining enzyme, DNA polymerase **β**. Specifically, we have previously shown that with **β**-pol haploinsufficiency, there is a lack of induction of **β**-pol and

an accumulation of SSB (60), much the same as perceived in folate deficiency (80).

Colon Cancer

In the US, colorectal cancer is the second leading cause of cancer-related death and accounts for approximately 25% of all cancer deaths in the Western world. It is believed that 85% of CRC cases are caused by sporadic mutation, that is, it occurs in people who have no (or very little) family history of the disease and therefore is due to diverse environmental risk factors. The remaining CRC cases are thought to be a result of genetic predisposition notably hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatosis polyposis (FAP) (88). Sporadic Colon Cancer occurs in people who have no (or very little) family history of the disease and generally occurs in individuals over age 50 years. It has been stated that inactivation of the *APC* gene is found in about 85% and inactivation of the mismatch repair genes, including *MSH2*, *MLH1*, and *PMS2,* is found in the remaining 15%of sporadic colon cancers (89). Hereditary nonpolyposis colorectal cancer (HNPCC) is the more common form of hereditary colon cancer. It is an autosomal dominantly inherited condition characterized by an early onset of either colon, endometrial, ovarian, urinary tract, stomach, small bowel, biliary tract, or brain cancers. The average age of diagnosis is 44 years for colon cancer. It is estimated that 5 genes related to mismatch DNA repair are responsible for the development of this disease (90). This condition gives rise to a modest number of polyps in the colon beginning in the third or fourth decade of life (90) and are described as highly villous and dysplastic with a rapid

progression to cancer (90, 91). FAP is an inherited condition that results from defects in the adenomatous polyposis coli (APC) gene. APC is a known tumor suppressor that prevents the uncontrolled growth of cells that may result in cancerous tumors. If this gene develops a mutation, the protein made by APC cannot suppress cellular overgrowth leading to the formation of polyps, which can overtime become cancerous. Polyp formation begins in early adolescence with the number and size increasing with time. The pathology of polyps in FAP is indistinguishable from sporadic colon cancers; however, due to the vast number and early development of polyps, most individuals with FAP will develop colon cancer (90).

Progression of colon cancer is thought to occur over a period of many years in a multistep process involving a series of pathological alterations ranging from discrete microscopic mucosal lesions, like aberrant crypt foci (ACF), to malignant tumors (92, 93). Subtle alterations in the regular pattern of ACF are among the first histologically detectable changes that may be linked with colorectal cancer development (CRC) (94, 95, 96). The growth, morphological and molecular features of ACF supports the line of reasoning that ACF are putative preneoplastic lesions (97). Bird (92) was the first to describe ACF in the colonic mucosa of rodents exposed to colorectal carcinogens. These ACF which have long been considered as preneoplastic lesions and precancerous lesions, appear larger, with a thicker epithelial lining than adjacent normal crypts and dilated irregular luminal openings rising above the surrounding mucosa. These lesions have been observed as early as two weeks after administration of the

colon carcinogen DMH (98). Evaluation of solitary crypts demonstrates the aberrant crypts divide by fission beginning near the base of the crypt (99) growing overtime to form a focus with multiple aberrant crypts (100). ACF in humans closely resembles the aberrant crypts detected in rodents treated with carcinogens (101). It should be noted that although the number of ACF increases with increased exposure to carcinogens little evidence supports tumor development with increasing numbers (102). In other words, aberrant crypts do not indicate that colon cancer is imminent.

Base Excision Repair, Folate Deficiency and Colon Cancer

The association between BER, dietary folate and colon carcinogenesis is probably linked to folate deficiencies role in the 'catastrophic repair' cycle that arises due to extensive DNA damage secondary to decreased synthesis of both thymidylate and purines. Under thymidylate duress, BER is inefficient in repair due to the resynthesis step where elevated dUTP causes reintroduction of genomic uracil. Uracil may arise in DNA, in small quantities as a result of spontaneous cytosine deamination or/and misincorporation of dUMP during DNA replication. Frederico et al. (103), using a genetic reversion assay conservatively estimated that this occurs approximately 50 times per cell per day in the human genome. With folate deficiency, however, the rate of cytosine deamination increases as much as 10,000-fold (78). This rate would likely stress or overwhelm the DNA repair pathway, leading to single strand breaks that if left unrepaired are clastogenic (104). This can give rise to sections of the

chromosome being deleted, added, or rearranged, hence the inception of mutagenesis which can further develop to carcinogenesis.

Introduction of other DNA repair pathways

NHEJ is considered to be responsible for resolving DSBs that occur in the G1 phase of the cell cycle, while HR is responsible for resolving DSBs during S and G2 phases. NHEJ is an error prone pathway, whereas HR is quite accurate (104). There have been reports of cross-talk between homologous recombination (HR) and BER. It is said that HR responds to persistent BER strand break intermediates (Wyatt et al., 2006) which would seem appropriate as SSB can give rise to DSB if left unrepaired. The demand for deoxynucleotides required for the resynthesis step of HR can extend up to thousands of nucleotides in comparison to the 1 to 5 nucleotides required to complete BER, presenting a problem with folate is deficient. Use of HR when nucleotide pools are imbalanced would appear to be deleterious, nevertheless it may be the only way to recover stalled and/or collapsed replication forks. Currently there is a lack of evidence for a role of NHEJ in folate deficiency,

Folate Deficiency and mismatch repair

Mismatch repair is a system for recognizing and repairing of damage incurred during DNA replication. Mutations in MMR genes results in microsatellite instability (MSI) which is instability of repeated sequences of DNA. Research has shown that MSI has a frequency of 10% to 20% in colon cancers which is attributed to both MLH1 promoter hypermethylation and germline mutations in MMR genes (MLH1, MSH2, MSH6, PMS1, and PMS2, (105, 106).

Poynter et al. (106) have also shown that 54% of population-based MSI cases had hMLH1 methylation. MSH2 and MSH6 detect DNA mismatches at which point they recruit MLH1 and PMS1to the repair sites. MLH1 proteins join with PMS2 to form the major complex for mismatch repair (107). This complex is responsible for the coordination subsequent repair events. Folate deficiency has also been observed to impair MMR, in nonneoplastic ulcerative colitis patients (108). It was believed that a defect in MMR in these patients might translate into an increased risk for mutations. Due to the fact that DNA methylation has a role in strand discrimination during postreplication MMR, aberrations in normal patterns of DNA methylation, which can occur with folate deficiency, might adversely affect this DNA repair mechanism. Both DNA hypomethylation which would impinge upon the methyl-directed MMR and methylation of CpG sites in the hMLH1 gene, which has been associated with MSI in colon and stomach cancer (109), can be induced by folate deficiency (110). Although BER and MMR have both been proven to have a basis in colon carcinogenesis, the causes are distinct defining the necessity to utilize **β**-pol-deficient animal model to directly test an effect of BER.

CHAPTER 2

Hypothesis and Specific Aims

Folate deficiency has been shown to result in low levels of nucleotide synthesis and in addition elevated levels of uracil being misincorporated in to DNA. Uracil incorporated into DNA is initially excised from DNA by uracil-DNA glycosylase at which time apyrimidinic endonuclease creates a nick in the DNA phosphate backbone. With massive uracil misincorporation as seen in folate deficiency it appears to create a 'futile' repair cycle in that there is an accumulation of transient single strand breaks that could result in a less repairable and more harmful double-strand breaks if two opposing nicks are formed. Accumulation of the aforementioned could result in chromosomal aberrations, increased mutational frequency and potentially the onset and progression of cancer, particular those of the colon. This scenario appears to be aped when **β**-pol is haploinsufficient. The objective of this research was, therefore, to elucidate the molecular mechanisms by which **β**-pol impacts colon cancer when folate is deficient.

β-pol, being the rate limiting enzyme in the BER pathway, was expected to result in significantly deleterious effects when folate was deficient. It was thought that if **β**-pol were deficient, with the removal of uracil from the DNA by uracil-DNA glycosylase and the generation of transient SSBs that occurs with folate deficiency, this **β**-pol haploinsufficiency would create an extremely toxic level of unrepaired BER intermediates that would progress to ACF development and colon cancer progression. Thus, I tested the hypothesis that the accumulation of

DNA damage, preneoplastic lesions and tumors in colon caused by folate

deficiency and carcinogen exposure will be accelerated by loss of **β**-pol. Using

heterozygous **β**-pol in which one allele was inactivated and wildtype C57bl mice,

I directly tested the impact of folate deficiency on BER activity in the **β**-pol

haploinsufficient mouse model when exposed to the colon carcinogen DMH.

Specific Aim 1: **To determine whether BER deficiency accelerates the**

induction of ACFs induced by DMH when folate is deficient. That is, to test

whether the accumulation of damages seen in folate deficiency results in the

acceleration in onset and progression of precancerous lesions when **β**-pol is

deficient. The accumulation of uracil,

abasic sites and DNA single strand breaks can translate into toxic repair

intermediates that create DNA damage in the form mutations when folate is

deficient. I predicted that folate deficiency would increase the accumulation of

DNA damage and mutational frequency in the **β**-pol$^{+/-}$ mice to a greater extent

than it would in the wildtype mice due to the deficiency in base excision repair

capacity. Both **β**-pol haploinsufficiency and folate deficiency on their own are

genotoxic so it seemed only rational to assume that when combined the effects

would be magnified with profound DNA damage and mutations and in contrast,

the folate supplemented wildtype mice would prove resistant even after treatment

with a colon carcinogen. This in turn would demonstrate that a folate deficit would

further impair an already deficient BER pathway when **β**-pol is haploinsufficient.

Specific Aim 2: To determine whether ββ-pol haploinsufficiency in a folate deficient environment accelerates the induction of tumors caused by DMH when folate is deficient. As stated previously, ACF development does not imply that colon cancer is forthcoming. in fact, a majority regress, however, it is well-established that an accumulation of DNA damage, mutations, or chromosomal abnormalities are initiating events in the genesis of cancer. Our

previous studies have determined an increase in tumor development in **β**-pσl mice. So this study aimed to determine if the combined effect of **β**-pol haploinsufficiency and folate deficiency would induce higher levels of ACF and if these putative markers were more likely to progress to neoplastic lesions. That

is I depicted that folate deficiency imposed in a BER deficient environment would result in a more advanced ACF where they had increased proliferative capacity and reduction of cell death secondary to the mutational effects of lack of repair by **β**-pol haploinsufficiency. It seemed plausible to assume that the transformation in the DNA from this stressful environment would result in increased cell number (hyperplasia) progressing ACF to neoplastic lesions.

Specific Aim 3: **To determine the impact of ββ-pol$^{+/-}$ on gene expression when folate was deficient.** Looking at gene expression analysis would help to elucidate the molecular mechanisms by which **β**-pol heterozygosity would influence ACF formation and colon tumorogenesis. Changes in expression of key genes could depict a more global indication of cellular mechanistic changes due to effects of **β**-pol heterozygosity in an environment deficient in folate. From this data we could not only elucidate changes in gene function but as well it may

highlight changes in key cellular pathways of colon carcinogenesis, such as cell cycle, apoptosis and colon carcinogenesis.

CHAPTER 3

Folate deficiency provides protection against colon carcinogenesis in DNA polymerase ββ haploinsufficient mice.

Lisa Ventrella Lucente[1], Archana Unnikrishnan[1], Amanda B. Pilling[1], Hiral V. Patel[1], Deepa Kushwaha[1], Alan Dombkowski[2], E.M. Schmelz[3], Diane C. Cabelof [1], Ahmad R. Heydari[1].

[1]Department of Nutrition and Food Science, Wayne State University, Detroit, MI, United States. [2]Institute of Environmental Health Sciences, Wayne State University, Detroit, MI, United States. [3]Department of Human Nutrition, Foods and Exercise, Virginia Tech, Blacksburg, VA, USA.

Running Title: Folate deficiency, **β**-pol and Colon Carcinogenesis

Reprint Requests: Ahmad R. Heydari, Department of Nutrition and Food Science, Wayne State University, Detroit, MI, United States. Phone: (313)577-2459, Fax: (313) 577-8616. E-mail: ahmad.heydari@wayne.edu.

Abstract

Aging and ββ-pol deficiency (ββ-pol$^{+/-}$) interact to accelerate the development of malignant lymphomas and adenocarcinoma and increases tumor bearing load in mice. Folate deficiency (FD) has been shown to induce DNA damage repaired via base excision repair (BER) pathway. We anticipated that FD and BER deficiency will interact to accelerate aberrant crypt foci (ACF) formation and tumor development in ββ-pol haploinsufficient animals. FD resulted in a significant increase in ACF formation in wildtype (WT) animals exposed to 1, 2-dimethylhydrazine (DMH), a known colon and liver carcinogen; however FD reduced development of ACF in ββ-pol haploinsufficient mice. Prolonged feeding of the FD diet resulted in advanced ACF formation and liver tumors in wild type mice. However, FD attenuated onset and progression of ACF and prevented liver tumorigenesis in ββ-pol haploinsufficient mice, i.e., FD provided protection against tumorigenesis in a BER deficient environment in all tissues where DMH exerts its damage. Here we show a distinct downregulation in DNA repair pathways, e.g., BER, NER and MMR, and decline in cell proliferation, as well as an upregulation in PARP, proapoptotic genes and apoptosis in colon of FD ββ-pol haploinsufficient mice. Moreover, we report that FD BER deficient mice show a significant downregulation in mTOR pathway, which is implicated in cell proliferation and survival. Our data indicate, therefore, that FD in BER deficient mice

downregulates mTOR, inhibiting s-phase transit perhaps secondary to depletion of cellular energy by PARP, promoting apoptosis and providing protection against tumorigenesis.

INTRODUCTION

Folate deficiency is an important public health concern because of the role folate plays in the development of many different health problems, including neural tube defects, cardiovascular disease, Alzheimer's disease and cancer, specifically colon cancer. It has been proposed that the carcinogenic properties of folate deficiency may be related to a decrease in DNA methylation, perhaps as a function of reduced *S*-adenosylmethionine (SAM) levels, an increase in the uracil content of DNA or an increase in oxidative stress by alterations in thiol switches. Folate deficiency has also been shown to increase (in cells, animal models and humans) levels of single strand breaks (84, 111, 112, 85, 113), micronucleus formation, (114, 115), chromosomal aberration (116, 117) and mutation frequency (82, 83), all potentially downstream effects of high levels of uracil in DNA and oxidative damage to DNA.

The DNA repair pathway for removal of uracil and oxidized bases is the base excision repair (BER) pathway. The BER pathway is believed to repair small, non-helix-distorting lesions in the DNA. It has been estimated to be responsible for the repair of as many as one million nucleotides per cell per day (47), stressing its importance in the maintenance of genomic stability. It has been suggested that BER has evolved in response to *in vivo* exposure of DNA to ROS and endogenous alkylation, and that this pathway suppresses spontaneous mutagenesis (117). In the initial elucidation of the BER pathway the following steps were involved: i) removal of the damaged base by a DNA glycosylase; ii) incision of the phosphate backbone by an endonuclease; iii) synthesis of new

DNA by a polymerase; iv) excision of the deoxyribose phosphate (dRp) moiety; and v) ligation. This remains the predominant BER pathway and is clearly the pathway for repair of uracil. DNA polymerase **β** (**β**-pol) performs the polymerization steps in the predominant short patch BER pathway and appears to perform the rate-limiting step, by virtue of its dRp lyase activity (53). In the process of uracil removal and repair of oxidized bases, a transient formation of a

DNA single strand break occurs. Our laboratory has shown that haploinsufficiency in **β**-pol would result in persistence of the DNA single stand breaks, where this persistence could result in DNA double strand breaks and chromosomal aberration (60).

Based on current understanding of the role of a **β**-pol-dependent BER pathway on removal of uracil, alkylated bases and oxidized bases, it is interesting to determine the impact of **β**-pol haploinsufficiency on tumorigenesis. While the homozygous **β**-pol knockout is embryonic lethal, the **β**-pol heterozygous knockout mice are viable and fertile. We previously characterized this mouse model as expressing 40-50% less **β**-pol mRNA and protein in various tissues and determined that this mouse has 40-50% reduced *in vitro* BER activity in all tissues tested (60). In addition, these animals exhibit an accumulation of spontaneously arising single strand breaks and chromosomal aberrations with age. Furthermore, in response to alkylating and oxidizing agents, an even greater accumulation of single strand breaks and chromosomal aberrations, as well as an increase in mutation frequency are observed in these mice (60).

Moreover, **β**-pol haploinsufficient mice display an acceleration of normal, age-related tumors, e.g., lymphomas, developing alongside an increased susceptibility to epithelial tumors, e.g., adenocarcinomas, which do not typically occur at a high incidence in C57BL/6 mice (77).

Based on the striking similarities between DNA damage induced by folate deficiency and that induced by a reduction in BER capacity, we suggest a strong association between BER and folate. We have reported previously that folate deficiency overwhelms the capacity of BER through the inhibition of upregulation of **β**-pol (80). It is feasible that the inability to induce **β**-pol when folate is deficient results in a functional BER deficiency, providing a logical explanation for the phenotype induced by folate deficiency. While a tight correlation between DNA damage and cancer exists, it is necessary to evaluate preneoplastic lesions and tumors arising in response to the interaction between **β**-pol loss and folate deficiency. The purpose of this study is to determine whether **β**-pol haploinsufficiency accelerates the development and/or aggressiveness of these lesions in colon and liver in response to carcinogen, 1,2-dimethylhydrazine (DMH). This study has important human health implications, as polymorphisms within the human population may render individuals haploinsufficient for **β**-pol and increase cancer risk by reducing their DNA damage tolerance.

METHODS

Animals- Experiments were performed in young 4 to 6 month old C57BL/6-specific pathogen free male mice and mice heterozygous for the DNA polymerase **β** gene (**β**-pol$^{+/-}$) (77). All practices performed on animals were in agreement with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. Mice were backcrossed to the C57BL/6 background. The Wayne State University Animal Investigation Committee approved the animal protocol. Mice were maintained on a 12-h light/dark cycle and given water *ad libitum*.

Diets and Carcinogenic Treatment- After acclimation for 7 days, wildtype (WT) and **β**-pol$^{+/-}$ mice were randomly assigned to two dietary groups; a folate adequate (FA) or folate deficient (FD) AIN93G-purified isoenergetic diet (Dyets, Inc., Lehigh Valley, PA) as described previously (80). The FA group received a folate adequate diet containing 2 mg/kg folic acid. The FD group received a folate-deficient diet containing 0 mg/kg folic acid. Diets were stored at –20℃. 1% succinyl sulfathiazole was added to all diets. One week after commencement of food ingestion, randomly selected mice from both FA and FD were injected i.p. with 1,2-Dimethylhydrazine HCL (DMH, 30 mg/kg body weight) in 10mmol/L of NaHCO$_3$ (Fisher Scientific, Fair Lawn, NJ) once a week for 6 wks (Figure2, panel A). Both food intake and body weights were checked twice weekly to monitor for signs of of toxicity, e.g., weight loss, and the diets were continued for 12 weeks.

Aberrant colonic crypt (ACF) analysis- Animals were anesthetized under CO_2 asphyxiation, the abdominal cavity was opened and the colon excised, rinsed with cold PBS, cut longitudinally, and fixed flat overnight in 10% neutral buffered formalin. The colonic crypts were stained with 2g/L of methylene blue in PBS for 5 min. The number of ACF and aberrant crypts per foci were determined by light microscopy at 10X magnification in a blinded man ner.

