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Long-term dietary folate deficiency and intestinal tumor development in mice

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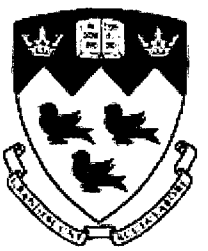
September 2008

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy



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This thesis is dedicated to anyone from a small town who doesn't think they can ever make it out in the big world. You can.

ACKNOWLEDGEMENTS

"'I shan't call it the end, till we've cleared up the mess,' said Sam gloomily. 'And that'll take a lot of time and work.'"

Excerpt from "The Lord of the Rings, The Return of the King" by J.R.R. Tolkien[1]

I would like to thank my supervisor, **Rima Rozen**, for all of her help and direction over the years. I hope to adopt your high standards in my career to come. The many lessons I learned from you will always stay with me.

To my supervisory committee I would like to say thank you for all your encouragement and guidance. To **Morag Park**: your challenging questions always got to the heart of the matter and helped me see the real meaning of my results. Thank you also for helping me feel at ease, I enjoyed all the Running Room talk. To **Jacquetta Trasler:** special thanks for always taking the time to listen to my questions and respond with thoughtful answers. I especially appreciate all your career advice which was given freely and with a generous spirit.

I cannot count all the ways I have been supported by **Laura Benner**, **Marlene Aardse**, **Danuta Rylski** and **Aileen Lebofsky**. I thank you not just for helping me navigate through the jungle of bureaucracy, but also for taking the time to stop and chat and laugh. A special thanks to **Eric Simard** for always running to the rescue when something expensive and necessary was broken (but not by me, of course). I would also like to thank **Kandance Springer** and **Thomas Leslie** for helping me through each step towards graduation.

This project would never exist without the endless work and tireless patience of **Liyuan Deng** and **Qing Wu**, who bred limitless mice and sat through hours of dissections. Ladies, you were a pleasure to work with and, don't worry,

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[1] Tolkien, J.R.R., *The Lord of the Rings, The Return of the King*, 1999, Harper Collins Publishers. p. 354

my mandarin will improve one day. My thanks also to **Xiao-Ling Wang** for her superior sectioning skills; if I could only be a fraction as good! I will be eternally awed by the intellect of **Daniel Leclerc**, your logic would put Spock to shame! I have never met anyone who is so right so much of the time and yet so humble about it. I would also be remiss not to thank **Ying Li** for showing me the ropes in my first few days and always keeping me laughing as well as **Jijun Shen**, **Nelly Sabbaghian** and **Jitka Stankova** for their help in the first few years.

To my fellow students over the past five years, what can I say? A huge thanks goes to **Andrea Lawrance**, who taught me how to know a tumor from a lymph node, how to time step, BECM, always keep an eye on my timer, and who always made me feel "appreciated". Thanks to **Laura Pickell** for being a good bench neighbor, sharing a good time on the town with me on more than one occasion, and for just being an all-around great person to spend five years with. I will never forget **Basak Celtikci** who always knew the best sales and for being our Komshu; thank you for helping me learn to say "I like it" more than "it is sucks". Thank you to **Karen Christensen** for all the pathway talk, answering my innumerable stupid questions about biochemistry, and being willing to "geek it up" with me in the lab despite that we were already pretty geeky to begin with. Thanks also to **Jessica Chan** (for your enthusiasm), **David Garcia-Crespo** (for putting up with all the noise in the "hen house"), **Deqiang Li** (for your work-ethic), **Leonie Mikael** (for an example of true determination), and **Marc Roy** (for your fun-loving attitude).

To all the rest of the Place Toulon gang, past and present, who have made me feel welcomed in the big city and only sometimes laughed at my small town "colloquialisms", a big thank you. I would like to thank **Tamara Kelly** in particular for getting me involved in HGSS and introducing me to the Running Room. For many, many good times and long laughs I would like to thank **Oana Neaga**, **Leslie Ribeiro**, **Amanda Fortier**, **Donovan Chan**, **Flavia Lombardi-Lopes**, **Serge McGraw**, **Kirsten Niles**, **Katia Nadeau**, **Isabel Mandeville**, **Salomon Cornejo**, **Tala Achkar**, **Karine Jacob**, **Caroline Sollier**, **Takrima Haque**, and **Inga Murawski**. To **Michelle Collins**, **Natalie Magnus**, **Nafisa**

Jadavji, **Nicole Carp**, and **Tim Bao** I bequeath the recycling program and student seminars, enjoy!

To my friends and family who have watched all my rises and falls and listened to all my gripes and glories, thank you. Thank you to my mom, **Sue McKean**, and dad, **Bill Thurston**, for believing in me and thanks to my in-laws, **Barb** and **John Knock**, for taking an interest in whatever it is I do. Thanks to my brothers, **Adam** and **Alexander** and my sister **Elizabeth Thurston**, for not making too much fun of me for being a science nerd. Thank you to my grandparents, **Hector** and **Gladys McKean**, for always calling to check up on me. Thank you to my friends **Vanessa Fells** and **Tamara Hamilton** for sharing the joys and woes of the grad school experience with me. Finally, I would like to thank my husband, **Matthew Knock**, for illicit gel-loading help, for reading "Biology for Dummies" so you would know what I was complaining about when I got home, for not getting too bored as you sat and watched me write this thesis, and for all the love and understanding you've shown over the past 10 years, even when I dragged you here to the city with me. You are my best friend.

ABSTRACT

Epidemiological evidence linking dietary folate deficiency and risk for colorectal cancer is conflicting. Studies using animal models indicate that timing, dose and presence of pre-malignant lesions will influence whether folate deficiency prevents or promotes tumor formation. In this thesis a new model of spontaneous tumor formation due to long-term dietary folate deficiency alone, in non-transgenic mice and without carcinogen induction, is developed. The mechanisms by which folate deficiency might influence cancer risk are also examined.

BALB/c mice, with or without a null allele in a key folate-metabolizing enzyme, *Methylenetetrahydrofolate reductase* (*Mthfr*), develop intestinal tumors due to dietary folate deficiency alone. On folate-deficient (FD) diets, 12.5% of *Mthfr*+/+ mice and 28.1% of *Mthfr*+/- mice developed tumors; mice on control diet (CD) did not. C57Bl/6 mice (a strain resistant to other methods of tumor induction) placed on the same diets for the same amount of time did not develop any tumors. To investigate possible mechanisms the levels of DNA damage (dUTP/dTTP ratio and p-H2AX staining) and DNA methylation (thin layer chromatography) were examined. FD BALB/c, but not C57Bl/6 mice, had a trend towards increased dUTP/dTTP and DNA double-strand breaks and decreased global DNA methylation compared to CD mice. To determine why the FD diet affects the BALB/c and not the C57Bl/6 strain, the expression of genes involved in folate metabolism was examined. Several changes in gene expression were observed. In particular, BALB/c mice had increased *Mthfr* expression and MTHFR activity compared to C57Bl/6 mice. Increased MTHFR activity may deplete 5,10-methylenetetrahydrofolate supplies for the dTMP synthesis, increasing the dUMP levels and, possibly, DNA damage. The levels of several DNA repair genes were also examined. Two genes involved in base excision repair, *Thymine DNA glycosylase* (*Tdg*) and *Apurinic/apyrimidinic endonuclease 1* (*Apex1*), were increased in FD C57Bl/6 compared to FD BALB/c mice suggesting increased DNA repair capacity.

These results support the evidence that dietary folate deficiency promotes intestinal tumor formation possibly through increased DNA damage, with subsequent defects in DNA repair.

RESUMÉ

Les études épidémiologiques reliant un déficit en folates au risque pour le cancer colorectal ont généré des résultats contradictoires. Des études utilisant des modèles animaux ont, elles, indiqué que l'effet des folates dépend du moment d'ingestion et de la quantité ingérée. D'autre part, la présence de lésions pré-malignes influence l'effet des folates sur le développement et la croissance des tumeurs. Dans cette thèse, un nouveau modèle de tumorigénèse est présenté. Il implique une diète déficitaire en folates, sans nécessiter l'initiation par une prédisposition génétique ou par un produit chimique. Des mécanismes pouvant expliquer l'effet des folates sur le risque de développer un cancer sont discutés.

La méthylènetétrahydrofolate réductase (gène *Mthfr*) est une importante enzyme régulatrice du métabolisme des folates. Les souris BALB/c ont développé des tumeurs intestinales lorsqu'elles étaient soumises à une diète déficitaire en folates et la proportion des souris affectées dépendait du niveau d'expression du gène *Mthfr*. En présence d'une diète déficitaire en folate (DF), 12,5% des souris *Mthfr*+/+ et 28,1% des souris *Mthfr*+/- ont développé des tumeurs ; les souris non-traitées (diète contrôle, DC) n'en ont pas développé. Les souris C57Bl/6 (qui sont résistantes à plusieurs inducteurs de tumorigénèse) n'ont pas développé de tumeurs lors d'expériences menées en parallèle. Afin de comprendre ce qui cause une différence entre ces lignées de souris, l'intégrité de l'ADN (rapport dUTP/dTTP et détection de p-H2AX) et sa méthylation (chromatographie sur couche mince) ont été examinées. Les souris BALB/c nourries avec la diète DF, mais pas les souris C57Bl/6, ont présenté une augmentation modeste du rapport dUTP/dTTP et du bris d'ADN double-brin ainsi qu'une diminution du taux de méthylation de l'ADN en comparaison avec les souris exposées à la diète DC. Afin de comprendre la raison pour laquelle la diète DF a affecté les souris BALB/c et pas les souris C57Bl/6, l'expression des gènes reliés au métabolisme du folate a été examinée. Plusieurs changements ont été observés. En particulier, le taux d'expression de *Mthfr* et le taux d'activité de MTHFR est plus élevé pour les souris BALB/c que pour les souris C57Bl/6. Une augmentation de MTHFR peut diminuer la disponibilité du 5,10-méthyltétrahydrofolate, lequel est requis

pour la synthèse de dTMP. Ceci peut résulter en des dommages à l'ADN. L'expression des gènes reliés à la réparation d'ADN a été examinée. Les souris C57Bl/6 nourries avec la diète DF semblent avoir une augmentation de *Thymine DNA glycosylase (Tdg)* et *Auprinic/apyrimidinic endonuclease1(Apex1)* en comparaison avec les souris BALB/c, ce qui peut contribuer à une meilleure capacité de réparer l'ADN.

Ces résultats suggèrent qu'un déficit en folates peut initier la formation de tumeurs intestinales, possiblement à cause d'une augmentation de dommages à l'ADN associée à une diminution des processus de réparation.

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THESIS FORMAT

This thesis comprises five chapters. Chapter I is a review of the literature pertaining to this thesis. Chapters II through IV are data chapters in the format in which they were published or will be submitted for publication and are linked by connecting text.

CONTRIBUTIONS OF THE AUTHORS

For chapters II, III and IV, the candidate designed experiments, analyzed and interpreted the data, and wrote the manuscripts in collaboration with her supervisor.

In Chapter II, the candidate killed the mice and inspected the intestines for tumors with aid from Liyuan Deng. She analyzed the microarray data, which had been previously obtained by Daniel Leclerc. She performed all nucleic acid extractions, laser capture microdissection, RT-PCR and immunostaining. Qing Wu and Liyuan Deng maintained the animal colony. Xiao-Ling Wang sectioned the fixed intestines and performed the histological characterization of the tumors.