Realtime PCR- Total RNAs were isolated from the colon mucosa of mice using the RNeasy Mini Kit (Qiagen, Valencia, CA) per manufacturer's protocol. cDNAs were synthesized from 1 µg RNA using random hexamer primers (Promega, Madison, WI) and purified with the QIAquick PCR Purification kit (Qiagen). The levels of cDNAs were quantified using a LightCycler real-time PCR machine (Stratagene, La Jolla, CA). PCR reactions contained 3 µL purified cDNA, 12.5 µL qPCR master mix, and 0.5 µmol/L each of sense and antisense primers (Roche)(Table I). For all amplifications, PCR conditions consisted of an initial denaturing step of 95℃ for 5 minutes followe d by 40 cycles of 95℃ for 10 seconds, and 60℃ for 30 seconds, with a melting curve a nalysis from 60℃ to 95℃ to confirm specificity. External standards were pre pared by amplification of cDNAs for each gene. The amplicons were cloned into pGEM-T Easy vector, linearized with appropriate restriction enzyme, and used to prepare external standard curves. The level of each transcript was normalized to GAPDH. Results are expressed as mean values from five animals per experimental group.

Western blot analysis- Western blot analysis was performed using 200**μ**g nuclear protein as previously described (118). Upon completion of SDS–PAGE, the region containing the protein(s) of interest was excised and prepared for Western blot analysis, whereas the remaining portion of the gel was stained with GelCode blue stain reagent (Pierce Biotechnology) to ensure equal protein loading. Western analysis was accomplished using affinity purified polyclonal antisera developed against mouse **β**-pol. As an internal control for protein loading, membranes were reprobed with anti-Lamin B antibody (Santa Cruz Biotechnology, Santa Cruz, CA) The bands were visualized and quantified using a ChemiImager System (AlphaInnotech, San Leandro, CA) after incubation in SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). Data are expressed as the integrated density value (I.D.V.) of the band per ∝g of protein loaded.

Microarray assays- Total RNAs were isolated from the colon mucosa of mice using the RNeasy Mini Kit (Qiagen, Valencia, CA) per manufacturer's protocol. RNA samples were quantified with NanoDrop ND-1000 (NanoDrop Technologies, Inc, Wilmington, DE) and 260/280 ratio in the range of 2.0-2.2 was defined as acceptable. A quality check of the total RNA was performed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). One micro liter of total RNA sample was applied on RNA 6000 NanoChip, and the assay was run on the Bioanalyzer to determine if the 18S and 28S ribosomal bands are defined and to ensure no RNA degradation was present. Optimal concentration used to check the RNA quality is 250ng/∝l.

Microarray expression profiling was conducted by Microarray & Bioinformatics Facility Core at Wayne State University (Institute of Environmental Health Sciences, Detroit, MI) according to the manufacturer's protocol. A balanced block experimental design was used: 4 microarrays were completed for each comparison, with each microarray representing 1 randomly selected labeled mucosal sample from each experimental group paired with 1 randomly selected FA WT labeled mucosal sample i.e. in total 4 mucosal RNA samples from FDWT cohybridized with 4 FAWT, 4 FDWT DMH treated cohybridized with 4 FAWT and 4 FD β-pol$^{+/-}$ DMH cohybridized with 4 FAWT treated, representing 4 micorarrays for each experimental comparison. Samples on a given array were oppositely labeled with Alexa 647 and Alexa 555 dyes. The four microarrays for a given group (i.e., WT untreated, WT DMH or β-pol$^{+/-}$ DMH treated) represent samples from eight separate mice, providing consideration of biological variation. In total, 12 arrays representing 24 mice (8 WT, 8 WT DMH-treated, 8 β-pol$^{+/-}$ DMH-treated) were completed. Dye swaps were used to account for dye bias effects such that of the four arrays in a given phenotype group, two had FD treated samples labeled with Alexa 647 co-hybridized with control samples labeled with Alexa 555 while the other two arrays within the same phenotype group had opposite dye orientations. Microarrays were scanned using the Agilent dual laser DNA micorarray scanner model G2565AA, with 10 micron resolution.

Microarray gene profile analysis- Microarray image analysis was performed with Agilent Feature Extraction software, version A.5.1.1. Hierarchal Clustering analysis was performed using GeneSpringGX V10 (Agilent

Technologies) software, and the parameters were set for centroid linkage which calculates the euclidean distance between the respective centroids of two clusters. False discovery rate for FDWT was 5.1%, 1.7% for FDWT DMH treated and 0.39% for FD β-pol$^{+/-}$ DMH-treated experimental groups. Heatmaps were created by inputting accession numbers of differentially expressed genes, at p<0.001, for FDWT and FD β-pol$^{+/-}$ DMH-treated experimental groups through DAVID functional annotation. Outputs revealed gene onotologies of differentially expressed genes, which were than compared to created differentially expressed gene lists of DAVID biological processes, such as apoptosis and DNA repair. Data sets were then combined for FDWT and FD β-pol$^{+/-}$ DMH-treated to 1 large data set in order to create heatmaps for each biological function. Gene ontology analysis was performed using Gene Ontology Tree Machine (GOTM), (Bioinformatics,Vanderbilt University), applying differentially expressed genes as depicted in the heatmaps`. We chose a single gene set analysis, where GOTM compares the distribution of single gene set in each GO category to those in an existing reference gene list from the mouse genome, identifying GO categories with statistically significant enriched gene numbers as determined by the hypergeometric test (p<0.01) (119, 120). Real time quantitative RT-PCR was used to confirm the data obtained for selected genes in DNA repair pathways as described above.

Terminal deoxynucleotidyl transferase-mediated nick-end labeling of apoptotic cells in situ- Colon tissues were dissected, opened longitudinally, fixed in 10% neutral buffered formalin, embedded in paraffin wax then cut into 5 μm-

thick sections. Sections were put on slides for the in situ terminal deoxynucleotidyl tranferase-mediated nick-end labeling (TUNEL) (CHEMICON International Inc., Temecula, CA) as per manufacturer's protocol. Cells with positive staining (brown staining) were considered as apoptotic cells and the number of apoptotic cell**s** was determined as the percentage per crypt.

5-Bromo-2-deoxyuridine (BrdU)-staining of proliferating cells in situ- Two hours prior to CO_2 asphxyation, mice were injected i.p. with a BrdU solution (10 mol/L, 1 mL per 100 g body weight) (5-Bromo-2'-deoxy-uridine Labeling and Detection Kit ll, Roche Diagnostic, Mannheim, Germany) to immunostain proliferating cells, following manufacturers instructions for paraffin-embedded tissues. Briefly, after CO_2 asphyxiation, as mentioned above, colons were excised, and fixed in formalin. The tissue was then embedded in paraffin and sections of 3 – 5 ∝m were cut longitudinally the full length of the colon. Tissues were incubated in 10 ∝mol anti-BrdU monoclonal antibody for 1 hr at 37℃ followed by incubation with anti-mouse-Ig-alkaline phosphatase. The labeling index was used to quantify cell proliferation, defined as the percentage of BrdU-positive cells per crypt.

Tumor Analysis- All animals were sacrificed at 40 weeks after the last dose of DMH, by asphyxiation with carbon dioxide, and all organs and tissues were examined for grossly visible lesions. Liver and colon tissues, including gross abnormalities, were fixed in 10% neutral buffered formalin, trimmed, embedded in paraffin, sectioned to a thickness of 5 to 6 µm, stained with H&E, and examined microscopically. Preparation of slides for histopathology

evaluation was performed by pathologists at the Department of Pathology, Wayne State University.

Statistical analysis- Statistical significance between means was determined using ANOVA, followed by the Fisher's least significant difference test where appropriate (121). P-values less than 0.05 were considered statistically significant.

RESULTS

Induction of aberrant crypt foci (ACF) formation in DNA polymerase β haploinsufficient mice in response to dimethyl hydrazine (DMH)- The purpose of this study was to determine the impact of BER deficiency on colon carcinogenesis in an animal model. In the first series of experiment, we

determined the impact of **β**-pol heterozygosity on the development and progression of ACF in DNA polymerase **β** heterozygous knockout mice of C57BL/6 background, characterized previously by our laboratory (122). We have previously observed a deficiency in **β**-pol gene expression in various tissues of **β**-pol$^{+/-}$ mice (brain, liver, spleen and testes) with a concurrent decline in BER capacity (122). As shown in Figure 1A, in this study we have confirmed similar decline in expression of **β**-pol gene in the colon mucosa of the **β**-pol$^{+/-}$ mice suggesting a parallel decline in BER activity in the colon of these mice.

It is well established that carcinogen-induced ACF are early indicators of initiation of colon cancer in animal models (92, 123, 124). In this study, we utilized 1,2-dimethylhydrazine (DMH), an established colon and liver carcinogen. It is proposed that DMH converts to the active metabolites azoxymethane and methylazoxymethanol in the liver, which are then transported to the colon via blood and bile (125). DMH in these tissues exerts its damage to DNA by induction of alkylation damage (O6-meG and N7-meG) as well as oxidative damage. We have previously demonstrated that the **β**-pol$^{+/-}$ mouse is not sensitive to the O6-meG lesion (removed by direct reversal), but is sensitive to the N7-meG lesion processed by BER pathway (126). Additionally, we have

observed increased accumulation of DNA single strand breaks in the **β**-pol$^{+/-}$ mouse in response to oxidative stress, as compared to its wildtype counterpart (60). Thus, we postulated that **β**-pol haploinsufficiency may predispose animals to increased colon carcinogenicity and induce development of ACF in response to DMH. At the outset of the current study, mice were injected with 30 mg/kg body weight DMH for 6 weeks to induce ACF. Six weeks after the final injection, the mice were sacrificed and the level of ACF in colon was determined. As shown in Fig. 1B, wildtype and **β**-pol haploinsufficient untreated mice did not display any ACF in their colon. Thus, **β**-pol haploinsufficiency and subsequent deficiency in BER capacity is not enough to induce ACF in mice, suggesting that **β**-pol is a low-penetrance gene, requiring a high penetrance environmental insult, e.g., DMH or nutritional deficiency, for the damage to accumulate. Furthermore, as shown in Fig. 1B, **β**-pol $^{+/-}$ DMH-treated mice exhibited a significantly higher level of ACF formation (67% higher) as compared to wildtype counterparts (15.4±1.8 versus 29.7±1.4, for wildtype and **β**-pol$^{+/-}$ mice, respectively, p<0.01). These findings indicate that **β**-pol haploinsufficient mice are more sensitive to DMH, i.e., **β**-pol$^{+/-}$ animals show an inability to respond to oxidative stress and alkylation damage as compared to their wildtype littermates. Interestingly, no significant differences in ACF size and aberrant crypts per focus were observed in these animals. Having confirmed these findings, we were interested in the effect of folate deficiency in a BER deficient environment on the response to DMH.

*Impact of folate deficiency and **β**-pol haploinsufficiency on ACF formation in colon-* Folate deficiency has been suggested to impact SAM/SAH ratio (126), increase uracil incorporation into DNA (a substrate for BER pathway) and induce oxidative stress in animals. Subsequently, to elicit the role of folate deficiency on ACF formation in wildtype and **β**-pol$^{+/-}$ animals, animals were fed either a folate adequate (FA, 2 mg/kg folic acid) or folate deficient (FD, 0 mg/kg folic acid) diets as outlined in Figure 2A. To induce severe folate deficiency, 1% Succinyl Sulfathiazole was added to the diet deficient in folic acid to prevent synthesis of folic acid in the gut by bacteria. The animals' food intake and body weight were monitored weekly. Folate deficiency did not affect body weight while it reduced the plasma folate level by 90% as determined by a SimulTRAC-SNB radioassay kit for vitamin B$_{12}$ (^{57}Co) and folate (^{125}I) per the manufacturer's protocol (ICN Diagnostics, Orangeburg, NY) as described previously (80). After one week on respective diet, the wildtype and **β**-pol$^{+/-}$ mice were injected with DMH once per week for 6 weeks (Figure 2A). Six weeks after the final injection DMH-treated and control mice were sacrificed and the levels of ACF in colons were determined. Interestingly, no ACF was observed in control mice fed a folate adequate and/or folate deficient diet. Thus, **β**-pol haploinsufficiency in conjunction with folate deficiency was not enough to induce ACF formation in untreated mice. However, DMH-induced ACF were observed in all DMH-treated animals. As observed by other laboratories (127, 128), folate deficiency resulted in a significant increase in ACF formation in wildtype animals treated with DMH as compared to the FA counterpart (15.4±1.8 versus 37.6±5.2, for FA and FD

mice, respectively, p<0.01), i.e., folate deficiency predisposes mice to increased colon carcinogenesis in response to DMH. As shown in Figure 2B, DMH-induced ACF was confirmed and significantly higher in FA β-pol$^{+/-}$ mice as compared to their wildtype littermates. Based on these findings and in view of the fact that FD resulted in further decline in β-pol expression in colon mucosa (Figure 3), we anticipated that β-pol haploinsufficient mice would display a dramatic development of ACF when folate is deficient. However, we observed a significant reduction in the formation of ACF in β-pol$^{+/-}$ mice when compared to their wildtype counterparts in a folate deficient environment (50% reduction; 37.6±5.2 versus 18.3±4.1, for wildtype and β-pol$^{+/-}$ mice fed a folate deficient diet, respectively, p<0.01, Figure 2B). It has been proposed that the size of ACF and number of aberrant crypts per focus could be a more appropriate indicator of colon tumorigenesis. As shown in Figure 2C, no significant differences in the number of aberrant crypts per focus were observed in all experimental groups treated with DMH. These data indicate that β-pol haploinsufficiency attenuate carcinogen-induced ACF development when folate is deficient.

To determine whether prolonged folate deficiency increases ACF progression or results in ACF regression, wildtype mice were maintained on their specific diets for 40 weeks post DMH treatment. After sacrifice, colons were macroscopically examined for the development of ACF and the number of aberrant crypts per focus (Figure 4A). As shown in Figure 4B, as has been reported elsewhere, there was a lack of further increase in ACF numbers, but rather a regression of ACFs as well as, a further development to larger ACF with

increased aberrant crypts per focus in these mice. Although we observed no significant change in crypt multiplicity at 6 weeks post treatment for any group studied, the number of crypts per focus significantly increased in FD mice as compared to their FA counterparts after 40 weeks in wildtype mice (7.63 ± 0.4 verses 6.33 ± 0.4, for FD and FA mice, respectively, $p<0.05$). Moreover, ACF in

FD mice displayed a well defined elevation above the surrounding mucosa compared to its FA counterpart which appeared flat (Fig. 4A, Panels IV). Thus based on the notion that the size of ACF and the number of aberrant crypts per focus are better indicator of tumor formation, folate deficiency does not only increase the number of ACF in response to DMH early on, but its adverse impact persist resulting in further development of aberrant foci and formation of microscopic adenoma, while this phenomenon could be hampered where **β**-pol is deficient. Accordingly, it was important to evaluate the experimental groups for tumor analysis. Determining tumor incidents in these mice is important as these data potentially shed light on the impact of BER deficiency on colon tumors in a folate deficient environment, and determine potential continuity between ACF development and formation and metastasis of tumors.

*Analysis of pathology in **β** pol haploinsufficiency and wildtype counterparts in response to folate deficiency and DMH treatment*- Having established the

DMH-induction of ACF in colon of **β**-pol$^{+/-}$ mice and their wildtype counter parts fed a FA and/or FD diet, we studied the impact of folate deficiency and DMH treatment on tumor formation in these animals, based on the known impact of DMH on colon and liver tissues. In this study, we followed animals for 40 weeks

after the last DMH treatment, to determine the incidence of tumor formation as outlined in Figure 5A. Analysis of formalin-fixed and methylene blue stained colons provided evidence of advanced ACF in the colon of wildtype FD (Figure 4, IV) mice treated with DMH as compared to FA mice (Figure 4, III). In other words, while a more defined elevation above the surrounding mucosa was observed in ACFs of FD fed mice, the FA mice displayed less developed ACF. Upon dissection of animals, we perceived gross changes in the pathology of the

liver in FD DMH-treated mice (50% tumor formation) and FA DMH-treated **β**-pol$^{+/-}$ (100% tumor formation). As shown in Figure 5B, panels II and III, the

architecture of the liver tissue in all the FD WT and **β**-pol$^{+/-}$ fed a FA diet, demonstrated an atypical morphology depicting progression of tumors. In contrast, there were no visible changes in the liver for wildtype mice consuming a

FA diet and **β**-pol$^{+/-}$ animals fed a FD diet. Taken together, FD WT and **β**-pol$^{+/-}$ fed a FA diet with DMH treatment show more developed ACF and tumor

formation than FA WT DMH-treated and FD **β**-pol$^{+/-}$ animals. This is suggestive, yet again, of haploinsufficiency conferring protection when exposed to oxidative/alkylation stress induced by DMH treatment in a folate deficient environment. Based on these data, it is inviting to suggest that **β**-pol haploinsufficiency in combination with folate deficiency might impact cell cycle arrest and apoptosis in response to DMH, thus impacting ACF development and consequent tumor formation. Accordingly, we wanted to determine the impact of **β**-pol haploinsufficiency and folate deficiency on gene expression in mucosal tissue of the colon.

Effects of folate deficiency on gene expression in colon mucosal cells- Having seen a difference in colon pathology, as aforementioned, we wanted to determine differential expression of genes in colon tissues of **β**-pol$^{+/-}$ mice subjected to DMH in a folate deficient environment. In order to ascertain possible mechanisms of reduced ACF and tumor formation, we decided to

conduct a microarray analyses on FD **β**-pol$^{+/-}$ DMH-treated and FD WT DMH-treated colon mucosa, relative to FA WT. As depicted in the scatter plots of differentially expressed genes, there was a marked difference in expression of many genes in DMH treated **β**-pol haploinsufficient mice subject to a folate

deficient environment (4621 upregulated, 5757 downregulated in FD **β**-pol$^{+/-}$ DMH-treated versus 528 upregulated, 557 downregulated in FD WT mice) (Figure 6A). In other words, **β**-pol haploinsufficiency and DMH-treatment resulted in more extensive differential expression of genes as compared to wildtype untreated mice. To authenticate the outcome of microarray analysis, we performed quantitative RT-PCR using FullVelocity™ SYBR ® Green QRT-PCR

Reagents (Stratagene) on candidate genes depicted as differentially expressed through microarray analysis. The candidate genes studied included UNG, MPG,

β-pol, XRCC1, ligase 3 and RAD51li. qRT-PCR results for the 6 genes selected were consistent with microarray data. All were significantly downregulated in the

FD **β**-pol $^{+/-}$ DMH-treated mice ($p<0.05$) (Table IV). Having confirmed the validity of microarray findings, we wanted to identify the inter-relationships that existed between the abundance of differentially expressed genes.

Based on our assessment of a possible role of DNA repair and apoptosis we used DAVID biological function to determine the changes in individual gene expression related to these two pathways. Once identified, we generated a series of hierarchal clustered heatmaps based on intensity of gene expression from raw data files (Figure 6B). Hierarchal clustering assisted in exploring the relationships that exist amongst the statistical data identified in the microarray analysis. Here we show that upon conducting a clustered analysis, all the data demonstrates a thrust toward a reduction in DNA repair and an upregulation in apoptotic related gene activity in the FD **β**-pol $^{+/-}$ DMH-treated mucosal tissue. In contrast, WT-untreated counterparts had a propensity toward either no difference in or slight upregulation in DNA repair and a downregulation in proapoptotic gene expression. Furthermore, FD WT DMH-treated groups showed either no difference or an upregulation in DNA repair capacity and an upregulation in apoptotic.

To further characterize the differences in gene expression, we input differentially expressed genes, from the raw data files, into Directed acyclic Graphs (DAG) view of Gene ontology to acquire clusters of statistically ($p<0.01$) enriched differentially expressed genes according to their gene ontology. In this analysis, all gene names from raw data files were input into GOTM without reference to significance or intensity levels. Here, again, even with limited data input, we confirmed enrichment in DNA repair response and apoptotic activity FD **β**-pol **β**-pol$^{+/-}$ DMH-treated. As shown in Figure 7, the ontology groups enriched at program cell death and apoptosis, as well as, in BER, NER and recombination

repair all converging on DNA damage response, signal transduction and induction of apoptosis.