In Chapter III, the candidate bred the majority of the BALB/c mice; the rest of the BALB/c and all of the C57Bl/6 mice were bred by Qing Wu. The candidate designed and implemented the betaine supplementation experiment. She killed the mice and inspected the intestines for tumors with the aid of Liyuan Deng. She performed laser capture microdissection, all nucleic acid extractions and immunostaining. The HPLC method was optimized for the lab by Andrea Lawrance and the candidate performed the HPLC sample preparation, running and analysis under her supervision. TLC was performed by Liyuan Deng. Plasma homocysteine was measured by the Institut de Recherches Cliniques de Montréal as a service.

In Chapter IV the candidate performed all nucleic acid and protein extractions using tissues collected during work for the previous chapters. She optimized and performed the Thymidylate Synthase activity assay. She prepared the samples and analyzed the data from the aCGH, which was performed as a service by Empire Genomics. The MTHFR activity assay was performed by Qing Wu. The MTR activity assay was performed by C. Lee Elmore under the

supervision of Rowena Matthews. The free intestinal amino acids were measured by Sapna Gupta under the supervision of Warren Kruger. TLC was performed by Liyuan Deng.

ABBREVIATIONS

$^{5\text{-me}}C$	5-methylcytosine
aCGH	array comparative genomic hybridization
AMP	adenylate monophosphate
AOM	azoxymethane
Apc	adenomatous polyposis coli
APE1/ Apex1	apurinic/apyrimidinic endonuclease 1
ATM	ataxia-telangiectasia mutated
ATR	ATM and Rad3 related
BHMT	betaine homocysteine methyltransferase
CBS	cystathionine-beta-synthase
CDC2	cell division cycle 2
CDC25C	cell division cycle 25c
CD	control diet
CDP	cytidylate diphosphate
CHK1	checkpoint kinase 1
CHK2	checkpoint kinase 2
CHO	Chinese hamster ovary
CIMP	CpG island methylator phenotype
CRC	colorectal cancer
CTP	cytidylate triphosphate
dAMP	deoxyadenylate monophosphate
dATP	deoxyadenylate triphosphate
dCMP	deoxycytidylate monophosphate
dCTP	deoxycytidylate triphosphate
DEPC	diethylpyrocarbonate
dGMP	deoxyguanylate monophosphate

dGTP	deoxyguanylate triphosphate
DHF	dihydrofolate
DHFR	dihydrofolate reductase
DMH	dimethylhydrazine
DNMT	DNA methyltransferase
dNTP	deoxynucleotide triphosphate
dsb	double-strand break
dTMP	deoxythymidylate monophosphate
dTTP	deoxythymidylate triphosphate
dUMP	deoxyuridylate monophosphate
dUTP	deoxyuridylate triphosphate
FAD	flavin adenine dinucleotide
FAP	familial adenomatous polyposis
FDB	folate-deficient betaine-supplemented diet
FD	folate-deficient diet
Foxo3	forkhead box O3
FPGS	folylpoly-gamma-glutamate synthase
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
GMP	guanylate monophosphate
GNMT	glycine-N-methyltransferase
HNPCC	hereditary non-polyposis colorectal cancer
HPLC	high pressure liquid chromatography
Igf2	insulin-like growth factor 2
IMP	inositol monophosphate
MAT	methionine adenosyltransferase
MBD4	methyl-binding domain 4
Mlh1	mut-L homolog 1
Msh2	mut-S homolog 2
MSI	microsatellite instability

MTHFD1	methylenetetrahydrofolate dehydrogenase, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase 1
MTHFD1L	formyltetrahydrofolate synthetase 1L
MTHFD2	methylenetetrahydrofolate dehydrogenase 2
MTHFR	methylenetetrahydrofolate reductase
MTR	methionine synthase
MTRR	methionine synthase reductase
MYC	myelocytomatosis oncogene
MYT1	*myt*-1 kinase
NI	normal intestine
OR	odds ratio
PCFT	proton-coupled folate receptor
p-H2AX	phosphorylated histone H2AX
PLA2G2	secretory type II phospholipase A2
PLK1	polo-like kinase 1
PMS2	post mitotic segregation increased 2
QAMP	quantitative analysis of methylation by PCR
QPCR	quantitative PCR
RAS	retrovirus associated sequence
RBC	red blood cell
RFC1	reduced folate carrier 1
RLGS	restriction landmark genomic scanning
RR	relative risk
SAH	S-adenosylhomocysteine
SAHH	SAH hydrolase
SAM	S-adenosylmethionine
Sec63	translocation protein SEC63 homolog
Ses1	sestrin 1
SHMT1	serine hydroxymethyltransferase 1
SHMT2	serine hydroxymethyltransferase 2

SMUG1	single-strand selective monofunctional uracil DNA glycosylase
Snx3	sorting nexin 3
Ssb	single-strand break
TDG	thymine DNA glycosylase
THF	tetrahydrofolate
TLC	thin layer chromatography
TS	thymidylate synthase
UDP	uridylate diphosphate
UMP	uridylate monophosphate
UNG	uracil DNA glycosylase
UTP	uridylate triphosphate
WEE1	*wee*-like homolog 1
Zbtb24	zinc finger 24

CONVENTIONS

In this thesis the names of genes and transcripts are italicised; uppercase for human (*i.e. MTHFR*) and titlecase for mouse (*i.e. Mthfr*). The names of proteins are not italicised; both human and mouse proteins are uppercase (*i.e.* MTHFR).

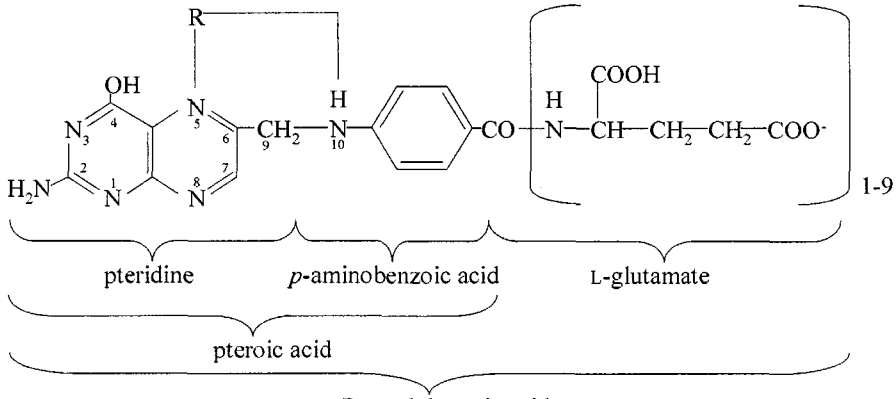
CHAPTER I

Literature Review

1.1 Folate metabolism

1.1.1 Folate intake and absorption

Folate is a B-vitamin composed of a pteridine ring attached to *p*-aminobenzoic acid by a methylene bridge with 1 to 9 glutamate residues connected by γ-peptide bonds (**Figure 1.1**)[1; 2].



R: CH_3 (N^5), CHO ($N^{5,10}$), CH=NH (N^5), CH_2 ($N^{5,10}$), and CH= ($N^{5,10}$)

Figure 1.1 The molecular structure of folate and derivatives.
Folate can contain between 1 and 9 glutamate residues. Folic acid is oxidized at carbons 5, 6, 7 and 8. Dihydrofolate is reduced at carbons 5 and 6, tetrahydrofolate is reduced at carbons 5, 6, 7, and 8. The N5 and N10 positions (R) of tetrahydrofolate can be substituted with methyl, formyl, formimino, methylene, or methenyl one-carbon groups. *Based on Figure 1 of Stokstad and Koch, 1967*[1] *and Kim YI, 2007*[2]

The pteridine ring is readily reduced to form the dihydro (C 7 and 8) or tetrahydro (C 5, 6, 7, 8) derivatives. The R-group, attached to the nitrogen at position five, varies in the different folate metabolic derivatives. A methyl group side chain on the fully reduced tetrahydrofolate (THF) form creates 5-methylTHF, the major circulating form of folate in the body[1]. Other metabolically active forms include 5,10-methyleneTHF (R= CH_2), 5 and 10-formylTHF (R= CHO), 5,10-methenylTHF (R= CH) and 5-formiminoTHF (R= CH=NH). The fully

oxidized form of the vitamin, folic acid, occurs in the monoglutamylated form and is synthesized for use in vitamin supplements and fortified foods[2].

Mammals cannot synthesize folate; therefore it must be obtained through diet or supplement use. Foods with high folate content include leafy green vegetables, citrus fruits, yeast extracts, liver and kidney. Naturally occurring folates, however, are easily oxidized and unstable and food preparation methods can reduce folate bioavailability[3]. It is estimated that only about 50% of naturally occurring folate in food is bioavailable for metabolic processes compared to 100% of folic acid from supplements[4]. This may be due to the difficulty of removing the polyglutamate residues of folate, a process necessary for intestinal absorption[5]. In addition to food sources, the naturally occurring intestinal microflora are capable of synthesizing folate,[6] which can be absorbed by the large intestine[7].

Folate absorption occurs largely in the duodenum and upper jejunum[8]. The enzyme γ-glutamyl hydrolase is located on the brush border of intestinal enterocytes and removes the polyglutamate tail from dietary folate for absorption to occur[9]. Monoglutamylated folates are then taken up by cells through the action of folate transporters, mainly the reduced folate carrier (RFC1) and the proton coupled folate transporter (PCFT). Both transporters have been found to be expressed in the intestinal brush border. RFC1 is an anion exchange transporter with optimal activity at a neutral pH and an increased affinity for reduced and decreased affinity for oxidized folates[8]. The optimal pH for the uptake of folate by intestinal enterocytes, however, is pH 6 and oxidized forms of folate are favored for uptake by the intestine[8; 10]. It has since been shown that the PCFT is the major intestinal folate transporter due to its optimal activity at pH 5.5 with similar affinities for the oxidized and reduced forms of folate[11; 12]. Further evidence comes from a study of a family with hereditary folate malabsorption which was found to be due to an inactivating mutation in PCFT[12].

Once absorbed by the intestine, folates enter the hepatic portal system where they are delivered to the liver. Both RFC1 and PCFT1 are thought to be involved in folate uptake by extra-intestinal tissues, along with the folate

receptors (α and β), which can take up folate by an endocytotic mechanism[8; 12]. Folates stored in the liver are kept intracellular by the addition of a polyglutamate tail by the enzyme folylpoly-gamma-glutamate synthetase (FPGS)[13; 14]. The liver is the main site of folate metabolism after which folate is delivered back into circulation in the form of 5-methylTHF and is taken up by the specific transporters in other tissues[12].