Analyzing expression data with DAVID biological processes highlighted several genes related to promotion of apoptosis and DNA repair activity (Table II, III, IV). Interestingly, we observed a distinct decline in overall DNA repair activity, including but not exclusive to BER (UNG, Apex, and **β**-pol), mismatch repair (MSH2 and MSH3), and nucleotide excision repair (ERCC2 and Xpc) (Table I). In addition, we see a downregulation in FRAP1 (mediates cellular response to DNA damage) in **β**-pol haploinsufficient and FD WT mice, with no change in gene expression in FD WT DMH-treated colonic mucosa. In contrast, there was an enhanced expression of several proapoptotic genes, including genes involved in intrinsic/extrinsic apoptotic pathways (CASP 4 and CASP8), TNF signaling (Tnfsf12 and Tnfrsf26), as well as, GAS1 and Trp63 (Table II). All of these data suggest enhanced programmed cell death, either based on enhanced apoptotic activity, forgoing DNA repair, or lack of response of DNA repair pathways to cellular damage, increasing the sensitivity of apoptotic related pathways. Interestingly, the differential expression of genes in the WT FD DMH-treated colonic mucosa was either not differentially expressed or upregulated for DNA repair, with the exception of **β**-pol and upregulated in apoptosis, apart from a downregulation in CASP 3. In addition, FD WT colonic mucosa were either not differentially expressed or for the most part, slightly upregulated for DNA repair and showed reduction in apoptotic activity suggesting that in addition to folate deficiency, a compromised BER pathway is required to trigger apoptosis in DMH-

treated mice. In view of these findings, we wanted to further confirm these results through a series of immunohistological experiments.

Evaluation of apoptotic and proliferative activity in colonic mucosal cells-

Having determined the impact of **β**-pol haploinsufficiency on colon and liver tissue; we wanted to establish why BER insufficiency attenuates development of lesions in the face of deleterious surroundings of DMH-induced carcinogenesis and folate deficiency. We, therefore, conducted both apoptotic and proliferative assays because maintenance of mucosal integrity is reliant on the regulation of these two entities, i.e., development of ACF may arise if this integrity becomes compromised. To determine apoptotic activity in our experimental groups, we measured apoptosis in colon using the TUNEL assay. Firstly, folate deficiency increased apoptotic body in wildtype animals (data not shown). Interestingly, folate deficiency induced a greater level of apoptotic activity in **β**-pol haploinsufficient mice as compared to wildtype counterparts (Figure 8A, IV).

Next, to characterize the effect of **β**-pol heterozygosity and folate deficiency on cell proliferation in response to DMH treatment, we examined the proliferative activity of colon tissues examining BrdU incorporation. As shown in Figure 8D, I, FA **β**-pol$^{+/-}$ mice showed significantly more proliferation (BrdU incorporation) as compared to FD **β**-pol$^{+/-}$ mice. Thus, the TUNEL and BrdU assays confirmed the differential expression observed in microarray analysis, suggesting that folate deficiency provide protection against tumorigenesis in DMH-treated **β–**pol$^{+/-}$ mice by altering the balance between DNA repair and apoptotic pathway favoring apoptosis.

Figure 1
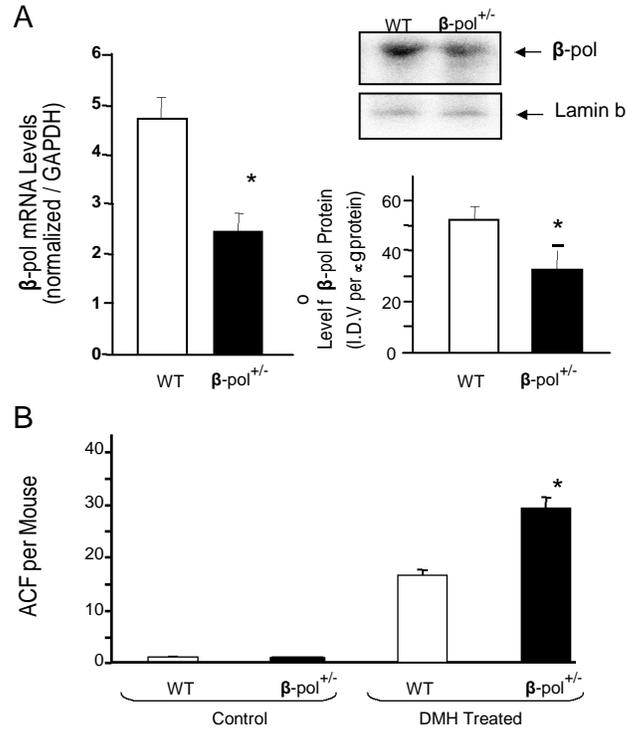
Fig 1. *Impact of β-pol heterozygosity on expression of **β**-pol in colon mucosa and ACF formation in colon of mice treated with DMH.* (A) Analysis of **β**-pol expression, mRNA and Protein levels, in mucosa from wildtype and **β**-pol haploinsufficient mice. Expression of **β**-pol gene was determined using a real time PCR technique and the level was normalized base on GAPDH expression. Protein level was quantified using a western blot analysis and normalized based on the Lamin b protein level. (B) Wildtype (WT) mice and β-*pol* haploinsufficient mice received either no treatment (controls) or i.p. treatment with DMH for 6 weeks at 30 mg/kg body weight (DMH treated). Colons were processed after CO_2 asphyxiation of mice as described in materials and methods. Colons were analyzed under light microscopy to visualize the number of ACF per mouse colon (ACF/mouse). *Bars,* SE, * , P<0.01.

Figure 2

A

DMH Treatment
(30 mg/kg)

Sacrifice for
analysis of ACF

Time (Wk) 0 1   6   12

Genotype (no.)

WT (n=6)   ◄————— Folate (2 mg/kg) —————►

WT (n=6)   ◄————— Folate (0 mg/kg) —————►

β-pol⁺/⁻ (n=6) ◄————— Folate (2 mg/kg) —————►

β-pol⁺/⁻ (n=6)   Folate (0 mg/kg) —————►

B

C
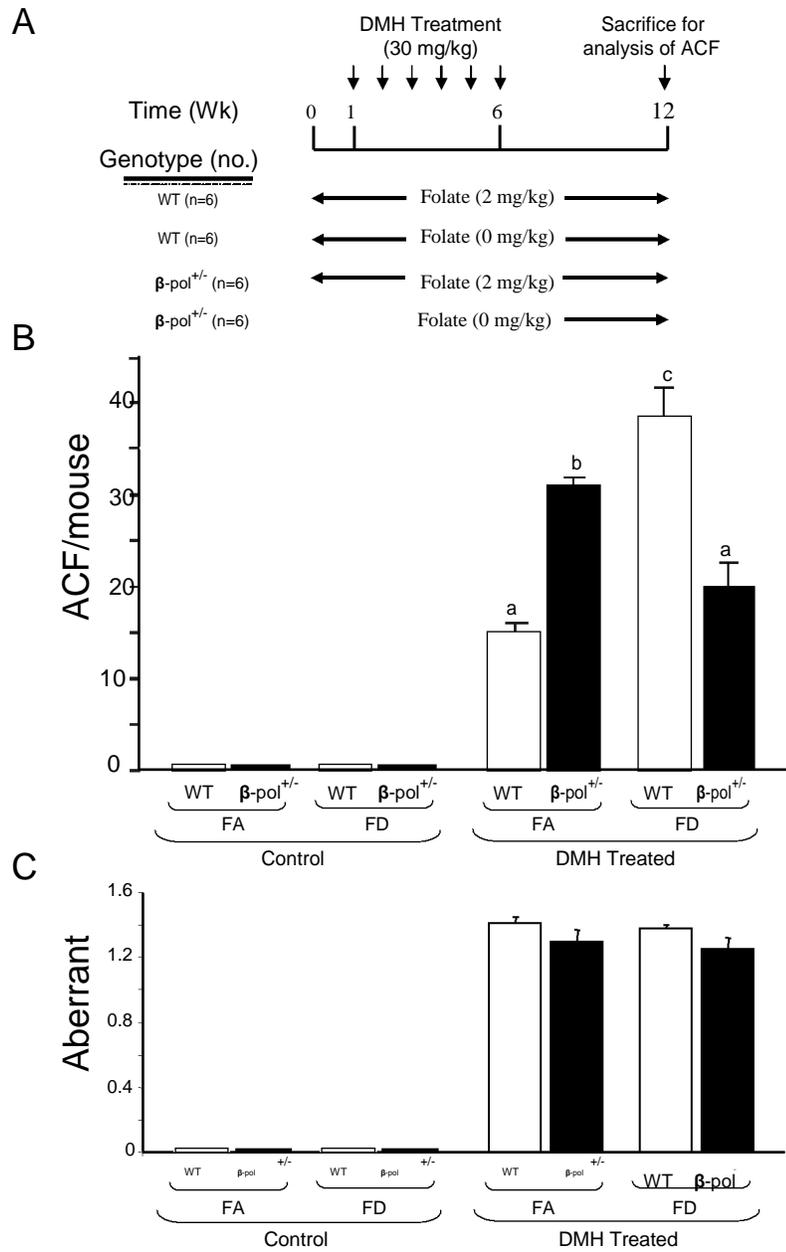
Fig 2. *Panel A: Experimental Design*: WT and β-pol$^{+/-}$ mice were fed either a folate adequate (2 mg/kg, FA) or a folate deficient (0 mg/kg, FD) diet for 12 weeks. After one week of ingestion of respective diets, mice were injected with 30 mg/kg body weight DMH for 6 weeks. Six weeks after final injection, animals were sacrificed by CO_2 asphyxiation. *Panel B and C: ACF formation and crypt multiplicity in colon of* β-pol$^{+/-}$ *mice consuming a folate deficient diet.* WT and β-pol$^{+/-}$ received either a FA diet or FD diet and subjected to either no treatment (control) or i.p. treatment with DMH for 6 weeks at 30 mg/kg body weight (DMH treated). After sacrifice colons were processed as per materials and methods. Colons were analyzed under light microscopy to visualize the number of ACF per mouse colon (ACF/mouse) (**B**) and the number of crypts per focus (**C**). Bars with different letters indicate significant differences at *P* < 0.05.

Figure 3
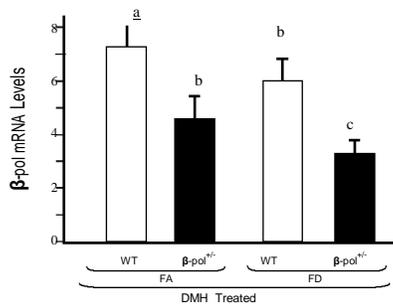
Fig 3. *Impact of folate deficiency and DMH treatment on expression of β-pol in colon mucosa of* β-*pol*^{+/-} *mice.* Expression of **β**-pol gene was determined using a real time PCR technique and the level was normalized base on GAPDH expression as described in materials and methods. Bars with different letters indicate significant differences at *p* < 0.01.

Figure 4

A

B

C

53
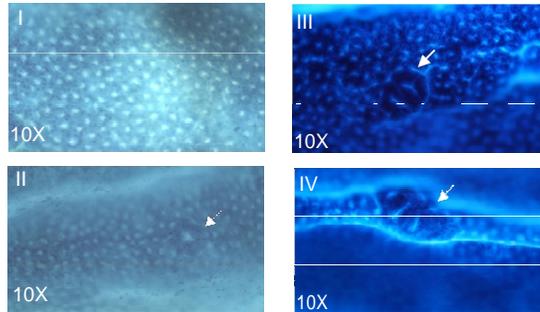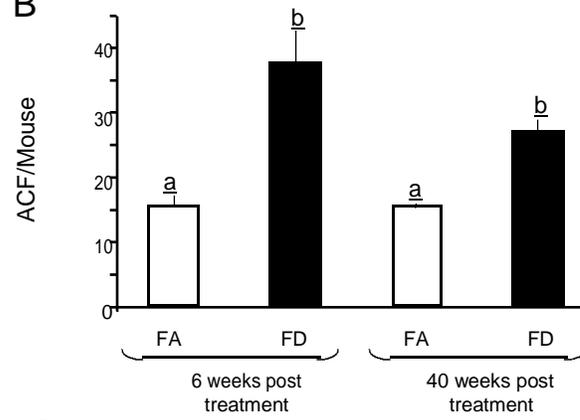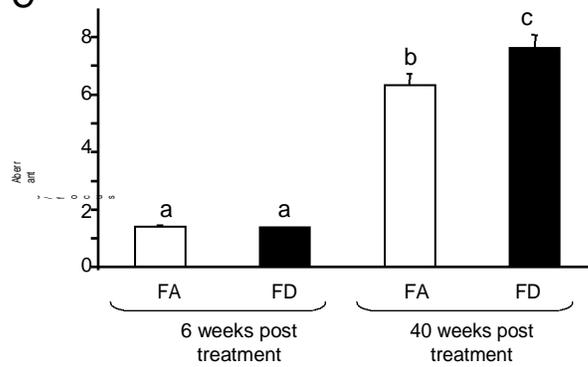
Fig 4. *Impact of long term feeding on ACF formation and Crypts Multiplicity.* Comparison of ACF formation and crypt multiplicity in mice fed either a FA or FD diet at 6 weeks versus 40 weeks post DMH treatment. Panel **A: (I)** Normal colonic crypts, (**II**) ACF formation in FD environment 6 weeks post DMH, (**III**) ACF formation in mice fed FA diet 40 weeks post DMH, and (**IV**) number of ACF of mice fed a FD diet 40 weeks post DMH treatment. Arrows depict area of aberrant crypt formation. Panel **B:** Relative number of ACF/mouse in mice fed either a FA or FD diet 6 weeks or 40 weeks post DMH treatment, respectively. Panel **C:** Number of aberrant crypts per focus in mice fed either a FA or FD diet 6 weeks or 40 weeks post DMH treatment, respectively. Bars with different letters indicate significant differences at $P < 0.05$.

Figure 5

A

DMH Treatment
(30 mg/kg)

Sacrifice for analysis of
ACF and cancer

Time (Wk)   0   1   6   46

Groups

WT   Folate (2 mg/kg)

WT   Folate (0 mg/kg)

β-pol$^{+/-}$ Folate (2 mg/kg)

β-pol$^{+/-}$ Folate (0 mg/kg)

B

FA WT DMH, 0% visible tumor

FD WT DMH, 50% visible tumor

FA ββ-pol$^{+/-}$ DMH, 100% visible tumor

FD ββ-pol$^{+/-}$ DMH, 0% visible tumor
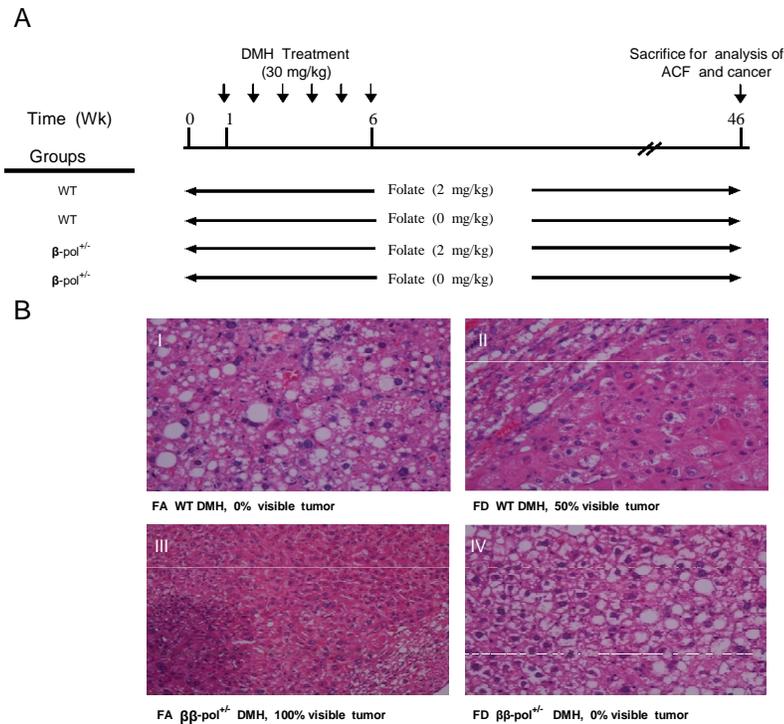
Fig 5. *Impact of β-pol$^{+/-}$ and folate deficiency on induction of tumors in DMH-treated mice.* **A.** Feeding study was conducted as depicted in Figure 2. The mice were sacrificed 40 weeks after last treatment by CO_2 asphyxiation and incidence of tumor progression was assessed. **B.** Exemplary H&E micrographs showing tumor formation in liver sections from (**I**) WT FA (**II**) WT FD, (**III**) FA β-pol$^{+/-}$ and (**IV**) FD β-pol$^{+/-}$ mice treated with DMH. The % value represents the percent of mice with visible liver tumor formation. All wildtype animals fed the FD diet showed liver tumors through H&E analysis.

Figure 6
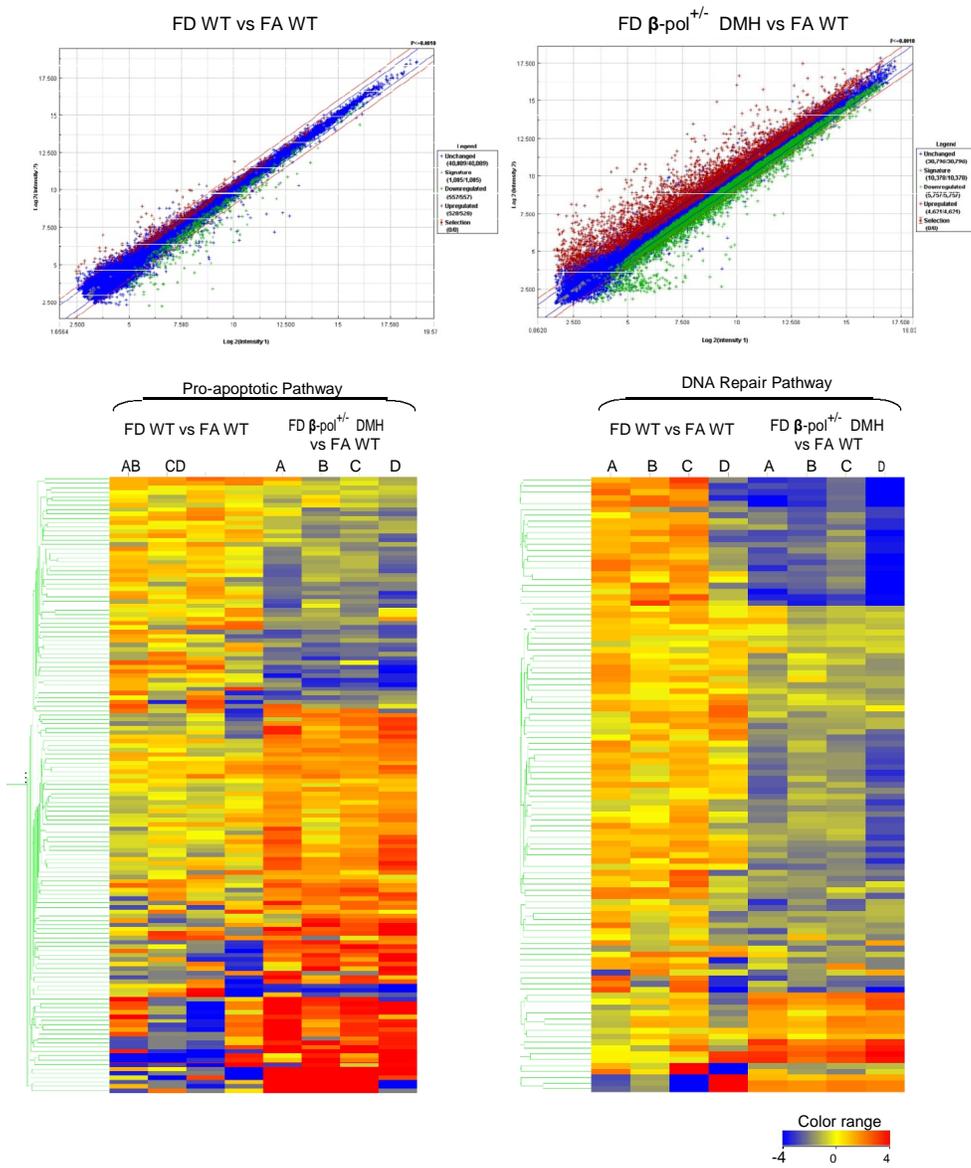
Fig 6. *Scatter plot of differentially expressed genes and heat map representation of microarray data for proapoptotic and DNA repair differentially expressed genes.* **A.** Scatter plot of differentially expressed genes from colon mucosa from FD β-pol$^{+/-}$ relative to FA β-pol$^{+/-}$ tissues and WT FD relative to WT FA tissues included in the Agilent Whole Mouse Genome oligonucleotide micorarray containing probes for over 41,000 well characterized genes. **B.** Hierarchal Clustering analysis was performed using GeneSpringGX V10 (Agilent Technologies) software, and the parameters were set for centroid linkage which calculates the euclidean distance between the respective centroids of two clusters. A heat map in quadruple (A, B, C, and D) for each condition representing proapoptotic and DNA repair genes are shown.

Figure 7
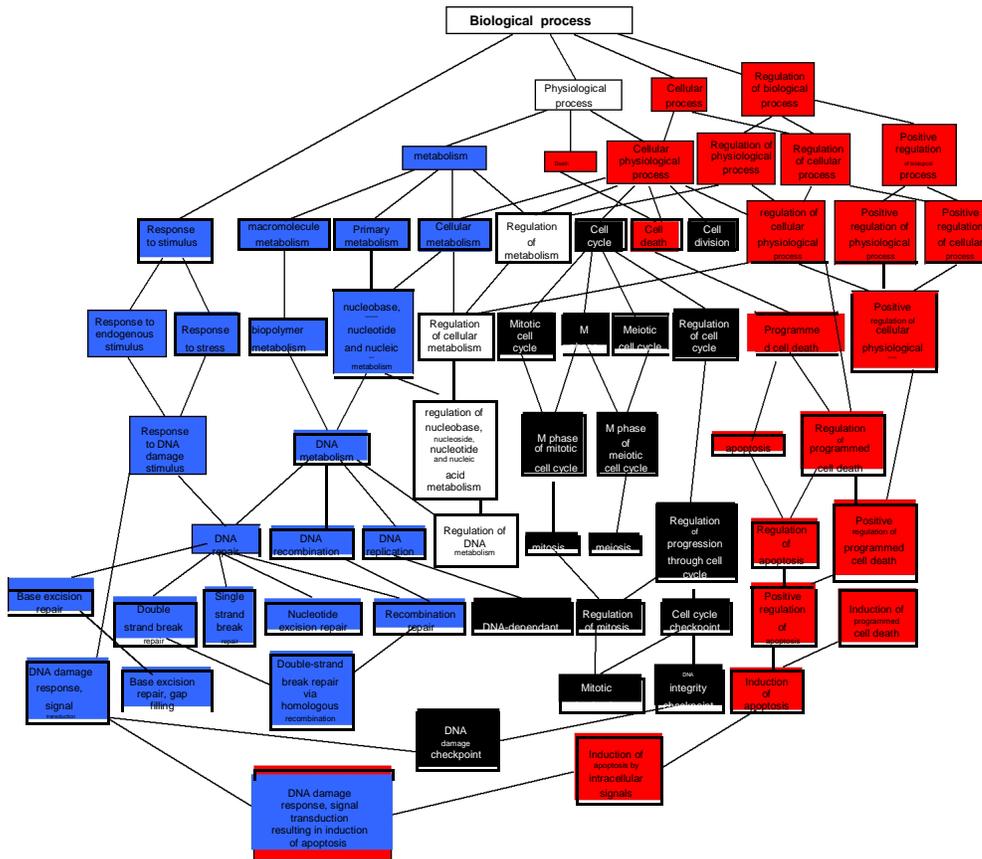
Fig 7. *DAG view of gene ontology analysis, of colonic mucosal tissue in FD_β-pol^{+/-}_versus FA* WT. Blue boxes indicate enrichment of expression of genes related to DNA repair. Red boxes indicate enrichment of expression of genes related to apoptosis.

Figure 8

A

FA WT DMH   FD WT DMH

FA **β**-pol$^{+/-}$ DMH   FD **β**-pol$^{+/-}$ DMH

C

FA **β**-pol$^{+/-}$ DMH   FD **β**-pol$^{+/-}$ DMH

FA **β**-pol$^{+/-}$ DMH   FD **β**-pol$^{+/-}$ DMH

B

D
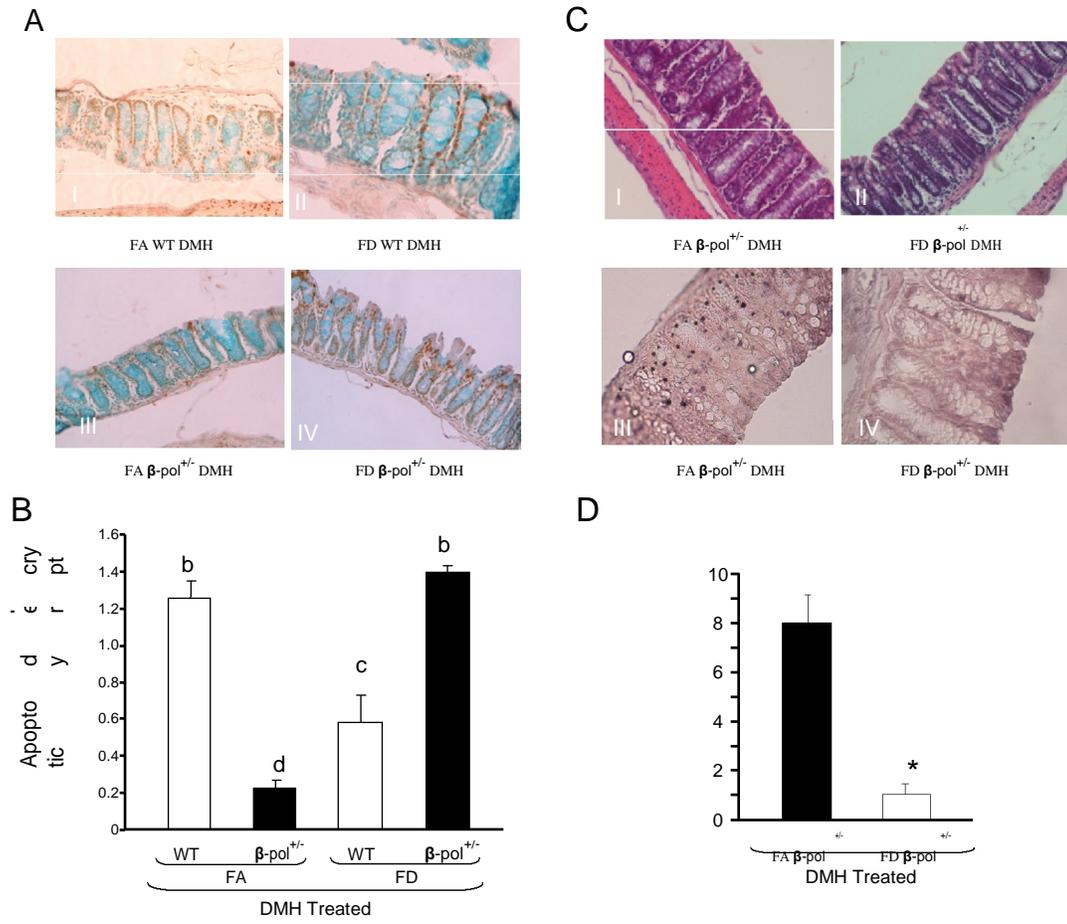
Fig 8. *Impact of* β-pol$^{+/-}$ *and folate deficiency on apoptotic activity and induction of proliferation in colonocytes*. **A**. Representative photomicrographs showing TUNEL-positive staining in cells (*brown*) of colonic mucosa. (**I**) WT FA, (**II**) WT FD, (**III**) FA β-pol$^{+/-}$ and (**IV**) FD β-pol$^{+/-}$ mice treated with DMH. **B**. Tally of the TUNEL-positive apoptotic cells. WT and β-pol$^{+/-}$ were fed either a FA or FD diet and subjected to DMH treatment. Colon tissue was processed, TUNEL assay conducted and percent apoptotic cells were calculated as described in methods. Means without a common letter differ, $P < 0.01$. **C**. Representative photomicrographs showing H&E of colonic mucosa. (**I**) FA β-pol$^{+/-}$ and (**II**) FD β-pol$^{+/-}$ mice treated with DMH. Representative micrographs showing BrdU staining *(brown)* of colonic mucosa. (**III**) FA β-pol$^{+/-}$ and (**IV**) FD β-pol$^{+/-}$ mice treated with DMH. Proliferation Analysis: **β**-pol haploinsufficient mice were treated as described in materials and methods. Briefly, 2 hours prior to sacrifice mice were injected i.p. with BrdU (2.16mg/kg body weight). Colon segments were fixed in 10% formalin and embedded in paraffin. BrdU incorporation was detected by immunostaining as described in materials and methods. **D**. Enumeration of positive proliferation. Percent cell proliferation was conducted as detailed in materials and methods. * indicate differences at P<0.01.

Gene	Sense Primer Sequences	Antisense Primer Sequences
UNG	5'-AACAGGCTTGGTTGGCGAAGG-3'	5'- CGGCTGGCATCCTAAGACATC-3'
ββ-pol	5'-TACGACCAGAGGCATACAGGGACT-3'	5'-ACCAACTGGGACGACATGGAGAAG-3'
Lig3	5'-AAGTTCGCATTCAGGTCTAAGC-3'	5'-AGAGGCTCACAAGTTCCATCC-3'
MPG	5'-GGAGACTGAGGCATACTTGG-3'	5'-GACTAGAGACATTCAAGCAGA-3'
Rad51l1	5'-ATCTCTCACCGGCATTCCTGTCTACTACCC-3'	5'-CCGCCATCTCAACCAGTCTCTCAGCAG-3'
GAPDH	5'-GCCAGCACGCAAAAGGTCACATTGT-3'	5'-AGTTCCGGAAAGCACAGCCAGGATG-3'

Table I: Primer Sequences for Real-Time PCR

Table II. Summary of the effect of folate deficiency on DNA repair pathway genes in wildtype, wildtypeDMH-treated and **β**-pol$^{+/-}$ DMH-treated mice*

Accession no.	Gene name	WT -	WT DMH	B-POL$^{+/-}$ DMH
NM_009764	Breast Cancer 1	-1.7	ns	ns
NM_009765	Breast Cancer 2	-1.6	1.4	1.5
NM_178309	BRCA1 interacting protein C-terminal helicase 1	-1.8	ns	ns
AF236887	Ataxia telangiectasia and Rad3 related	-1.3	1.2	ns
AK048970	Ataxia telangiectasia mutated homolog (human)	-1.6	ns	ns
NM_021385	RAD18 homolog	-1.7	ns	ns
NM_011236	RAD52 homolog (S. cerevisiae)	ns	1.4	1.2
NM_009015	RAD54 like	-2.0	ns	ns
NM_011234	RAD51 homolog	-1.7	ns	1.6
NM_009013	RAD51 associated protein 1	-1.9	1.4	2.0
NM_009014	RAD51-like 1	-2.4	ns	1.3
NM_011677	Uracil DNA glycosylase	-1.6	ns	ns
NM_133250	MutY homolog (E. coli)	-1.5	ns	ns
NM_181569	N-methylpurine-DNA glycosylase	-1.4	ns	ns
NM_009687	Apurinic/apyrimidinic endonuclease 1	-1.7	ns	1.4
AK047252	Polymerase (DNA directed), beta	-1.5	ns	ns
NM_021498	Polymerase (DNA directed), epsilon 3 (p17 subunit)	-1.3	ns	ns
NM_010715	Ligase I, DNA, ATP-dependent	-1.3	ns	1.5
NM_010716	Ligase III, DNA, ATP-dependent	-1.3	ns	ns
AK048442	Ligase IV, ATP-dependent	-1.4	ns	ns
NM_008628	MutS homolog 2	-1.5	ns	ns
NM_010829	MutS homolog 3	-1.4	ns	ns
NM_013600	MutS homolog 5	-1.8	ns	ns
NM_146235	Excision repair cross-complementing complementation group 6 - Like	-2.0	ns	1.8
NM_007949	Excision repair cross-complementing complementation group 2	-1.3	ns	ns
NM_009531	Xeroderma pigmentosum, complementation group C	-1.4	ns	ns
NM_016925	Fanconi anemia, complementation group A	-1.4	1.4	ns
NM_175027	Fanconi anemia, complementation group B	-1.6	ns	ns
NM_007985	Fanconi anemia, complementation group C	-1.3	ns	ns
NM_178912	Fanconi anemia, complementation group M	-1.4	ns	ns
NM_018736	Meiotic recombination 11 homolog A	-1.3	ns	ns
NM_011045	Proliferating cell nuclear antigen	-1.3	ns	ns
NM_009532	X-ray repair complementing defective repair in Chinese hamster cells 1	-1.2	ns	ns
NM_028875	X-ray repair complementing defective repair in Chinese hamster cells 3	-1.7	ns	ns
NM_009533	X-ray repair complementing defective repair in Chinese hamster cells 5	-1.4	ns	ns
NM_010247	X-ray repair complementing defective repair in Chinese hamster cells 6	-1.5	ns	ns

* P<0.01; ns, no significant difference

Table III. Summary of the effect of folate deficiency on apoptotic pathway genes in wildtype, wildtypeDMH-treated and **β**-pol^+/- DMH-treated mice*

Accession no.	Gene name	WT -	WT DMH	B-POL^+/- DMH
NM_008086	Growth arrest specific 1	8.8	ns	ns
NM_011641	Transformation related protein 63	27.4	ns	ns
NM_009367	Transforming growth factor, beta 2	11.4	ns	ns
NM_008337	Interferon gamma	6.2	ns	ns
NM_008329	Interferon activated gene 203	5.2	ns	2.1
NM_009812	Caspase 8	1.4	ns	ns
NM_007609	Caspase 4, apoptosis-related cysteine peptidase	2.1	ns	1.5
XM_139295	Caspase recruitment domain family, member 6	1.4	ns	ns
NM_175362	Caspase recruitment domain family, member 11	3.6	ns	ns
NM_011817	Growth arrest and DNA-damage-inducible 45 gamma	1.5	ns	ns
AK010701	Death effector domain-containing DNA binding protein 2	1.6	ns	ns
NM_025858	Scotin gene	1.5	ns	ns
NM_013693	Tumor necrosis factor	3.9	ns	ns
NM_010177	Fas ligand (TNF superfamily, member 6)	2.1	ns	ns
NM_009425	Mus musculus tumor necrosis factor (ligand) superfamily, member 10	2.0	ns	ns
NM_011614	Tumor necrosis factor (ligand) superfamily, member 12	2.2	ns	ns
NM_178589	Tumor necrosis factor receptor superfamily, member 21	1.4	ns	ns
NM_175649	Mus musculus tumor necrosis factor receptor superfamily, member 26	4.1	ns	ns
NM_183322	Nur77 downstream gene 1	2.5	ns	3.8
NM_009810	Caspase 3	ns	ns	-1.4
NM_134111	ELL associated factor 2	2.6	ns	ns

 * $P<0.01$; ns, no significant difference

Table IV. Summary of the effect of folate deficiency on antiapoptotic pathway genes in wildtype, wildtypeDMH-treated and β-pol$^{+/-}$ DMH-treated mice*

Accession no.	Gene name	WT -	WT DMH	$\beta\beta$-pol$^{+/-}$ DMH
NM_134138	Tumor necrosis factor superfamily, member 5-induced protein 1	-1.4	ns	1.3
AK046479	Apoptosis, caspase activation inhibitor	-1.7	ns	ns
NM_172729	Caspase recruitment domain 4	-1.4	ns	ns
AK018453	Transforming growth factor, beta receptor associated protein 1	-1.6	ns	ns
NM_009743	Bcl2-like 1	-1.4	ns	ns
AK041357	BCL2-like 12 (proline rich)	-1.3	1.8	ns
NM_009736	Bcl2-associated athanogene 1	-1.2	ns	ns
AJ250687	Bcl2-associated athanogene 3	-2.8	ns	ns
BC027637	Transmembrane BAX inhibitor motif containing 4	-1.3	ns	ns
NM_009689	Baculoviral IAP repeat-containing 5	-2.8	ns	ns
NM_007891	E2F transcription factor 1	-2.0	ns	ns
NM_134092	Mdm2, transformed 3T3 cell double minute p53 binding protein	-1.5	1.3	1.6
BC043920	FK506 binding protein 12-rapamycin associated protein 1	-1.8	ns	ns

* $P < 0.01$; ns, no significant difference

Table V. Confirmation of differences observed in Gene Array using real-time PCR for the effect of folate deficiency on DMH-treated knockout mice*

Accession no.	Gene name	Array	Realtime PCR
AK047252	Polymerase (DNA directed), beta	-1.5	-1.8
NM_011677	Uracil DNA glycosylase	-1.6	-1.9
NM_181569	N-methylpurine-DNA glycosylase	-1.4	-1.3
NM_010716	Ligase III, DNA	-1.4	-7.0
NM_009014	RAD51-like 1	-2.4	-4.4

* $P < 0.01$

DISCUSSION

In this study we examined the impact of BER deficiency on colon carcinogenesis in a mouse model. BER processes endogenous damage, as such, our interest has been in developing model systems to test the processing of endogenous damage and determine the impact of BER deficiency on tumorigenesis in vivo. Our laboratory has previously characterized DNA polymerase **β** heterozygous knockout mice displaying haploinsufficiency in **β**-pol gene expression in various tissues (Brain, Liver, spleen and testes) with a concurrent decline in BER capacity (122). These animals exhibit an accumulation of spontaneously arising single strand breaks and chromosomal aberrations with age (60). Additionally, in response to alkylating and oxidizing agents, an even greater accumulation of single strand breaks and chromosomal aberrations, as well as an increase in mutation frequency are observed in these mice (60). In this study, we show a significant decline in **β**-pol expression in the colon of the knockout mice as compared to their wildtype littermates, suggesting a BER deficiency in the colon of these mice. Using the same animal model, Allen et al. (129) showed that normal **β**-pol activity is necessary for maintaining low germline mutation frequency. We previously observed in n **β**-pol haploinsufficient mice, an acceleration of normal, age-related tumors, e.g., lymphomas, developing alongside an increased susceptibility to epithelial tumors, e.g., adenocarcinomas, which do not typically occur at a high incidence in C57BL/6 mice. Additionally, we have shown that in **β**-pol$^{+/-}$ mice, levels of single strand breaks accumulate in

response to oxidative stress, to a greater extent than they do in the wildtype mice (122). Interestingly, in this study despite compromised BER capacity, the **β**-pol mice do not display any evidence of spontaneously induced preneoplastic lesion in their colon confirming **β**-pol's low penetrance. Noting this, we wanted to know the impact of exposure of animals to DMH, an established inducer of colon and liver carcinogenesis. DMH is a DNA damaging agent that induces alkylation damage (O6-meG and N7-meG) (130) as well as oxidative damage (131). We have previously demonstrated that **β**-pol$^{+/-}$ mice are not sensitive to the O6-meG lesion (removed by direct reversal), but is sensitive to the N7-meG lesion processed by BER pathway (122). Intriguingly, in this study, when the **β**-pol$^{+/-}$ mice were treated with DMH a significant increase in ACF formation relative to wildtype counterparts were observed. Thus, herein, we have confirmed that the **β**-pol heterozygous mice are predisposed to preneoplastic lesions and cancer development, primarily by reducing tolerance to the DNA damage in response to DNA damaging agent, DMH. As such, these manipulations gave rise to an animal model of colon carcinogenesis for future study.