1.1.2 Folate metabolic pathway: overview

Figure 1.2 shows a simplified schematic overview of the folate metabolic pathway. As previously mentioned, folate enters the cells in one of two forms, oxidized (folic acid) or reduced (5-methylTHF), and is retained in the cell by the addition of polyglutamate tails. Oxidized forms of folate enter the metabolic pathway through reduction by the enzyme dihydrofolate reductase (DHFR) at C 7 and 8 to dihydrofolate (DHF) then at C 5 and 6 to THF. Reduced forms of folate, such as 5-methylTHF, are already metabolically active and do not need to be further modified for use as substrates in folate metabolism. The major role of reduced folates in the cell is to provide one-carbon groups for the synthesis of nucleotide precursors for DNA synthesis and cellular methylation reactions through formation of the universal methyl donor, S-adenosylmethionine (SAM). The enzyme methylenetetrahydrofolate reductase (MTHFR) is essential to folate metabolism in that it sits at a critical junction and determines whether one-carbon units will be distributed to nucleotide precursors or SAM synthesis. An in-depth discussion of MTHFR and the key enzymes involved in folate metabolism follows.

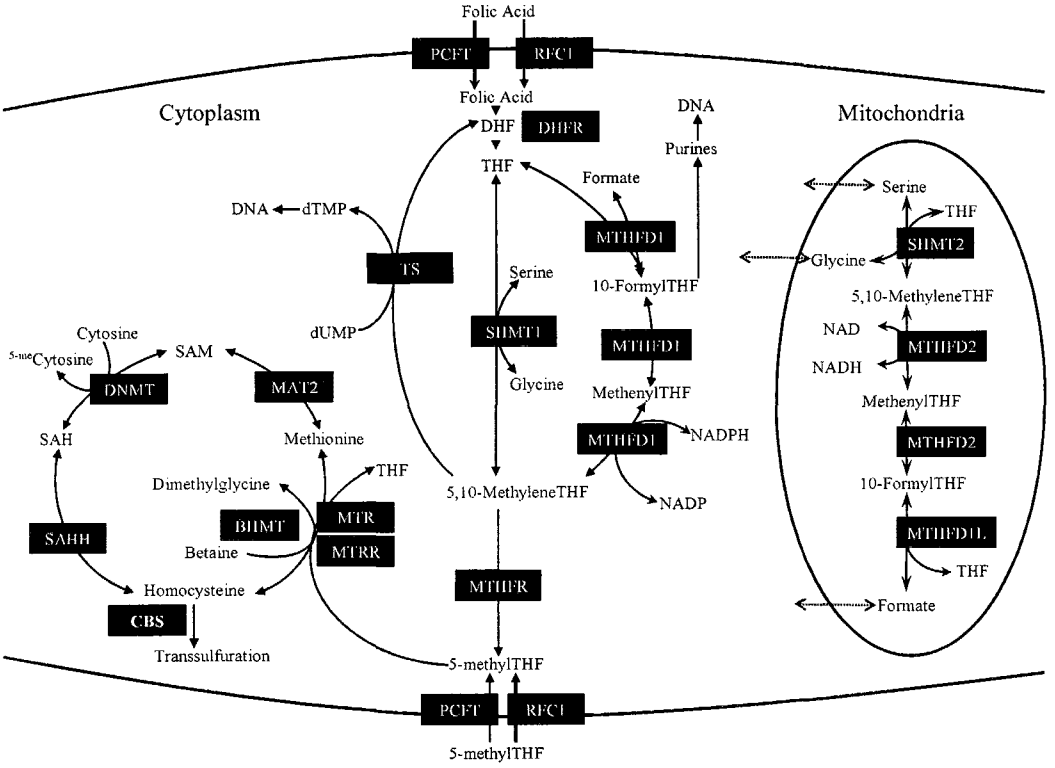

Folic Acid
PCFT
RFC1
Cytoplasm
Folic Acid
DHF
DHFR
DNA
Purines
Mitochondria
THF
Formate
Serine
THF
DNA
dTMP
MTHFD1
SHMT2
Glycine
TS
Serine
10-FormylTHF
5,10-MethyleneTHF
dUMP
SHMT1
NAD
MTHFD2
Cytosine
SAM
Glycine
NADH
5-meCytosine
DNMT
MAT2
MethenylTHF
MethenylTHF
SAH
Methionine
MTHFD1
NADPH
MTHFD2
Dimethylglycine
THF
5,10-MethyleneTHF
10-FormylTHF
NADP
BHMT
MTR
MTHFD1L
SAHH
Betaine
MTRR
THF
MTHFR
Homocysteine
Formate
CBS
Transsulfuration
5-methylTHF
PCFT
RFC1
5-methylTHF

Figure 1.2 The folate metabolic pathway

The thick lines represent the plasma and mitochondrial membranes. Folate in its common circulating form, 5-methylTHF crosses the plasma membrane mainly due to RFC1. Folic acid crosses mainly due to PCFT. Within the cell, folate metabolism is divided into the cytoplasmic and mitochondrial compartments. One-carbon units cross the mitochondrial membrane in the form of serine, glycine and formate (dashed arrows). The enzymes involved in folate metabolism are in boxes.

Abbreviations: **BHMT**: betaine homocysteine methyltransferase; **CBS**: cystathionine-β-synthase; **DNMT**: DNA methyltransferase; **DHF**: dihydrofolate; **DHFR**: dihydrofolate reductase; **dTMP**: deoxythymidylate monophosphate; **dUMP**: deoxyuridylate monophosphate; **MAT**: methionine adenosyltransferase; **MTHFD1**: trifunctional methylenetetrahydrofolate dehydrogenase; **MTHFD2**: bifunctional methylenetetrahydrofolate dehydrogenase; **MTHFD1L**: monofunctional formyltetrahydrofolate synthetase; **MTHFR**: methylenetetrahydrofolate reductase; **MTR**: methionine synthase; **MTRR** methionine synthase reductase; **PCFT**: proton coupled folate transporter; **RFC**: reduced folate carrier; **SAH**: S-adenosylhomocysteine; **SAHH**: S-adenosylhomocysteine hydrolase; **SAM**: S-adenosylmethionine; **SHMT**: serine hydroxymethyltransferase; **THF**: tetrahydrofolate; **TS**: thymidylate synthase.