While the mechanism by which folate deficiency increases cancer risk is not clear, it has been demonstrated to alter both DNA damage and DNA methylation. Folate deficiency has been shown to induce damage repaired via BER pathway (80). Based on this fact, we were interested in determining what impact folate deficiency would have on a BER deficient model. First, we needed to confirm folate deficiency as an inducer of ACF when wildtype mice are

subjected to a carcinogenic environment. Others, such as Branda et al. (82) have demonstrated that folate deficient animals are less able to repair damages induced by alkylating agents. For example, ENU was found to be more mutagenic within the context of folate deficiency. Interestingly, folate deficiency did not enhance the mutagenic effect of cyclophosphamide suggesting a lack of sensitivity to lesions repaired by the nucleotide excision repair pathway. As well, Duthie et al. (85) have further shown that human colon epithelial cells grown in the absence of folate are poorly able to repair damages induced by MMS (an alkylating agent) and hydrogen peroxide (an oxidizing agent). Growth in a low folate medium has also been shown to impair excision repair capacity of colonocytes (86). More oxidative damage, which is repaired by BER,

accumulates in response to amyloid β-peptide in neuronal cells depleted of folate

(87). In line with these studies, we show an increased incidence of ACF, microscopic adenoma and liver tumor formation in wildtype mice upon DMH treatment, when folate is deficient. These data indicate, in conjunction with our previous studies (80), that the pathway responsible for repairing these damages may be ineffective when folate is limiting. Based on the above findings, it is inviting to suggest that folate deficiency mimics BER deficiency perhaps by overwhelming the capacity of BER through inhibition of its rate determining enzyme, DNA polymerase β. In other words, we suggest that the inability to induce β-pol results in a functional BER deficiency, providing a logical explanation for the phenotype induced by folate deficiency. That is, if folate deficiency exerts its effects by inhibiting BER then the phenotype of folate

deficiency should mimic that of BER deficiency, as shown in our previous studies (80).

To address whether an underlying BER deficiency would exacerbate the effects of folate deficiency, we subjected **β**-pol haploinsufficient mice to folate deficiency. We have recently reported that aging and **β**-pol deficiency interact to accelerate the development of malignant lymphomas and adenocarcinoma and increases tumor bearing load in mice. We anticipated that folate deficiency and BER deficiency will likewise interact to accelerate ACF formation and tumor development in **β**-pol haploinsufficient animals. As expected, folate deficiency resulted in a significant increase in ACF formation in wildtype animals exposed to DMH, however folate deficiency reduced development of ACF in **β**-pol haploinsufficient mice. Interestingly, prolonged feeding of the folate deficient diet resulted in advanced ACF formation, microscopic adenoma and liver tumors in wildtype mice, while **β**-pol haploinsufficiency attenuated onset and progression of ACF to microscopic adenoma and prevented liver tumorgenesis. These findings are rather exciting because folate deficiency appears to provide protection against tumorigenesis in a BER deficient environment in all tissues where DMH exert its damage.

To enhance our understanding of the dynamics contributing to a reduction in ACF formation and liver tumorogenicity we conducted microarray analyses on colon mucosa of **β**-pol haploinsufficient and wildtype mice in both a folate adequate and folate deficient environment. Microarray analysis from our study produced a list of up and down regulated genes, which were classified by their

gene ontologies. The data show a distinctly compromised DNA repair capacity (expression of over 100 genes are downregulated), including, but not restricted to BER, NER and MMR, in FD **β**-pol$^{+/-}$ with DMH treatment. As previously mentioned, BER is responsible for the removal of endogenous alkylation, oxidized bases and uracil. Here we see a decrease in glycosylase, APEX, **β**-pol, and ligase activity in FD DMH treated **β**-pol haploinsufficient mucosal tissue. In addition to BER, NER which is responsible for the repair of bulky distortions in

DNA and MMR, which is accountable for the maintenance of normal Watson Crick base pairing are also impaired. Findings from FD WT DMH treated mice showed a significant upregulation in UNG, APEX, and ligase 1 activity, with a significant downregulation in **β**-pol. These aforementioned findings are in line with our previous studies (80), demonstrating FD ability to impair BER capacity.

We were also interested in the FK506 binding protein 12-rapamycin associated protein 1 (FRAP1), also known as mTOR, for it is activated in various types of neoplastic disease (132), and its inhibition makes cancer cells more sensitive to chemotherapeutic agents (133). FRAP1 is a central controller of cell proliferation, cell growth, and cell survival, becoming impaired in response to DNA damage and nutrient withdrawal (134). Relative to our study, we see that either damage as a result of a BER deficiency or deprivation of folate enhances cell survival as depicted by increased incidence of ACF and tumor formation. Fujishita et al. (135), however, have shown that inhibition of the mTOR pathway suppresses the formation of intestinal polyps and death rates in Apc min mice. As well, Shen et al. (136), showed in their study utilizing yeast cells, that the

inhibition of FRAP1 through TORC1 complex by rapamycin, enhanced the sensitivity to MMS treatment, resulting in increased cell death. Interestingly, we show a significant downregulation in FRAP1, in FD DMH $\tilde{\beta}$pol haploinsufficiency.

These findings are similar to that of Fujishita (135) and Shen (136), although here we show FD inhibiting an already BER deficient environment, suggesting a likeness of rapamycin inhibiting TORC 1 complex and as such, enhances the lethality of DMH. We suggest, therefore, that the downregulation of FRAP1 in the FD DMH **β**-pol haploinsufficient mouse model could account for the reduced incidence of ACF and tumor formation.

Observing the lack of response in DNA repair and reduction in FRAP1, we studied the expression of apoptotic gene on our microarrays to determine if cell death may be responsible for tending of imposed damage and thus reducing the level of ACF and tumor formation. Notably, there was an upregulation of 75 differentially expressed genes related to apoptotic activity. There was an upregulation in intrinsic/extrinsic apoptotic, and TNF signaling, as well as cell cycle arrest in **β**-pol haploinsufficient mice. Interestingly, we see a reduction in ATM, ATR and CHK2, which phosphorylate p53, rendering it active, however, a significant increase in p21 expression, a collective inhibitor of cell cycle progression, is detected (137). Both, CASP11, which is tightly regulated at the transcription level, and known to regulate both apoptosis and inflammation (138), and CASP8, an initiator of apoptosis (139) are elevated in **β**-pol haploinsufficient mice in a folate deficient environment. In addition, we see a highly significant up regulation in GAS1, a known tumor suppressor (140, 141) that mediates cell

cycle arrest and apoptosis (142, 143, 144, 145). Based on our results, we would suggest that colon cells in DMH treated **β**-pol haploinsufficient mice in a folate deficient environment would prefer to undergo cell death, rather than attempt repair. Interestingly, a distinct upregulation in PARP 3, 4, 8, 12 and 14 expression in FD, DMH treated **β**-pol haploinsufficient colonic mucosa is observed. In addition to mediating BER by recruiting BER intermediates like **β**-pol, PARPs are the key regulators of cell survival and cell death (146). As well,

Huang et al. (147), in their study utilizing $Bax^{-/-}Bak^{-/-}$ mouse embryonic fibroblasts, show depletion in ATP secondary to PARP-1 activation, which in turn inhibits FRAP1. We suggest that down regulation in expression of key enzymes in BER pathway, e.g., UNG, **β**-pol and APE1, Lig1 and XRCC1, renders available PARPs futile. In other words, elevation in PARPS would result in depletion of ATP/NAD(+) levels and subsequent down regulation in FRAP1/mTOR pathway.

Moreover, cleavage of PARPs by CASP3 (148) triggers apoptosis and induces cell death. Interestingly, while FD WT DMH treated animals show a significant decline in the level of CASP3, a key "executioner" of apoptosis (149). It should be noted that the activity in FD WT counterparts show either no change in differential expression or slight opposing expression of both apoptotic and DNA repair genes. These findings provide further evidence of defense by BER deficiency in the multi-step process of colon/liver cancer, when folate is deficient.

We propose that **β**-pol deficiency in a folate deficient environment results in increased accumulation of DNA repair intermediates that impacts the balance between cell survival and cell death, pushing cells toward apoptosis, thus

reducing onset and progression of preneoplastic lesions. In support of this notion, we provide evidence that the **β**-pol haploinsufficient mice fed a folate deficient diet display increased apoptotic body and decreased proliferation in their colon in response to DMH. Interestingly, in agreement with our findings,

Ochs et al (150) demonstrated that BER deficiency results in the accumulation of repair intermediates, along with increased DSB in **β**-pol null fibroblasts exposed to the alkylating agent, MMS. Interestingly, accumulation of DNA damage in these cells resulted in decline in *Bcl*-2 and increased Caspase 3/9 activation triggering apoptosis in a replication dependent manner. Moreover, Taverna et al (151) showed that BER inhibition by methoxyamine increased cytotoxicity of the methylating agent temozolomide through persistence in AP sites, increased DSB and increased apoptosis in HCT 116 colon cancer cell lines. Likewise, Rinne et al. (152) showed that breast cancer cell lines overexpressing *N*-methylpurine DNA glycosylase (MPG) exposed to the alkylating agents methyl methanesulfonate, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, methylnitrosourea, dimethyl sulfate, and the clinical chemotherapeutic temozolomide, significantly increases the sensitivity of these cells. Sensitivity is further increased through coadministration of the BER inhibitor methoxyamine making cells unable to complete repair initiated by the glycosylase, and resulting in an increase in the number of AP sites and SSBs. Furthermore, Fishel et al. (153) showed that overexpression of MPG, in addition to inhibition of BER pathway via methoxyamine, dramatically increases the sensitivity of ovarian cancer cells to the DNA methylating agent temozolomide via increase in apoptotic activity. In

other words, methylation of N^7 and O^6 positions of guanine and the N^3 of adenine by temozolomide, in addition to overexpression of MPG, resulted in initiation of BER. However, inhibition of BER pathway by methoxyamine downstream of glycosylase resulted in an accumulation of DNA repair intermediate, increased DNA DSB and triggered apoptosis in a p53 independent manner. Furthermore, Trivedi et al. (154) showed that elevated MPG expression in addition to **β**-pol knockdown would result in increased sensitivity of human breast cancer cells to temozolomide. Interestingly, Trivedi et al (154) showed that overexpression of

β-pol with mutation in its polymerase site, while maintaining it dRP lyase activity, resulted in restoration of resistance to temozolomide in **β**-pol knockdown human breast cancer cell lines.

The in vitro studies outlined above utilize specific glycosylases for the creation and persistence of abasic sites, while, methoxyamine potentiates the cytotoxicity of telozolomide through its binding to abasic sites, hampering BER, resulting in accumulation of repair intermediates and ultimately, DNA DSBs. These conditions generate a perfect environment for apoptosis to take place.

These studies add to the fascination of our own, for the aforementioned were conducted within cell lines, whereas, our in vivo data demonstrate protection against actual onset and progression of tumor, via similar mechanism. Our data shows that treatment of mice with DMH results in damage to bases, along with upregulation in UNG expression/activity (data not shown), that when combined with β̃pol haploinsufficiency, results in an accumulation of DNA repair intermediates and ultimately DNA DSBs, resulting in the onset of ACF,

progression of microadenoma and tumors, in colon and liver, respectively. We suggest that wildtype animals fed a folate deficient diet and subject to DMH treatment exhibit a similar scenario. In other words, folate deficiency and DMH treatment would result in the induction of DNA damage, upregulation in specific glycosylases, and downregulation in BER capacity all of which contribute to an accumulation of repair intermediates that create an environment favoring tumor formation. In contrast, BER haploinsufficiency combined with folate deficiency provide a protective environment against DMH carcinogenesis. Thus, folate deficiency sensitizes the **β**-pol haploinsufficient mice to DMH, generating even higher level of DNA SSBs and DSBs, mimicking the environment observed in in-vitro studies. These conditions are favorable to apoptosis, where DNA repair machineries are downregulated and proapoptotic pathways are induced.

In line with our findings, Lawrance et al. (155) showed that folate deficiency in *mthfr* deficient $Apc^{min/+}$ mice results in a reduction in adenoma formation in colon tissue, while an elevation in dUTP/dTTP ratio in DNA and an increase in apoptotic activity are observed. APC has been shown to interact with **β**-pol (156). Furthermore, haploinsufficiency in reduced-folate carrier gene ($Rfc^{+/-}$) creating a folate deficiency phenotype resulted in decreased adenomas and tumor load in $Apc^{min/+}$ mice. In addition, Branda et al (157) showed folate deficiency reduced mutation frequency in 3-methyladenine glycosylase null mice exposed to methyl methanesulfonate. The aforementioned, demonstrate much the same as our investigation in that modulating BER repair, through **β**-pol and exposure to folate deficiency, results in reduced repair activity while influencing

apoptosis. Additionally, research conducted by Duthie et al. (158), demonstrated that NCM460 immortalized cells in a folate deficient media exposed to hydrogen peroxide exhibit increased apoptotic activity, as well as, decreased proliferation. Furthermore, Crott et al. (159) showed an inverse correlation between expression of genes involved in cell cycle checkpoint and media folate levels (25 to 100nM) in NCM460, HCEC and NCM356 cell lines derived from human colon, irrespective of oxidative stress. These studies, much like our own, have significantly reduced levels of folate, rather than a complete folate deficit. In our experiments, despite use of a folate deficient diet and use of succinyl sulfathiazole, an 80 – 90% reduction in folate levels is observed relative to folate adequate diets. It is at this reduced level that we see protective effects.

In conclusion, our data indicate BER deficiency and DMH treatment interact synergistically to increase DNA repair intermediates and genomic instability, resulting in formation of preneoplastic lesions, while folate deficiency in BER deficient mice exposed to DMH provides protection, creating an environment preferential to apoptosis. While folate deficiency induces DNA damage in control/untreated mice (80), slight upregulation in DNA repair activity and maintaining normal or slightly lower levels of apoptotic activity prevents onset of tumorigenesis. In contrast, folate deficiency in response to DNA damaging agent would result in accumulation of genotoxic intermediates that are potentially mutagenic and thus, as shown here, prone to increased ACF and tumor formation. However, when a BER deficiency is present and subject to exposure of DMH, induction of a folate deficient environment results in a further

reduction of DNA repair capacity and increased level of apoptosis. This is suggestive that the damage is so extensive that is beyond repair, thus opting for cell death. We surmise that the damage induced blocks BER capacity, resulting in elevated PARP levels, which in turn deplete energy availability to the cell, signalling need for cell death. These conditions result in a reduction of ACF and tumor formation. This study has great therapeutic importance and human relevance as recent findings indicate BER inhibitors in combination with the DNA methylating agent temozolomide, function as an effective chemopreventive agent in colon cancer (160). In addition, these findings have relevant translational implications, since variants/polymorphisms in BER has been associated with increased cancer risk, and an alteration in micronutrients could potentially provide protections under the right conditions.

CHAPTER 4

Microarray Gene Expression Profiling of Dietary Folate Deficiency on Colon Carcinogenesis in DNA Polymerase ββ Haploinsufficient Mice

Introduction

Colorectal cancer is one of the most prevalent and lethal types of malignancies in Western countries and represents the second leading cause of cancer death in the US (161). It has been implicated that environmental factors relative to a Western lifestyle are responsible for this intestinal neoplastic transformation. Much research has focused on the strong connection between the carcinogenic properties of folate deficiency and increased colon cancer risk (162). It has been proposed that folate deficiency imparts high levels of uracil and oxidative damage to the DNA that may increase the risk of atypical DNA methylation, high levels of uracil misincorporation in DNA, and increased levels of DNA single strand breaks. Folate has also been shown to increase micronucleus formation and chromosomal aberrations all leading to increased mutational frequency and colon cancer progression.

It is well documented that the Base Excision Repair (BER) pathway is responsible for the removal of uracil and oxidized bases throughout the cell cycle. Its primary obligation is believed to be the repair of small, non-helix distorted lesions in the DNA. If damaged bases are left unrepaired it could cause mutations via mispairing and/or strand breaks in DNA during replication. Briefly, the removal of damaged bases by BER involves i) a specific DNA glycosylase binding to the affected base and hydrolyzing the N-glycosidic bond, releasing the damaged base while keeping the DNA backbone intact. ii) The abasic site is

subsequently recognized by an endonuclease, notably Ape 1, which nicks the phosphodiester backbone immediately 5' to the lesion leaving a strand break with a normal 3'-hydroxyl group and an abnormal 5'dRp residue. iii) synthesis of new DNA by incorporation of the correct nucleotide by DNA polymerase, iv) excision of the 5'dRp moiety, and v) ligation by a DNA ligase (Ligase 1 or Ligase 3/XCRR1 (127). Short patch BER is the major repair mode, where **β**-pol performs the polymerization steps and appears to be the rate-limiting step by virtue of its dRp lyase activity (53). With the removal of damaged bases, as would occur with uracil misincorporation, and repair of abasic sites, a transient formation of a DNA single strand break occurs. We have previously shown that **β**-pol$^{+/-}$ results in genomic instability as a result of persistence of these DNA single strand breaks which could result in DNA double stand breaks (60). Additionally, in response to alkylating and oxidizing agents, these animals display accelerated age-related tumor development such as lymphomas, which are atypical of C57BL/mice (77).

The **β**-pol haploinsufficient mouse has been shown to have a reduced ability to tolerate carcinogen exposure, demonstrating reduced BER capacity and this translates into concern regarding the development of certain cancers. In fact, mutations in the gene encoding **β**-pol have been previously identified in human colorectal, prostate, lung and breast carcinomas (68, 69, 70, 71). These mutations are found only in the tumor tissue and not in the adjacent normal tissue, thus corresponding to sporadic mutations (73). Based on these findings and our previous reports of **β**-pol haploinsufficiency on DNA (60), it would seem

prudent to assume that the high levels of uracil misincorporated into the DNA backbone, as a result of inadequate folate levels, would lead to an increase insingle strand breaks as a result of β-pol deficiency, overwhelming BER, proceeding to mutagenesis. Centered on these experimental findings, in our

study of β-pol$^{+/-}$ in a folate deficient environment, we expected that β-pol haploinsufficiency would result in extreme damage with a dramatic increase in the formation of the first histologically detectable change apparent in colon cancer, the aberrant crypt and potentially progressive colon cancer development. Astoundingly, we saw not only significantly less ACF development but additionally, those that did form were visually less advanced than wildtype mice exposed to 1,2 dimethylhydrazine (DMH) a known colon and liver carcinogen. As well, we saw no detectable pathological changes in liver tissue as noted in the wildtype folate deficient mice subject to DMH (Figure 5B). Based on our remarkable findings, the purpose of this study was to determine the potential mechanism behind the protective effect β-pol haploinsufficiency appears to confer with respect to colon cancer development in response to folate deficiency and DMH. Microarray technology has made accessible a powerful tool for identifying possible means of the effect of folate and DMH on the onset and progression of colon cancer in the colonic mucosa of β-pol haploinsufficient mice at the level of the transcriptome. These findings are invaluable for they offer direction, through pinpointing potential means of this disease, necessary for further studies.

Methods

Animals. Experiments were performed in young 4 to 6 month old C57BL/6-specific pathogen free male mice and mice heterozygous for the DNA polymerase **β** gene (**β**-pol$^{+/-}$) (77). All practices performed on animals were in agreement with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. Mice were backcrossed to the C57BL/6 background. The Wayne State University Animal Investigation Committee approved the animal protocol. Mice were maintained on a 12-h light/dark cycle and given water *ad libitum*.

Diets and Carcinogenic Treatment. After acclimation for 7 days, wildtype (WT) and **β**-pol$^{+/-}$ mice were randomly assigned to two dietary groups; a folate adequate (FA) or folate deficient (FD) AIN93G-purified isoenergetic diet (Dyets, Inc., Lehigh Valley, PA) as described previously (80). The FA group received a folate adequate diet containing 2 mg/kg folic acid. The FD group received a folate-deficient diet containing 0 mg/kg folic acid. Diets were stored at −20℃. 1% succinyl sulfathiazole was added to all diets . One week after commencement of food ingestion, randomly selected mice from both FA and FD were injected i.p. with 1,2-Dimethylhydrazine HCL (DMH, 30 mg/kg body weight) in 10mmol/L of NaHCO$_3$ (Fisher Scientific, Fair Lawn, NJ) once a week for 6 wks (Figure2, panel A). Both food intake and body weights were checked twice weekly to monitor for signs of of toxicity, e.g., weight loss, and the diets were continued for 12 weeks.

Microarray assays. Total RNAs were isolated from the colon mucosa of mice using the RNeasy Mini Kit (Qiagen, Valencia, CA) per manufacturer's

protocol. RNA samples were quantified with NanoDrop ND-1000 (NanoDrop Technologies, Inc, Wilmington, DE) and 260/280 ratio in the range of 2.0-2.2 was defined as acceptable. A quality check of the total RNA was performed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). One micro liter of total RNA sample was applied on RNA 6000 NanoChip, and the assay was run on the Bioanalyzer to determine if the 18S and 28S ribosomal bands are defined and to ensure no RNA degradation was present. Optimal concentration used to check the RNA quality is 250ng/∝l.

Microarray expression profiling was conducted by Microarray & Bioinformatics Facility Core at Wayne State University (Institute of Environmental Health Sciences, Detroit, MI) according to the manufacturer's protocol. A balanced block experimental design was used: 4 microarrays were completed for each comparison, with each microarray representing 1 randomly selected labeled mucosal sample from each experimental group paired with 1 randomly selected FA WT labeled mucosal sample i.e. in total 4 mucosal RNA samples from FDWT cohybridized with 4 FAWT, 4 FDWT DMH treated cohybridized with 4 FAWT and 4 FD $\boldsymbol{\beta}$-pol$^{+/-}$ DMH cohybridized with 4 FAWT treated, representing 4 micorarrays for each experimental comparison. Samples on a given array were oppositely labeled with Alexa 647 and Alexa 555 dyes. The four microarrays for a given group (i.e., WT untreated, WT DMH or $\boldsymbol{\beta}$-pol$^{+/-}$ DMH treated) represent samples from eight separate mice, providing consideration of biological variation. In total, 12 arrays representing 24 mice (8 WT, 8 WT DMH-treated, 8 $\boldsymbol{\beta}$-pol$^{+/-}$ DMH-treated) were completed. Dye swaps were used to account for dye bias effects

such that of the four arrays in a given phenotype group, two had FD treated

samples labeled with Alexa 647 co-hybridized with control samples labeled with

Alexa 555 while the other two arrays within the same phenotype group had

opposite dye orientations. Microarrays were scanned using the Agilent dual laser

DNA micorarray scanner model G2565AA, with 10 micron resolution.

   Microarray gene profile analysis. Microarray image analysis was

performed with Agilent Feature Extraction software, version A.5.1.1. Hierarchal

Clustering analysis was performed using GeneSpringGX V10 (Agilent

Technologies) software, and the parameters were set for centroid linkage which

calculates the euclidean distance between the respective centroids of two

clusters. Heatmaps were created by inputting accession numbers of differentially

expressed genes, at p<0.001, for FDWT and FD **β**-pol$^{+/-}$ DMH-treated

experimental groups through DAVID functional annotation. Outputs revealed

gene onotologies of differentially expressed genes, which were than compared to

created differentially expressed gene lists of DAVID biological processes, such

as apoptosis and DNA repair. Data sets were then combined for FDWT and FD

β-pol$^{+/-}$ DMH-treated to 1 large data set in order to create heatmaps for each

biological function. Gene ontology analysis was performed using Gene Ontology

Tree Machine (GOTM)**,** (Bioinformatics,Vanderbilt University), applying

differentially expressed genes as depicted in the heatmaps`. We chose a single

gene set analysis, where GOTM compares the distribution of single gene set in

each GO category to those in an existing reference gene list from the mouse

genome, identifying GO categories with statistically significant enriched gene

numbers as determined by the hypergeometric test (p<0.01) (119, 120). Real time quantitative RT-PCR was used to confirm the data obtained for selected genes in DNA repair pathways as described above.

Results

Experimental design

To elicit the role that folate plays in the onset of colon carcinogenesis in a BER deficient environment, wildtype and β-pol$^{+/-}$ animals were fed either a folate adequate (FA, 2 mg/kg folic acid) or a folate deficient (FD, 0 mg/kg folic acid) diet as outlined in Figure 2A. To induce severe folate deficiency, 1% Succinyl Sulfathiazole was added to the diet deficient in folic acid to prevent synthesis of folic acid in the gut by bacteria. The animals' food intake and body weight were monitored weekly. Folate deficiency did not affect body weight while it reduced the plasma folate level by 90% as determined by a SimulTRAC-SNB radioassay kit for vitamin B_{12} (^{57}Co) and folate (^{125}I) per the manufacturer's protocol (ICN Diagnostics, Orangeburg, NY) as described previously (80). After one week on respective diet randomly selected mice from each feeding study were injected with 30mg/kg body weight DMH once per week for 6 weeks (Figure 2A). We chose DMH as it is a known colon and liver carcinogen that gets converted to the active metabolites azoxymethane and methylazoxymethanol in the liver, which are then transported to the colon via blood and bile (125). Once in the tissues DMH induces alkylation (O6-meG and N7-meG) as well as oxidative damage impairing DNA. Although direct reversal is responsible for the repair of 06-meG, we have previously shown that the BER pathway processes N7-meG lesions

(60). Six weeks after the final injection DMH-treated and control mice were sacrificed. Mucosal scrapings were taken and used to perform a balanced block experimental microarray design to screen for differentially expressed genes in response to folate deficiency (FD) and 1,2- dimethylhydrazine (DMH) mice.

Microarray gene expression analysis

Our previous studies determined an increased level of ACF, increased cell proliferation, as depicted by BrdU analysis and a decrease in apoptotic activity, shown by the TUNEL assay, in wildtype mice exposed to DMH in a folate

deficient environment. In contrast, FD DMH **β**-pol$^{+/-}$ treated mice showed decreased formation of ACF, reduced proliferative capacity and an increase in the level of apoptosis (163). We wanted to know, therefore, the effect of folate and DMH on gene expression. In order to do so, we categorized genes according to their biological function as depicted through DAVID biological processes.

Gene Expression analysis of folate pathway

Knowing folates suggested role in colon carcinogenesis, we initially analyzed microarray gene expression data with respect to folate and its metabolism (Table VI). Here we see a down regulation in N^5, N^{10} methylene tetrahydrfolate reductase (MTHFR), an enzyme which reduces 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate using NAD(P)H as the reducing agent, in FA DMH, FD DMH treated and FD **β**-pol$^{+/-}$ DMH treated mice. As well, we see an upregulation of dihydrofolate reductase (DHFR), which

reduces dihydrofolic acid to tetrahydrofolic acid, using NADPH as electron donor, in FDWT, FA DMH, and FD DMH treated colonic mucosa, however it is

significantly downregulated in FD β-pol$^{+/-}$ DMH treated mice. Interestingly, we also see an increase in thymidylate synthase (TS), an enzyme which converts dUMP to dTMP, in FD DMH treated animals and no change in FD WT or FA WT

DMH treated mice, whereas FD β-pol$^{+/-}$ DMH treated mice show a significant downregulation. Correspondingly, there is a significant downregulation in dUTPase (5031412I06Rik) in β-pol haploinsufficiency which is either upregulated or not differentially expressed in other animal models. This indicates a lack of

pyrimidine synthesis in FD β-pol$^{+/-}$ with DMH treatment, in contrast to the other experimental models which are still capable of producing thymidine. From these findings one could conclude that availability of purines and pyrimidines would be significantly lowered in the β-pol haploinsufficient mouse when subject to DMH and a folate deficit. These results shed light on our previous findings of reduced

DNA repair capacity in FD β-pol$^{+/-}$ DMH treated mice.

Gene Expression analysis of DNA repair genes

Both FD DMH and FA DMH treated mice showed a 75% elevation in DNA repair expression (44 upregulated and 15 downregulated, 33 upregulated and 11 downregulated, respectively), whereas FD untreated animals demonstrated an 84% elevation (31 upregulated and 6 downregulated) in repair activity.

Interestingly, FD β-pol$^{+/-}$ DMH treated mice shows an 88% reduction in DNA repair activity (13 upregulated and 92 downregulated). These findings indicate

sufficient levels of purines and pyrimidines for DNA repair in FDWT, FA DMH, and FD DMH treated mice, however, deficiency in these nitrogenous bases

appears to hamper DNA repair in FD β-pol$^{+/-}$ DMH treated mice. It should be noted that genes expressed in any experimental model are characteristically, but not exclusively related to BER. NER, MMR NHEJ and recombination repair (Table VII). Denoting the reduced capacity in repair, determining repair machineries access to chromatin is important, for this access is needed in order for repair to proceed.

Gene expression analysis of histones

Noting the differential expression of DNA repair genes in all animal models studied, we wanted to know the impact of a BER deficiency on histone modification when folate is deficient, for it is the histones that unpackage the DNA in order for damage recognition. Chromatin remodeling allows for changes in histone-DNA interactions in the nucleosome. This is achieved by the action of chromatin-remodeling complexes, which are a family of ATP-dependent machines that participate in transcriptional regulation, DNA repair, and chromatin assembly. (164). Gene expression of these family subunits are significantly

downregulated in FD β-pol$^{+/-}$ DMH treated colonic mucosa, but show no differential expression in FD WT, FA WT DMH, and FD WT DMH treated mice (Table VIII). Our microarray analysis also shows a significant reduction in genes related to histone H2A, one of the core components of the nucleosome, in FD β-pol$^{+/-}$ DMH treated mice, whereas all other experimental groups showed no change in expression of H2A with the exception of one varied gene being

downregulated in each of the groups. Of particular interest is H2AX , for it is a receptive target for looking at DSBs in the cell. Once phosphorylated, H2AX lessens DNA condensation and increasing its availability for recruitment of repair machinery to DSBs (165). We have previously ascertained an increase in SSB in **β**-pol haploinsufficiency exposed to a folate deficit (80). If these SSB are not properly repaired, they may result in double strand breaks (DSB) in replicating DNA. DSB recognition involves activation of ATM which functions to control the rate at which cells grow. In turn, ATM phosphorylates H2afx. Both ATM (Table VII) and H2AFX are significantly downregulated in FD **β**-pol haploinsufficiency with i.p DMH treatment. All other experimental groups showed no difference in expression of these aforementioned genes. Although phosphorylation of H2AX is most dependent on ATM, phosphorylation can also be carried out via ATR (Table VII) and PRKDC (also known as DNA-pk)(Table VII) (165, 166, 167, 168) Both of these aforementioned genes are instrumental in NHEJ and HR, pathways that are significantly downregulated in FD **β**-pol$^{+/-}$ DMH treated mice, whereas they are either increased or not significantly different in FD WT, FA WT DMH, and FD WT DMH treated mice. These findings are suggestive of chromatin condensation an event which would favor apoptotic activity (169).

Gene expression analysis apoptotic activity

Based on our previous findings of apoptotic activity depicted by TUNEL assay (Figure 8A), we wanted to know the apoptotic gene expression of all experimental models. We saw a 51% upregulation (22 upregulated and 21 downregulated) in proapoptotic activity in FD DMH treated mice and 57% (69

upregulated and 53 downregulated) in FD β-pol$^{+/-}$ treated mice in contrast to FD WT untreated (70% reduction, 9 upregulated and 21 downregulated) and FA WT DMH treated (49% reduction, 19 upregulated and 20 downregulated). There was a 59% decline (15 upregulated and 19 downregulated), 67% decline (9 upregulated and 18 downregulated), 71% decline (11 upregulated and 27 downregulated) and 52% decline (23 upregulated and 25 downregulated) in antiapoptotic genes in FD WT DMH, FD untreated, FA WT DMH and β-pol$^{+/-}$ treated mice, respectively. Although apoptotic levels are inclined in FD WT DMH treated, CASP3, which is downstream in the apoptotic pathway, is significantly downregulated, whereas it is significantly upregulated in FD β-pol$^{+/-}$ DMH treated and unchanged in FD WT untreated or FA WT DMH treated mice (Table IX). Noting lack of DNA repair activity and elevation of apoptosis, specifically CASP3 in FD β-pol$^{+/-}$ DMH treated mice; we wanted to determine the impact of PARP activity on colonic mucosa.

Gene expression analysis of poly(ADP-ribose) polymerase (PARP)

We wanted to know the level of PARP activity as it plays a vital role in DNA repair and programmed cell death. The most widely studied PARP is PARP1, which is activated mainly by SSBs or DSBs (170). Inhibition of PARP enzyme activity interferes with DNA base excision repair and leads to increased genetic instability and recombination but, on the other hand, can sensitize cells to apoptotic stimuli and by this mechanism may prevent tumor formation. While FD untreated mice show reduced or unchanged PARP expression, WT FD DMH mice display increased levels, specifically PARP1 and PARP3, which associates

with components of BER and NHEJ (171) or no change in gene expression and FA WT DMH as well, show elevated levels of PARP1 or no significant change, as did FD β-pol$^{+/-}$ DMH treated mice with the exception of PARP1 which is downregulated (Table X). When PARPs become elevated they signal for apoptosis or necrosis, which then becomes cleaved by CASP3 in order to prevent depletion of NAD. In this current study their is a reduction in PARP1 in

FD β-pol$^{+/}$- DMH treated mice and it appears as though the reduction in PARP1 maybe related to its cleavage by CASP3. While PARP1 levels are elevated in both FD WT DMH and FA WT DMH treated mice CASP3 is either downregulated or unchanged in these animal models, respectively (Table IX). We suggest that

the level of cellular toxicity imposed by FD β-pol$^{+/}$- DMH treated mice causes a significant reduction in ATP levels hence the signaling for PARP1 cleavage by CASP3.

Gene expression analysis of energy sensors

Noting a reduction in ATP dependent chromatin remodeling machinery, DNA repair and PARP, as well as an increase in apoptotic activity, we wanted to know the impact of β-pol haploinsufficiency on energy sensing gene expression. Microarray analysis demonstrates a significant upregulation in energy sensing enzymes Cab39 (aka MO25), a scaffold protein that activates Stk11 (aka LKB1), and Stk11, a tumor suppressor kinase that phosphorylates and activates AMP-activated protein kinase (AMPK) when cellular energy levels are low (172), in FD β-pol$^{+/-}$ treated mice (Table XI). In addition, Prkab1 (aka AMPK), which inhibits cell growth and proliferation through multiple pathways (172) is significantly

elevated in FD **β**-pol$^{+/-}$ treated mice. This information provides confirmation of energetic stress, consequently resulting in inhibition of cell survival and support of cell death. In view of these findings we wanted to look at the gene expression of downstream targets of AMPK that either produce ATP or consume it.

Differential expression of genes in downstream events of AMPK

Based on significant upregulation in expression of genes related to energy sensing, we wanted to know the impact of FD **β**-pol$^{+/-}$ DMH treatment on ATP-producing versus ATP-consuming processes. Interestingly, we see a significant upregulation in expression of genes related to glucose uptake (GLUT4), glycolysis (Pfkfb3), and mitochondrial biogenesis (Ppargc1), all of which result in ATP-producing catabolic processes. In contrast, we see a downregulation in gene expression of enzymes involved in fatty acid synthesis (Acaca), sterol synthesis (Hmgcr), glycogen synthesis (Gys2) and protein synthesis (FRAP1), indicating a downregulation in ATP-consuming processes (Table XI). These findings suggest that amidst energy deprivation, cells react to try and conserve ATP. With the aforementioned, we decided to analyze gene expression levels related to the cell cycle pathway.

Expression analysis of cell cycle related genes

Knowing the differential expression apoptotic capacity in all animal models, we decided to study the effects of FD and DMH on cell cycle pathway gene expression to gather a further understanding of the series of events that take place in the cell leading to cell division and replication. We see relative little difference in gene expression related to cell cycle progression in FD DMH (67%

increase, 24 upregulated and 12 downregulated), FD untreated (65% increase, 25 upregulated and 13 downregulated) and FA DMH (68% increase, 32 upregulated and 15 downregulated), respectively). Conversely, FD **β**-pol$^{+/-}$ treated mice show a 65% downregulation in expression of genes related to cell cycle progression.(18 upregulated and 33 downregulated). Of interest, Cdk4 which is important for cell cycle G1 phase progression and Cdk 6 for both progression and G1/S transition, cyclin E1, which forms a complex with cdk2 also for cell cycle G1/S transition, are all significantly downregulated in FD **β**-pol$^{+/-}$ treated mice (Table XII). These cell cycle dependent genes are either significantly upregulated or not differentially expressed in all other experimental conditions. Additionally, we see a significant reduction in expression of genes related to cell cycle arrest in FD untreated (52%, 10 upregulated and 11 downregulated), FD DMH treated mice (52%, 10 upregulated and 11 downregulated) and FD **β**-pol$^{+/-}$ treated (55%, 13 upregulated and 16 downregulated), whereas, FA DMH treated (55%, 17 upregulated and 14 downregulated) shows significant increases in genes related to cell cycle arrest.

Although genes related to cell cycle arrest are downregulated, it should be noted that P15 which forms a complex with Cdk4 or Cdk6, and prevents the activation of the Cdk kinases, p21 which binds to and inhibits the activity of cyclin-Cdk2 or -Cdk4 complexes, p27, which binds to and prevents the activation of cyclin E-Cdk2 or cyclin D-Cdk4 complexes, are all downregulated in FD **β**-pol$^{+/-}$ treated mice (Table XII). All function as cell growth regulators that control cell cycle

G1/S progression. Having discovered all of these findings, we wanted to know the expression of key genes related to the colorectal cancer pathway

Gene expression analysis of genes related to colorectal cancer pathway

Using DAVID bioinformatics we looked at the colorectal cancer pathway gene expression. Interestingly, FD **β**-pol$^{+/-}$ DMH treated mice showed a decreased expression of genes downstream where these genes confer upon either proliferation or antiapoptotic activity. In particular, there is reduced expression of myc, which mediates the cellular response to growth factors and survivin, a member of the inhibitor of apoptosis, in **β**-pol haploinsufficiency exposed to DMH in a folate deficient environment. These genes are significantly upregulated or not differentially expressed in FDWT, FAWT DMH, and FDWT DMH (Table XIII).