1.1.2.1 Methylenetetrahydrofolate Reductase (MTHFR)

MTHFR catalyses the irreversible conversion of 5,10-methyleneTHF to 5-methylTHF *in vivo*[15]. Decreased MTHFR activity results in an accumulation of 5,10-methyleneTHF, which is necessary for nucleotide synthesis. Increased activity results in increased 5-methylTHF, which is necessary for methionine and, subsequently, SAM synthesis[16]. The gene encoding human *MTHFR* was mapped to chromosome 1p36.3 and encodes a 2.2kb cDNA comprised of 11 coding exons[17-19]. The mouse ortholog with similar structure was mapped to chromosome 4[18] and found to share 90% amino acid identity with the human protein[19]. *MTHFR* was found to have two active promoter regions. The characterization of these promoter regions led to the discovery of two major and two minor tissue specific cDNA isoforms, as well as evidence of alternate splicing of exon 1 resulting in the expression of 70 and 77kDa protein isoforms[20; 21]. It is currently unknown whether these two isoforms have different activity. The *MTHFR* gene is ubiquitously expressed with highest expression in testis, intermediate expression in the brain and kidney, lower levels in heart, liver, lung, skin, small intestine, spleen and thymus, and lowest levels in stomach and muscle[20].

The MTHFR protein contains an N-terminal catalytic domain and a C-terminal regulatory domain. Enzyme activity has been shown to be influenced by several modifications and inhibitors. Flavin adenine dinucleotide (FAD) is a covalently bound co-factor necessary for MTHFR activity and NADPH is required as a reducing agent[22]. SAM has been shown to be a potent inhibitor of MTHFR activity by reducing the FAD binding capacity[23]. This presumed feedback mechanism spares one-carbon groups for nucleotide synthesis when sufficient one-carbons are present for methylation reactions[16]. MTHFR is also inhibited by DHF and polyglutamated folate derivatives[22]. Phosphorylation of MTHFR on threonine 34 influences activity by reducing SAM inhibition[24]. It has recently been shown that endoplasmic reticulum stress results in up-regulation of MTHFR in a c-JUN mediated mechanism[25].

1.1.2.1.1 Human MTHFR deficiency:

Mutations in *MTHFR* have been found which reduce enzyme activity to as low as 0-20%[26-28] and represent the most common inborn error of folate metabolism. Patients present with hyperhomocystinuria, motor and gait abnormalities, thromboses, neurological disorders and seizures[29].

The most common polymorphism in *MTHFR* is a C-T transition at base pair 677 (*MTHFR* 677C→T), resulting in an amino acid change from alanine to valine[30]. The frequency of the *MTHFR* 677TT genotype is high in North America, Europe and China where about 30% of the population is *MTHFR* 677TT[31]. North Americans of south Mediterranean and Hispanic decent have a very high frequency of *MTHFR* 677TT individuals (~40%) with the lowest incidence in Africans and Americans of African descent (estimates range from 0 to ~10%)[32; 33].

MTHFR 677C→T results in a mild form of MTHFR deficiency with homozygous mutants retaining 30% and heterozygotes retaining 65% enzyme activity and displaying increased levels of plasma homocysteine[30]. The alanine to valine amino acid change disrupts the FAD binding site of the enzyme resulting in a less stable enzyme with decreased residual activity after heat inactivation (thermolabile)[34]. FAD dissociates from the thermolabile enzymefaster than from the wild-type enzyme. However addition of 5-methylTHF results in a slower release of FAD[35]. This fact may help explain why many differences between individuals with the polymorphism become less prominent when there is sufficient supply of 5-methylTHF. In one human study, *MTHFR* 677TT homozygotes had significantly higher plasma homocysteine compared to 677CC people when plasma folate levels were low; however when plasma folate was high plasma homocysteine did not differ between homozygotes and wild-types[36]. In another study, total DNA isolated from the peripheral blood monocytes of *MTHFR* 677TT individuals showed decreased methylation compared to 677CC people, but only when plasma folate levels were low[37]. Differences in cancer susceptibility between individuals with *MTHFR* polymorphisms may also depend on folate status, as will be discussed in later sections.

The *MTHFR* 677TT genotype results in an increased risk for neural tube defects (NTDs)[38-40]. Risk is increased when the maternal genotype is also *MTHFR* 677TT and folate intake is low[41]. The *MTHFR* 677TT genotype is associated with high homocysteine, an increased risk factor for heart disease[42-46]. A meta-analysis of 40 studies indicates that the *MTHFR* 677TT genotype is a moderate risk factor for venous thrombosis[47].

A second common polymorphism in *MTHFR*, an A→C transversion at base pair 1298, results in an amino acid substitution from glutamate to alanine in the regulatory domain of the protein. The frequency of the 1298CC genotype is similar to that of the 677TT in the populations examined[48]. The 1298CC genotype is not associated with increased risk for neural tube defects, however a trend towards an increased risk was found for compound heterozygotes compared to individuals who were wild-type for both loci. This is due to linkage disequilibrium between these two loci[49; 50]. The association of the 1298CC genotype and risk for cardiovascular disease has not been well established[31]. Although the 1298CC genotype results in decreased enzyme activity in lymphocytes, no change in plasma homocysteine was observed between wild-type and 1298CC homozygotes[49].