Table VI. Summary of the effect of folate deficiency on folate pathway genes in wildtype, wildtypeDMH-treated and $\boldsymbol{\beta}$-pol$^{+/-}$ DMH-treated mice*

Accession no.	Gene name	FDWT	FAWT DMH	FDWT DMH	FDβ-pol$^{+/-}$ DMH
NM_010840	5,10-methylenetetrahydrofolate reductase	ns	-1.2	-1.3	-1.3
L26316	Dihydrofolate reductase	ns	1.3	1.2	-1.8
AI323028	Thymidylate synthase	ns	ns	ns	-1.9
XM_138431	5-methyltetrahydrofolate-homocysteine methyltransferase	ns	ns	ns	-1.4
NM_023595	Deoxyuridine triphosphatase	ns	ns	1.5	-1.5
NM_145569	Methionine adenosyltransferase II, alpha	ns	ns	ns	1.2
AK038303	Methionine adenosyltransferase II, beta	ns	ns	-1.4	1.5
NM_010066	DNA methyltransferase (cytosine-5) 1	ns	ns	1.4	-1.5
NM_010067	DNA methyltransferase 2	-1.4	-1.4	-1.2	ns
NM_007872	DNA methyltransferase 3A	-1.2	ns	-1.3	ns
NM_010068	DNA methyltransferase 3B	ns	1.5	1.3	-1.5
NM_010321	Glycine N-methyltransferase	ns	ns	ns	3.4
BC015304	S-adenosylhomocysteine hydrolase	ns	ns	ns	-1.7

* P<0.05; ns, no significant difference

Table VII. Summary of the effect of folate deficiency DNA pathway genes in wildtype, wildtypeDMH-treated and **β**-pol$^{+/-}$ DMH-treated mice*

Accession no.	Gene name	FDWT	FAWT DMH	FDWT DMH	FDβ-pol$^{+/-}$ DMH
NM_009764	Breast Cancer 1	1.4	1.5	ns	-1.8
NM_009765	Breast Cancer 2	1.5	1.4	1.5	-1.4
AF236887	Ataxia telangiectasia and Rad3 related	1.2	1.3	1.2	-1.3
AK048970	Ataxia telangiectasia mutated homolog (human)	ns	ns	ns	-1.6
NM_009012	RAD50 homolog	ns	ns	ns	-1.2
NM_011236	RAD52 homolog (S. cerevisiae)	1.3	ns	1.2	-1.2
NM_009015	RAD54 like	1.4	1.8	ns	-2.0
NM_011677	Uracil DNA glycosylase	1.2	1.8	1.6	-1.6
NM_181569	N-methylpurine-DNA glycosylase	ns	ns	ns	-1.4
NM_011561	Thymine DNA glycosylase	ns	ns	ns	-1.2
NM_009687	Apurinic/apyrimidinic endonuclease 1	ns	ns	1.4	-1.7
AK047252	Polymerase (DNA directed), beta	ns	-1.8	-1.4	-1.5
NM_021498	Polymerase (DNA directed), epsilon 3 (p17 subunit)	ns	ns	ns	-1.3
NM_010715	Ligase I, DNA, ATP-dependent	1.2	1.4	1.5	-1.3
NM_010716	Ligase III, DNA, ATP-dependent	1.3	1.3	ns	-1.3
AK048442	Ligase IV, ATP-dependent	ns	ns	ns	-1.4
NM_008628	MutS homolog 2	1.3	ns	1.3	-1.5
NM_133250	MutY homolog (E. coli)	ns	ns	1.3	-1.7
NM_010829	MutS homolog 3	ns	ns	ns	-1.4
NM_146235	Excision repair cross-complementing complementation group 6 - Like	1.3	1.5	1.8	-2.3
NM_007949	Excision repair cross-complementing complementation group 2	ns	ns	ns	-1.3
NM_009531	Xeroderma pigmentosum, complementation group C	ns	ns	ns	-1.2
NM_016926	Fanconi anemia, complementation group A	1.3	1.6	1.6	-1.4
NM_175027	Fanconi anemia, complementation group B	1.3	1.5	ns	-1.6
NM_007985	Fanconi anemia, complementation group C	ns	ns	ns	-1.3
NM_018736	Meiotic recombination 11 homolog A	1.2	ns	ns	-1.3
NM_011045	Proliferating cell nuclear antigen	ns	1.3	ns	-1.3
NM_009532	X-ray repair complementing defective repair in Chinese hamster cells 1	ns	ns	ns	-1.2
NM_028875	X-ray repair complementing defective repair in Chinese hamster cells 3	ns	ns	ns	-1.7
NM_009533	X-ray repair complementing defective repair in Chinese hamster cells 5	ns	ns	1.2	-1.4

* P<0.05; ns, no significant difference

Table VIII. Summary of the effect of folate deficiency chromatin remodelling in wildtype, wildtypeDMH-treated and **β**-pol$^{+/-}$ DMH-treated mice*

Accession no.	Gene name	FDWT	FAWT DMH	FDWT DMH	FDβ-pol$^{+/-}$ DMH
NM_053123	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1	ns	ns	ns	-1.5
NM_009211	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1	ns	ns	-1.3	-1.5
NM_146235	Excision repair cross-complementing rodent repair deficiency complementation group 6 - like	1.3	1.5	1.8	-2.3
NM_144958	Eukaryotic translation initiation factor 4A1	ns	ns	ns	-1.4
AK033272	AT rich interactive domain 1B (Swi1 like)	ns	ns	ns	-1.2
NM_010436	H2A histone family, member X	ns	ns	ns	-1.6

* $P<0.05$; ns, no significant difference

Table IX. Summary of the effect of folate deficiency apoptosis pathway in wildtype, wildtypeDMH-treated and **β**-pol$^{+/-}$ DMH-treated mice*

Accession no.	Gene name	FDWT	FAWT DMH	FDWT DMH	FDβ-pol$^{+/-}$ DMH
NM_008086	Growth arrest specific 1	-2.9	ns	ns	8.8
NM_011641	Transformation related protein 63	ns	ns	ns	27.4
NM_009367	Transforming growth factor, beta 2	ns	ns	ns	11.4
NM_008337	Interferon gamma	ns	-3.9	ns	4.1
NM_008329	Interferon activated gene 203	1.4	2.2	2.3	4.0
NM_009807	Caspase 1	ns	ns	ns	1.3
NM_009812	Caspase 8	ns	ns	ns	1.4
NM_007609	Caspase 4, apoptosis-related cysteine peptidase	ns	ns	1.5	2.1
XM_139295	Caspase recruitment domain family, member 6	ns	ns	ns	1.4
NM_175362	Caspase recruitment domain family, member 11	ns	1.4	1.2	1.7
NM_011817	Growth arrest and DNA-damage-inducible 45 gamma	ns	ns	ns	1.5
AK010701	Death effector domain-containing DNA binding protein 2	-1.2	ns	ns	1.6
NM_025858	Scotin gene	-1.2	ns	1.2	1.4
NM_013693	Tumor necrosis factor	ns	ns	ns	3.9
NM_010177	Fas ligand (TNF superfamily, member 6)	ns	ns	3.2	2.1
NM_009425	Mus musculus tumor necrosis factor (ligand) superfamily, member 10	-2.4	ns	ns	2.0
NM_011614	Tumor necrosis factor (ligand) superfamily, member 12	ns	ns	ns	2.2
NM_178589	Tumor necrosis factor receptor superfamily, member 21	ns	ns	ns	1.4
NM_175649	Mus musculus tumor necrosis factor receptor superfamily, member 26	ns	ns	ns	4.1
NM_183322	Nur77 downstream gene 1	-2.5	ns	3.9	3.0
NM_009810	Caspase 3	ns	ns	-1.4	1.7

* P<0.05; ns, no significant difference

Table X. Summary of the effect of folate deficiency on Poly (ADP-ribose) polymerase (PARP) in wildtype and DMH treated wildtype and Knockout mice*

Accession no.	Gene name	FDWT	FAWT DMH	FDWT DMH	FDβ-pol$^{+/-}$ DMH
X14206	Mouse mRNA for poly (ADP-ribose) polymerase	ns	ns	ns	-1.4
NM_145619	Poly (ADP-ribose) polymerase family, member 3	ns	ns	1.4	1.5
BC025847	Poly (ADP-ribose) polymerase family, member 4	ns	ns	ns	1.6
NM_027272	Poly (ADP-ribose) polymerase family, member 8 (Parp8), mRNA	-2.7	ns	ns	2.9
NM_181402	Mus musculus poly (ADP-ribose) polymerase family, member 11 (Parp11), mRNA.	ns	ns	ns	1.5
NM_172893	Poly (ADP-ribose) polymerase family, member 12	ns	-1.2	ns	1.6
BC021340	Poly (ADP-ribose) polymerase family, member 14	ns	ns	1.4	3.7
NM_008904	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	ns	-1.8	-1.7	1.19
AK045690	Peroxisome proliferative activated receptor, gamma, coactivator 1 beta	ns	ns	ns	1.3

* $P<0.05$; ns, no significant difference

Table XI. Summary of the effect of folate deficiency on energy sensors, glucose uptake, glycolysis, fatty acid oxidation, mitochondrial biogenesis, fat acid synthesis, sterol synthesis, glycogen synthesis and protein synthesis in wildtype and DMH treated wildtype and Knockout mice*

Accession no.	Gene name	FDWT	FAWT DMH	FDWT DMH	FDβ-pol^{+/-} DMH
NM_133781	Calcium binding protein 39	-1.2	-1.2	-1.3	2.1
NM_011492	Serine/threonine kinase 11	1.2	ns	ns	1.2
NM_031869	Protein kinase, AMP-activated, beta 1 non-catalytic subunit	ns	ns	ns	1.2
BC043920	FK506 binding protein 12-rapamycin associated protein 1	-1.3	ns	ns	-1.8
NM_009204	Solute carrier family 2 (facilitated glucose transporter), member 4	ns	ns	ns	1.7
NM_133232	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	ns	ns	ns	2.0
AK032149	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	ns	ns	-1.7	1.4
BC023946	Acetyl-Coenzyme A carboxylase alpha	ns	ns	ns	-1.3
NM_008255	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	ns	ns	-2.1	-1.6
NM_145572	Glycogen synthase 2	ns	ns	ns	-2.1

* P<0.05; ns, no significant difference

Table XII. Summary of the effect of folate deficiency on cell cycle genes in wildtype and DMH treated wildtype and Knockout mice*

Accession no.	Gene name	FDWT	FAWT DMH	FDWT DMH	FDβ-pol^{+/-} DMH
NM_009870	Cyclin-dependent kinase 4	ns	ns	1.4	-1.6
NM_009873	Cyclin-dependent kinase 6	ns	2.2	ns	-1.4
NM_009874	Cyclin-dependent kinase 7 (homolog of Xenopus MO15 cdk-activating kinase)	ns	ns	ns	-1.3
NM_007633	Cyclin E1	ns	ns	ns	-1.5
NM_172301	Cyclin B1	ns	1.4	1.3	-2.6
NM_016756	Cyclin-dependent kinase 2	ns	1.4	ns	-1.8
NM_023284	Cell division cycle associated 1	ns	1.5	1.5	-1.7
NM_175384	Cell division cycle associated 2	1.3	1.5	ns	-2.1
NM_007659	Cell division cycle 2 homolog A (S. pombe)	ns	ns	1.4	-2.5
NM_013538	Cell division cycle associated 3	ns	1.2	ns	-2.4
NM_028023	Cell division cycle associated 4	ns	1.3	ns	-1.2
NM_026410	Cell division cycle associated 5	1.4	1.4	1.5	-1.9
NM_025866	Cell division cycle associated 7	1.5	1.5	ns	-1.7
AK076422	Cell division cycle associated 8	ns	1.3	1.4	-2.3
NM_010849	Myelocytomatosis oncogene	1.3	1.4	1.3	-1.2
NM_007670	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	-1.2	ns	ns	1.6
NM_007669	Cyclin-dependent kinase inhibitor 1A (P21)	-1.4	-1.5	-1.4	1.3
NM_009875	Cyclin-dependent kinase inhibitor 1B (P27)	-2.5	-3.5	-1.5	1.2
NM_007670	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	-1.3	ns	ns	1.6
NM_009876	Cyclin-dependent kinase inhibitor 1C (P57)	-1.7	-1.3	ns	1.4
NM_009029	Retinoblastoma 1	1.6	-1.2	ns	1.2
U59758	P53-variant (p53)	ns	ns	ns	-1.8
NM_134092	Mdm2, transformed 3T3 cell double minute p53 binding protein	1.3	1.5	1.4	-1.5
BI646741	MAD homolog 3 (Drosophila)	ns	-1.4	-1.4	1.6
NM_008540	MAD homolog 4 (Drosophila)	1.2	-1.4	-1.5	ns
NM_019827	Glycogen synthase kinase 3 beta	-1.7	-1.4	-2.4	1.3
AK048970	Ataxia telangiectasia mutated homolog (human)	ns	ns	ns	-1.6
NM_007691	Checkpoint kinase 1 homolog (S. pombe)	1.5	1.8	1.7	-1.6
NM_016681	Mus musculus CHK2 checkpoint homolog (S. pombe) (Chek2), mRNA.	ns	1.3	ns	-1.9

* P<0.05; ns, no significant difference

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Table XII. Summary of the effect of folate deficiency on colon cancer genes in wildtype and DMH treated wildtype and Knockout mice*

Accession no.	Gene name	FDWT	FAWT DMH	FDWT DMH	FDβ-pol^{+/-} DMH
BI646741	MAD homolog 3 (Drosophila)	ns	-1.4	-1.4	1.6
NM_008540	MAD homolog 4 (Drosophila)	1.2	-1.4	-1.5	ns
AK036317	V-raf-leukemia viral oncogene 1	ns	ns	-2.2	1.5
AK088784	Glycogen synthase kinase 3 beta	-1.6	ns	-2.4	1.3
NM_015732	Axin2	1.4	ns	ns	-1.7
AK039745	Adenomatosis polyposis coli	-1.6	ns	-1.3	1.7
NM_010234	FBJ osteosarcoma oncogene	-1.6	ns	-1.3	1.5
NM_010591	Jun oncogene	ns	ns	ns	1.9
NM_010849	Myelocytomatosis oncogene	1.3	ns	1.3	-1.4
NM_016700	Mitogen activated protein kinase 8	ns	ns	ns	-1.4
NM_009689	Baculoviral IAP repeat-containing 5	ns	ns	ns	-2.8

* $P<0.05$; ns, no significant difference

Discussion

We have previously shown the consequence of **β**-pol heterozygosity on the development and progression of ACF in DNA polymerase **β** heterozygous knockout mice of C57BL/6 background (122), characterized formerly in our lab (122). In these studies both wildtype and **β**-pol haploinsufficient mice did not display any ACF in their colon, indicating that **β**-pol haploinsufficiency and successive BER deficiency is not enough to induce ACF in mice on its own. This suggests that **β**-pol is a low penetrance gene requiring some other insult in the environment, such as an exposure to a chemical or a nutritional deficiency. Therefore, to induce ACF formation, we injected 30mg/kg i.p. 1,2-dimethylhydrazine (DMH), a known colon and liver carcinogen, for 6 weeks. DMH produces alkylation damage (06-meG and N7-meG) as well as oxidative damage, damages we have shown (60) to be processed normally by the BER pathway. As expected, **β**-pol haploinsufficient mice, sacrificed 6 weeks after final DMH injection, showed a significant 67% higher level of ACF formation

(29.7$\pm$1.4) as compared to their wildtype counterparts (15.4$\pm$1.8), indicating that

β-pol$^{+/-}$ mice are more sensitive to both alkylation and oxidative damage. Noting these changes and having previously established that **β**-pol haploinsufficiency appears to mimic the effects of folate deficiency on the BER pathway, we then proceeded to look at the effect of folate deficiency (FD) on ACF formation in the

β-pol$^{+/-}$, for deficiency in this B vitamin has been implicated in colon carcinogenesis. Interestingly, FD in wildtype mice subject to DMH showed a

significant increase in ACF formation (37.6$\pm$5.2) relative to their wildtype counterparts, however, when FD was imposed in the **β**-pol$^{+/-}$, we saw a 50% decline in ACF formation (18.3$\pm$4.1) after DMH treatment. This indicated that **β**-pol haploinsufficiency attenuated the carcinogen-induced ACF formation when folate was deficient. There was no significant difference in the number of aberrant crypts per focus 12 weeks after sacrifice. When mice were macroscopically observed 40 weeks post treatment there was an apparent regression in the number of ACF in DMH treated mice, however, there was a significant increase in the number of aberrant crypts per focus in FDWT mice relative to their WT counterparts (7.63$\pm$0.4 versus 6.33$\pm$0.4, respectively), indicating a progression of this disease. In addition, methylene blue staining of formalin-fixed colon tissue showed defined elevation of ACF above the surrounding mucosa in FD fed mice, while the FA mice displayed less developed ACF, providing further evidence of advanced ACF formation. Based on the progression of ACF, we decided to conduct proliferative and apoptotic assays to further visualize the growth changes of these aberrations. Interestingly, TUNEL assay results showed a significantly greater level of apoptotic activity, while BrdU assays showed significantly more proliferation (BrdU incorporation) in FD **β**-pol$^{+/-}$ DMH treated colon mucosa compared to FAWT counterparts. Noting all of the aforementioned changes in colon morphology, we wanted to determine the impact of **β**-pol haploinsufficiency and FD on gene expression in colonic mucosal tissue.

Sequentially, to determine possible mechanisms of reduced ACF formation and progression in **β**-pol$^{+/-}$, we, in our current study, decided to conduct microarray analyses' on FDWT, FAWT DMH treated, FDWT DMH treated and

FD **β**-pol$^{+/-}$ DMH treated relative to FAWT colon mucosa. The genome-wide gene expression pattern in the colonic mucosa of these mice was investigated using the Affymetrix G4122A, Whole Mouse Genome and G4122F, Whole Mouse Genome.

The present data support our previous findings of **β**-pol haploinsufficiency conferring a protective effect in the development of colon cancer. Firstly we

determined the reduced expression of MTHFR, DHFR and TS in FD **β**- pol$^{+/-}$ DMH treated colonic mucosa. In a study conducted by Tsourouflis et al., (173) in human colon cancer primary tumor specimen, they discovered a significant number of samples testing positive for TS, defining TS as an independent prognostic risk factor for colon cancer. Shimoda et al., (174), in their investigation on humans with pancreatic and biliary cancers likewise identified higher levels of TS in cancerous than noncancerous tissues. TS is necessary for

DNA synthesis and has become therapeutic target for a number of cancers, particularly rapidly dividing cells that make vital use of their nucleotide synthesis machinery. Inhibition of TS results in an increase in dUTP pools, and incorporation of uracil into DNA. BER is responsible for the removal of uracil; however, when thymidylate is deficient and dUTP levels are elevated, uracil continually becomes misincorporated into the DNA leading to 'catastrophic' BER, with subsequent elevated levels of DSB and eventual cell death (175, 176, 177).

Peters et al., (178) in there study utilizing high doses of 5-fluorouracil (5-FU), a TS inhibitor, on 5-FU-sensitive and -resistant human colon cancer cell lines demonstrated enhanced TS inhibition, which also prevented TS induction and increased the antitumor effect. In this study we determined a significant

reduction in gene expression of MTHFR and DHFR in FD **β**- pol$^{+/-}$ DMH treated mice. Both of these enzymes catalyze reactions that require the use of energy in the form NADPH.

Denoting the irregularity in folate metabolism, we studied the effects of **β**-pol haploinsufficiency on the expression of DNA repair genes when DMH was injected in a folate deficient environment. We first looked at BER for BER has the primary role in processing the damage created by this environment. Genes related to BER were significantly downregulated in this animal model. Due to folate restriction there is a soaring elevation in uracil misincorporation. **β**-pol haploinsufficiency would lead to deficient BER activity giving rise to SSB which would eventually lead to DSB formation and the triggering of eventual cell death. In addition, we see an elevation in PARP gene expression, with the exception of

PARP1 in **β**-pol$^{+/-}$. PARPs are known to assist in the repair of single-strand DNA nicks, however, if significantly elevated, PARPs deplete ATP levels. Peterman et al., (179) using synthetic oligonucleotides, have shown a lack of short patch BER under conditions of ATP shortage. They also report maintenance of long patch BER through strand displacement by XRCC1 when ATP levels are reduced. Others, in their observations, show poly(ADP-ribose) being converted to ATP by pyrophsphorolytic cleavage using the pyrophosphate generated from dNTPs

during DNA repair synthesis (180). In our studied we see a reduced capacity of both short and long patch BER. Reduction in short patch repair could be explained by **β**-pol haploinsufficiency, as well as, a nucleotide and ATP shortage, whereas the altered ability for long patch repair may lie in the fact that, in addition to aforementioned, there is a downregulation in XRCC1, as well, there is a reduction in PARP1, a gene necessary for recruitment of BER enzymes to sites of DNA damage.

Homologous Recombination (HR) and non-homologous end-joining (NHEJ) are instrumental in the repair of DSB that can incur as a result of persistent BER intermediates. Yang et al., (181) studied the effect of thymidylate deprivation, through raltitrexed (RTX), in H-29 human colon cancer cells and HeLa human cervical cancer cells. Their results show an induction of HR which influences a cellular resistance to RTX. Using RTX treated cell line 9-2, which was derived by stably transfecting GM00637 cells with pTNeo99-7, Waldman et al., (182) demonstrated adequate NHEJ. In our study we see a reduction in a number of HR/NHEJ related genes with **β**-pol haploinsufficiency, implying that another causative effect is occurring other than thymidylate deprivation for reduced HR/NHEJ repair capacity. We propose that in addition to lack of thymidine, nucleotide and ATP shortage, as well as reduced PARP1 activity may result in the deficiency of DSB repair. It should be noted that **β**-pol haploinsufficiency in our damage inducible environment shows significant downregulation in genes related to HR/NHEJ, ATM, ATR and DNA-pk expression.

Recent evidence has suggested that a histone code, which involves phosphorylation, ubiquitylation, sumolation, acetylation and methylation is involved in DNA damage detection and DNA repair. It is predicted that this code allows for modification on a specific histone tail that can be read by other chromatin associated proteins (183). Chromatin modification is accomplished by a chromatin-remodeling complex, which are a family of ATP-dependent molecular machines that participate in transcriptional regulation, DNA repair, homologous recombination and chromatin assembly (164). The chromatin-remodeling complex can destabilize histone-DNA interactions in reconstituted nucleosomes in an ATP-dependent manner, though the exact nature of this structural change is unknown. This code becomes important for DNA repair, for histone modification is necessary for all repair machineries to gain access to

DNA in order to proceed (183). In this study FD **β**- pol$^{+/-}$ DMH treated mice show significant downregulation in genes relative to the ATPase dependent chromatin remodeling complex.

In addition to a potential lack of exposure of DNA in chromatin, our study shows a distinct downregulation in H2A related gene expression. A specific subtype of H2A, called H2afx (aka H2AX), which plays a very important role in the cellular response to DNA damage and participates in DNA double-strand break repair is significantly downregulated in **β**-pol haploinsufficiency upon exposure to DMH in a folate deficient environment. This enzyme is normally phosphorylated by ATM/ATR (involved in HR) or by DNA-pk (involved in NHEJ) (183, 184, 184). These 3 aforementioned genes, as previously stated, are

downregulated in FD **β**- pol$^{+/-}$ DMH treated mice. Upon phosphorylation of H2AX, then called **γ**-H2AX, it co-localizes to nuclear foci at sites of DNA damage (186). **γ**-H2Ax is believed to then recruit checkpoint proteins such as BRCA1, MRE11/RAD50/NBS1 complex, MDC1 and 53BP1 to the site of DNA damage (187). Interestingly, with the exception of MDC1, all the aforementioned genes

are significantly downregulated in FD **β**- pol$^{+/-}$ DMH treated mice. Hong et al.(188), in there study utilizing WTHBF-6 cells derived from primary human bronchial fibroblasts, demonstrated a co-localization of Mre11 to gamma-H2A.X and ATM when cells were exposed to Hexavalent chromium, an agent that induces DNA double strand breaks. Here we see lack of induction of ATM/ATR or DNA-pk inferring inhibition of phosphorylation of H2AX disallowing DNA repair to occur via HR and NHEJ.

Our current study shows a lack of expression of PARP1. PARP1 catalyzes the conversion of NAD^{+} into nicotinamide and poly(ADP-ribose) and is shown to be required for genome repair, DNA replication and the regulation of transcription (189). Liu et al. (190), in their study using HeLa cells treated with MNNG and ABT-888, a potent PARP inhibitor, facilitated the induction of apoptosis without a reduction in the level of ATP. Similarly, Ghosh et al. (191), showed PARP1 inhibition through Benzamide in HeLa cells resulted in an induction of apoptosis under mild oxidative stress. They also demonstrate that reduced PARP1 results in an increase in nuclear fragmentation and increased caspase 3 activity along with an increase in cellular NAD^{+} levels. In addition, a decrease in general poly(ADP-ribosyl)ation is seen. It should be noted that the

extensive DNA damage caused overactivation of PARP1 followed by severe ATP depletion which is hence causative of necrotic cell death (192). Interestingly, FD

β-pol$^{+/-}$ DMH treated mice show significant downregulation in Apoptosis-inducing factor (AIR)-like mitochondrion-associated inducer of death and Endonuclease G, genes involved in necrosis. Boulares et al. (193), using human osteosarcoma cells show that PARP1 cleavage during apoptosis is required for both the prevention of excessive depletion of NAD and ATP and to release of DNase1L3. DNase1L3 is inhibited by poly(ADP-ribosyl)ation. DNase1L3 is upregulated in

FD **β**-pol$^{+/-}$ DMH treated mice indicating a lack of its ribosylation and instead acts to mediate the breakdown of DNA during apoptosis. In a study on primary myeloid leukemic cells and myeloid leukemic cell lines which have double strand DNA repair defects, Gaymes et al. (194) used PARP inhibitors, KU-0058948 and

PJ34, which induced cell cycle arrest and apoptosis. This study depicts a downregulation in PARP1. It is currently unknown if this effect is either via cellular signaling to reduce its expression or as a result of CASP3 activity. Regardless, this reduced expression appears to enlist apoptosis. In this study,

FD **β**-pol$^{+/-}$ DMH treated mice show a significant upregulation in expression of apoptotic genes. The lack of expression of necrotic genes would indicate that

the activity occurring in the mucosal cells of FD **β**-pol$^{+/-}$ DMH treated mice is of low cellular energetic stress, however, not to the degree where death is induced via necrosis.

FD **β**-pol$^{+/-}$ DMH treated mice show an increase in expression of cellular energy stress sensors, including CAB39 and STK11 confirming energetic stress

in this environment. In response to energy stress, cells activate AMPK downstream of the tumor suppressor, STK11. We also see an increase in expression of APRT which catalyzes the formation of AMP and inorganic pyrophosphate from adenine and 5-phosphoribosyl-1-pyrophosphate (PRPP) indicating an elevation in the level of AMP/ATP ratio. Interestingly, Prkab1

(AMPK), which is elevated in FD β-pol$^{+/-}$ DMH treated mice, is activated when intracellular levels of ATP decline and intracellular levels of AMP increase (195), the former allosterically activating Prkab1 (196). These findings seem to mimic the effects of AMP-mimetic AICAR, which is a pharmacological target of human cancers that suppress cancer cell growth (197). In addition to elevated energy sensing genes there is also an increase in expression of CaMKK (CaMKK2), another physiological activator of AMPK (198). STK11 and CaMKK are two kinases that phosphorylate AMPK for its activation (199). Yee et al. (200), in there study on cervical cancer cell lines reveal that, A23187, an alternative pharmacological AMPK activator inhibits cancer cell growth through CaMKK.

Increased intracellular Ca^{2+} causes an increase in CaMKK (199). Chern et al. (201), utilizing human hepatoma Hep G2 cells, showed an overproduction of hydrogen peroxide as a result of the oxidative stress imposed by folate deficiency which ultimately led to cell death through apoptosis. Satu et al. (203), in their study on rat hepatocytes demonstrate H_2O_2 impairment of mitochondrial function via oxidative stress, that reduces intracellular ATP production, and in turn opens ATP-sensitive, non-specific cation channels, leading to Ca2+ influx which can trigger apoptosis. CaMKK is upregulated in FD β-pol$^{+/-}$ DMH treated

mice and is downregulated in FD WT untreated animals. These findings would indicate that the oxidative stress imposed by FD in **β**-pol haploinsufficiency would result in increased intracellular calcium levels leading to CaMKK activation of AMPK, as well as, the induction of apoptosis.

AMPK is known to maintain a balance between ATP production and its consumption in all eukaryotic cells. In this current study FD **β**-pol$^{+/-}$ DMH treated mice show compromised energy status thus activating catabolic pathways while diminishing macromolecule biosynthesis, cell growth and proliferation through AMPK. We see an increase in GLUT4 expression which induces glucose uptake, Pfkfb3, upregulating glycolysis and Ppargc1, implicated in increased mitochondrial biogenesis. Additionally we see a decrease in Acaca expression, indicative of a reduction in fatty acid synthesis, Hmgcr, involved in sterol synthesis, GYS2, causing a decrease in glycogen synthesis (FD **β**-pol$^{+/-}$ DMH treated mice show an upregulation of GSK3b which inhibits GYS2) and an increase in EF2K which inhibits both protein synthesis and mTOR activity (203). All of these findings are not significantly altered in all other animal models with the exception of an increase in Acaca and downregulated expression of mTOR in FD WT mice. This would indicate that **β**-pol haploinsufficiency subject to prolonged FD subsequent to DMH treatment activates AMPK, switching on ATP on ATP-generating pathways, like glycolysis, but also switches off ATP-consuming biosynthetic pathways.

In this study we demonstrate an inhibition of gene expression related to cell growth and an upregulation in expression of cell cycle arrest genes in FD **β**-

pol$^{+/-}$ DMH treated mice. We show that **β**-pol haploinsufficiency 1) induces G1 and G2/M arrest; 2) downregulates cdk4/6, cdk2, ccne1, cdc2a and ccnb1 expression; and 3) upregulates gene expression of cdkn1a (p21), cdkn1b (p27), cdkn2b (p15) and cdkn1c (p57). Autonomy of growth signals and avoidance of cell death has been described by Hanahan et al. (204) as two of the six necessary changes in cell physiology that dictate malignant growth. Therefore, our animal model has the ability to induce apoptosis and inhibit cell cycle progression in colon cancer, and as such these mechanisms may potentially be utilized in the chemoprevention of colon cancer.

Lastly, we looked at expression of genes relative to the colorectal cancer pathway. Interestingly, FD **β**-pol$^{+/-}$ DMH treated mice show a reduction of expression of genes downstream of this pathway. Namely, there is a reduction in survivin which is an antiapoptotic gene, as well as, Myc which encourages proliferation. Expression of these are either unchanged or show opposite correlation to FD **β**-pol$^{+/-}$ DMH treated in all other animals tested.

In January of 1998, the United States imposed mandatory fortification of enriched cereal-grain products with folic acid to help prevent pregnancies affected by a neural tube defect (NTD) and potentially other malformations in their babies like cleft palate and lip and cardiac defects. Previous research suggested that folate deficiency appeared to play an important pathogenic role in the development of many disease states beyond NTDs including cardiovascular disease, Alzheimer's disease and cancer (205). Due to the fact that the prevention effort targeted preconceptional women of child-bearing age, the

mandate exposed the entire population to this enrichment. This seemed feasible for folate is by and large regarded as safe and advantageous for disease prevention. The premise of this endeavor is working for the data from the 1999 to 2000 National Health and Nutrition Examination Survey (NHANES) revealed a considerable increase in median serum folate concentrations in nonpregnant women of childbearing age compared to 1988 to 1994. Our current findings suggest that this massive effort to eradicate folate deficiency maybe detrimental under certain conditions, particularly certain cancers. Here we show that a deficiency in this B-vitamin may confer a protective effect in colon carcinogenesis. These findings seem sensible for if one considers the needs of rapidly dividing preneoplastic lesions like the ACF, folate would supply the necessary environment, principally to repair machinery, which ultimately may inflict more damage due to erroneous repair. Our findings imply that folate deficiency is needed in conjunction with a BER deficiency in order to reduce repair of damage created by this vitamin deficit, not only by BER but as well, repair by other pathways.

Summary and Future Directions

Folate has emerged as an important nutritional element in the pathogenesis of several malignancies, including cancers of the colorectum, lung, pancreas, esophagus, stomach, cervix, and breast, as well as neuroblastoma and leukemia. Studies have collectively implied an inverse association between both dietary and serum folate and the risk of malignancy (1, 2); the most evidence relating to colorectal cancer (CRC) (3, 4). This information would appear, in light of these current studies, to be counterintuitive. Folate is especially important during periods of rapid cell division and growth such as seen in neoplastic cells. In addition, folate deficiency reduces intracellular nucleotide precursor pools; increasing uracil misincorporation and DNA strand breaks (5, 6) measures that would seem beneficial for the suppression of cancer cells.

Here we show that folate deficiency not only suppresses onset and progression of ACF but in addition appears to reduce the level of cellular energy such that cells do not have the power to maintain viability. This study demonstrates an inhibition of nucleotide synthesis through an interruption in the folate pathway, chromatin condensation, an arrest in cell cycle progression and cellular signaling to energy sensors when **β**-pol is haploinsufficient in a folate deficient environment. In addition, we show reduced DNA repair and increased apoptotic activity. We propose that the oxidative and alkylation damage imposed by folate and DMH treatment resulted in the recruitment of PARPs to the sites of DNA damage. With a lack of **β**-pol, damage created would continually signal for repair, bringing in an abundance of PARP. This in turn would result in a

reduction in the mitochondrial membrane potential and ATP depletion leading to energy failure and cell death. PARP-1 activation leads to ATP depletion and necrosis (205, 206). Here we propose that as a result of PARP1 abundance, the apoptotic pathway is signaled leading to activation of caspases which cleave PARP1 and thereby preserve ATP. As seen in this current study, PARP1 is downregulated, switching the mode of death from necrosis to apoptosis, as seen by lack of response by necrotic enzymes but a significant increase in apoptotic activity. Here we suggest that after immediate exposure to DMH, PARP1 is recruited to sites of DMH damage, however, a deficient BER leads to elevated PARP activity. This elevation creates cellular energetic stress, signaling apoptosis or necrotic cell death. Having theorized this, PARP levels immediately after final injection such be ascertained. Six weeks after the final DMH injection, however, PARP1 activity is reduced leading to apoptosis signifying the level of ATP depletion governs the mode of cell death. Similar findings were reported by Ha et al. (206) in their study using fibroblasts of PARP$^{-/-}$ animals after exposure to either MNNG or H_2O_2. Other modes of DNA repair are reduced possibly secondary to apoptotic cell signaling, lack of induction of PARP activity, reduced cellular energy levels to mediate repair and by the lack of sufficient purine and pyrimidine synthesis. Lastly, current chemotherapeutic regimens for all causes of colon cancer utilize the same therapeutic agents; oxaliplatin, an alkylating agent proposed to prevent DNA synthesis, 5-fluorouracil (5-FU), an inhibitor of thymidylate synthase, and leucovorin, which enhances the binding of 5-FU in the cell while providing a source of folic acid for normal cells. While the premise is

understandable to some degree, questions arise regarding 5FU in the cancer cell and the continuance of 'catastrophic' repair by sufficientBER machinery. Thus, our findings appear to have both therapeutic and protective implications against the development and progression of colon cancer by means of an alteration in micronutrients when **β**-pol variants/polymorphisms exist.

Future Directions

Having elucidated the potential mechanism behind colon cancer prevention, future studies would include a repeat of experimental procedures on mice sacrificed immediately after final DMH injection and after approximately 40 weeks to denote significant changes that occurs at these times as compared to our current 6 week after final injection sacrifice. This would assist in determining cellular performance immediately after exposure to and after an extensive time period without DMH. Studies should also be conducted on both

ACF and normal adjacent tissue extracted through laser micro-dissection to delineate if there is a distinction in these tissues with respect to the key findings of our current study. We propose that ACF have greater metabolic needs than

normal tissue. FD **β**-pol$^{+/-}$ DMH treated mice would have such a high level of metabolic stress that energy levels become so depleted that apoptosis would ensue. In contrast, with efficient BER, PARP levels would remain stable, allowing for repair and thus no lack ATP levels accelerating mutational frequency. As well, PARP1 cleavage should be assessed, for high levels of this enzyme without BER would cause severe cellular energy depletion and signal for its cleavage via caspases. In addition, it seems relevant to test the effects of

caloric restriction (CR) in **β**-pol haploinsufficiency when subject to DMH, for research has shown CR to increases life expectancy but as well, it would help delineate if what is found in our current study is a result of folate in a **β**-pol deficient environment or is it that folate depletes cellular energetic stores through the various aforementioned mechanisms and that CR could in fact mimic these findings. As well, it would be interesting to test the effects of **β**-pol haploinsufficiency in both the APC min mouse and MMR deficient mouse models fed a folate deficient diet, to test our current findings of protection when BER is deficient.

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ABSTRACT

**EFFECT OF DIETARY FOLATE RESTRICTION ON COLON
CARCINOGENESIS IN DNA
POLYMERASE ββ HAPLOINSUFFICIENT MICE**

by

LISA VENTRELLA LUCENTE

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Advisor: Dr. Ahmad R. Heydari

Major: Nutrition and Food Science

Degree: Doctor of Philosophy

The data presented in this research is central to establishing the role that the base excision repair pathway (BER) plays in the development and progression of colon cancer when dietary folate is deficient. Both cellular folate restriction and BER deficiencies have been shown to result in the accumulation of endogenous damage and lesions that could eventually develop into carcinogenesis. In this study, a dietary folate deficiency (FD) resulted in a significant increase in aberrant crypt foci (ACF) formation and triggered liver tumorogenesis in wildtype (WT) animals, as did a BER deficiency in DNA

polymerase **β** haploinsufficient (**β**-pol$^{+/-}$) mice exposed to 1, 2-dimethylhydrazine (DMH), a known colon and liver carcinogen. We combined both folate restriction and a BER deficiency to determine the fate of colon tissue after exposure to DMH. Of interest, we show that this model supports a protection against colon carcinogenesis. FD attenuated onset and progression of ACF and prevented liver tumorigenesis in **β**-pol haploinsufficient mice. Analysis of the data suggests

that the mechanism by which this phenomenon occurs appears to be through an elevation in DNA damage that signals recruitment of PARP enzymes to the site of damage, however, with a deficiency in BER, PARP function in DNA repair is futile leading to a depletion of cellular energetic levels. This energetic stress is sensed by cell death machinery and as such apoptosis is invoked.

AUTOBIOGRAPHICAL STATEMENT

LISA VENTRELLA LUCENTE

Education

Wayne State University, Detroit MI		Coordinated Program in Dietetics 1991
University of Western Ontario, London, Ontario, CANADA	HBSc.	Nutrition and Food Science 1988

Professional Experience:

2008 - present	Instructional Assistant Wayne State University, Detroit, MI
2003-2007	Graduate Teaching Assistant Wayne State University, Detroit, MI
1993-1994	Consulting Dietitian, The Elliott Home for the Aged, Guelph, Ontario Dr. Keith Bovell's Office, Gastroenterologist, Guelph, Ontario Outpatient Clinical Dietitian, Guelph General Hospital, Guelph, Ontario
1991-1992	Pediatric Dietitian, Pediatric Metabolic Diabetes Center, Hotel Dieu of St. Joseph Hospital, Windsor, Ontario
1991	Clinical Dietitian, Windsor Western Hospital, Windsor, Ontario

Publications

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Presentations

Nutrition intervention and the molecular mechanism of colon cancer: The role of the Base Excision Repair (BER) pathway. The 11th Annual Midwest DNA Repair Symposium, Ann Arbor, MI, May 16-17, 2009. Poster presentation.

Nutrition intervention and the molecular mechanism of colon cancer: The role of the Base Excision Repair (BER) pathway. The 9th Annual Midwest DNA repair symposium, Northpointe Conference Center Columbus, Ohio. May 19-20, 2007. Oral presentation.