1.1.2.1.2 Murine MTHFR deficiency

A mouse model which reflects the various levels of human MTHFR deficiency was created by Chen *et al.*[51] through disruption of *Mthfr* exon 3 by homologous recombination insertion of the neo^r gene. Homozygous mutant mice (*Mthfr*-/-) have no enzyme activity, severely increased plasma homocysteine (10-fold above wild-type values), decreased survival at five weeks of age (25% on the BALB/c background, 76.4% on the C57Bl/6 background) with slower development and neurological abnormalities. Heterozygous mice (*Mthfr*+/-) have 60% of the wild-type enzyme activity and moderately increased plasma homocysteine (1.6 fold above wild-type values). They show no phenotypic abnormalities at birth which would distinguish them from wild-type littermates[51]. *Mthfr*-/- mice have decreased levels of 5-methylTHF in plasma, liver, and brain compared to wild-type mice. Although not significant in *Mthfr*+/- mice, a trend

was observed towards decreased 5-methylTHF in plasma, liver, and brain[52]. Decreased levels of SAM were found in the testis and ovaries of both *Mthfr*+/- and -/- mice, as well as in brain of *Mthfr*-/- mice. Both *Mthfr*+/- and -/- mice showed decreased levels of total (or global) DNA methylation in brain and ovary[51]. Due to comparable enzyme activity and plasma homocysteine increases in the *Mthfr* mutant mice with humans who display MTHFR deficiency due to the 677 variant, these mice are considered are good models for examining the role of MTHFR in cardiovascular disease, birth defects, and other folate dependent disorders[51; 53-55].

1.1.2.2 Nucleotide precursor synthesis: overview

Nucleotides are composed of a purine (adenine or guanine) or pyrimidine (cytosine, thymine, or uracil) nitrogenous base attached to a ribose or deoxyribose sugar and 1 to 3 phosphate groups. Nucleotides are generally abbreviated according to the reduction state of the sugar ("d" if the sugar is deoxyribose, no prefix for ribose sugar), the nitrogenous base (adenylate=A, cytidylate=C, thymidylate=T, guanylate=G, uridylate=U) and the number of phosphate groups attached (one, monophosphate=MP, two, diphosphate=DP, or three, triphosphate=TP).

Folate metabolism is essential for nucleotide precursor synthesis because it provides one-carbon groups in the form of 5,10-methyleneTHF for dTMP synthesis and in the form of 10-formylTHF for dAMP and dGMP synthesis. However, *de novo* nucleotide synthesis occurs mainly during development and growth and may not play a large role in adult tissues with the exception of the liver, kidney or in neoplastic growth (i.e. cancer)[56]. In most adult tissues the "salvage" pathway is sufficient to meet the nucleotide requirements of dividing cells. This pathway involves kinases which can regenerate dNTPs from DNA degradation products[57]. Further discussion will focus on *de novo* nucleotide synthesis (**Figure 1.3**), since it is directly related to folate metabolism.

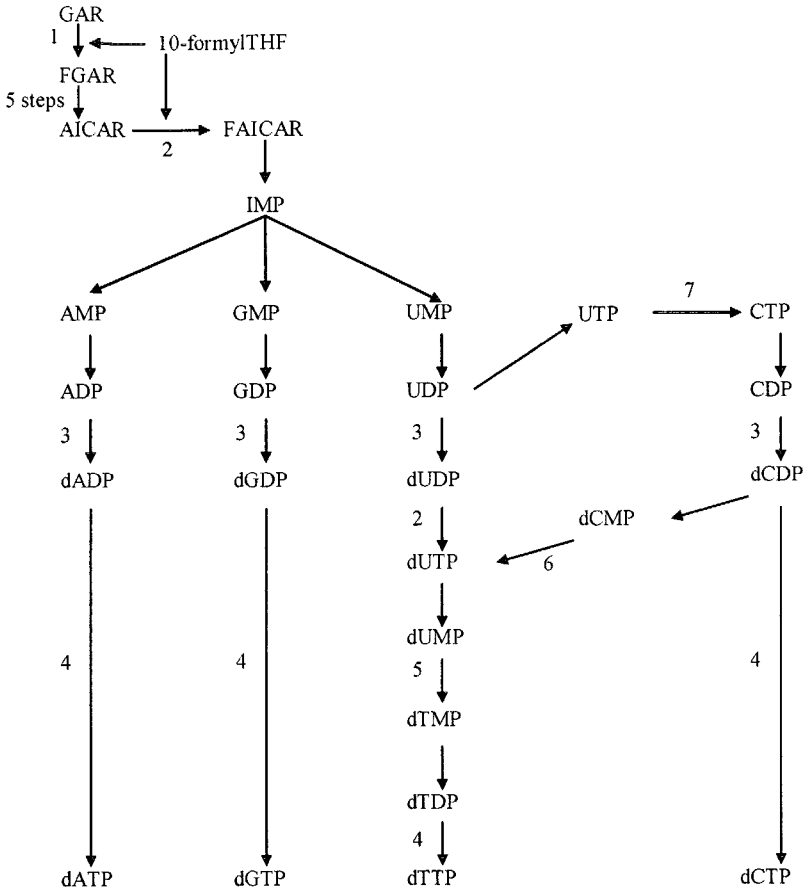

GAR
1
10-formylTHF
FGAR
5 steps
AICAR
2
FAICAR
IMP
AMP
GMP
UMP
UTP
7
CTP
ADP
GDP
UDP
CDP
3
3
3
3
dADP
dGDP
dUDP
dCDP
dCMP
2
dUTP
6
dUMP
4
4
5
4
dTMP
dTDP
4
dATP
dGTP
dTTP
dCTP

Figure 1.3 *De novo* nucleotide synthesis.

One-carbon units from 10-formylTHF are used in IMP synthesis through intermediates formed by reactions 1 and 2. IMP is the pre-cursor for purine synthesis (Left) and UMP synthesis. UMP is the pre-cursor for pyrimidine synthesis (Right). Reaction 5 also connects nucleotide synthesis to folate metabolism as one-carbon units from 5,10-methyleneTHF are required for this reaction.

Enzymes: 1-GAR transformylase; 2-AICAR transformylase; 3-ribonucleotide reductase; 4-deoxynucleotide diphosphate kinase; 5-thymidylate synthase; 6-dCMP deaminase; 7-CTP synthase.

Abbreviations: **AICAR**: 5'-aminoimidazole-4-carboxamine ribonucleotide; **ADP**: adenylate diphosphate; **AMP**: adenylate monophosphate; **CDP**: cytidylate diphosphate; **CTP**: cytidylate triphosphate; **dADP**: deoxyadenylate diphosphate; **dATP**: deoxyadenylate triphosphate; **dCDP**: deoxycytidylate diphosphate; **dCMP**: deoxycytidylate monophosphate; **dCTP**: deoxycytidylate triphosphate; **dGDP**: deoxyguanylate diphosphate; **dGTP**: deoxyguanylate triphosphate; **dTDP**: deoxythymidylate diphosphate; **dTMP**: deoxythymidylate monophosphate; **dTTP**: deoxythymidylate triphosphate; **dUDP**: deoxyuridylate diphosphate; **dUMP**: deoxyuridylate monophosphate; **dUTP**: deoxyuridylate triphosphate; **FAICAR**: formyl5'-aminoimidazole-4-carboxamine ribonucleotide; **FGAR**: formylglycinamide ribonucleotide; **GAR**: glycinaminde ribonucleotide; **GDP**: guanylate diphosphate; **GMP**: guanylate monophosphate; **IMP**: inositol monophosphate; **THF**: tetrahydrofolate; **UDP**: uridylate diphosphate; **UMP**: uridylate monophosphate; **UTP**: uridylate triphosphate; *Based on Figure 1 of Mathews 2006.*

1.1.2.2.1 Thymidylate Synthase (TS)

UMP is the precursor for both dTTP and dCTP synthesis (**Figure 1.3**). dUMP from the dCMP deaminase reaction, or from the reduction of UDP by ribonucleotide reductase, becomes the substrate for the thymidylate synthase (TS) reaction[57]. The TS enzyme catalyses the addition of a one-carbon unit from 5,10-methyleneTHF to dUMP to form dTMP and DHF. Nucleoside monophosphate and diphosphate kinases add the final phosphate groups to form dTTP[57]. As the main requirement for dTTP is during DNA synthesis (S phase of cell cycle), it is not surprising that *TS* expression varies over the cell cycle and is increased in proliferating, as opposed to quiescent cells. Control of *TS* expression occurs at the transcriptional, posttranscriptional and translational levels[58]. At the transcriptional level, a 28 base pair tandem repeated sequence in the *TS* enhancer is necessary for proper gene expression[59]. Additionally, a CpG rich region is located in the promoter region, suggesting that regulation by DNA methylation could be possible[60]. At the post-transcriptional level, cell cycle dependent regulation of expression is controlled by a mechanism whereby the essential promoter region regulates the proper splicing of introns to form the transcript[61]. Further regulation at the translational level occurs due to the ability of the TS enzyme to bind its own mRNA and inhibit its own proper translation[62]. The TS enzyme also has the ability to bind to the mRNAs of important growth regulatory genes such as *c-MYC* and *p53*[58].

The TS enzyme competes with MTHFR for available 5,10-methyleneTHF. The Km value of TS for 5,10-methyleneTHF is higher than that of MTHFR demonstrating that, under normal conditions, MTHFR is saturated and the activity of TS is dependent on cellular concentrations of 5,10-methyleneTHF. This is supported by the observation that, under increasing cellular concentrations of 5,10-methyleneTHF, purine and pyrimidine synthesis is increased[15]. When folate levels are low, MTHFR will out-compete TS and favor 5-methylTHF production over dTMP synthesis[16].

1.1.2.2.2 Serine Hydroxymethyltransferase 1 (SHMT1)

In order to synthesize dTMP, TS requires a one-carbon unit. This unit is provided by 5,10-methyleneTHF. The enzyme serine hydroxymethyltransferase (SHMT1) uses THF and serine to produce 5,10-methyleneTHF and glycine in a reversible reaction. Under normal physiological conditions, serine has been proven to be the major donor of one-carbon units for DNA synthesis[63; 64]. *SHMT1* is expressed in adult liver and kidney with very low levels of expression in other tissues[65]. *SHMT1* has also been found to have increased expression in tumors[66; 67]. The *SHMT1* gene has been observed to have multiple splice variants resulting in tissue specific protein forms[65]. Studies of the mouse protein (91% identity with human), however, suggest that any splice variants produced are not catalytically active[68]. Direct binding of c-MYC to the *SHMT1* promoter, resulting in increased expression, has been observed[67].

Since the SHMT1 reaction is reversible it has been postulated that, in addition to MTHFR, SHMT1 has the ability to direct one-carbon units towards *de novo* pyrimidine synthesis or towards SAM synthesis[63]. Studies show that increasing concentrations of glycine *in vitro* favor the production of 10-formylTHF at the expense of 5-methylTHF[69]. This would suggest increased purine synthesis and decreased SAM synthesis. In support of this, increased expression of *SHMT1* resulted in decreased SAM and increased *de novo* dTMP synthesis[69]. Based on these data it was proposed that two pools of SHMT1 exist; one which associates with DHFR and TS to supply one-carbons for nucleotide synthesis in a glycine independent manner and one which actively competes with MTHFR for 5,10-methyleneTHF in a glycine dependent manner[69]. A recent animal model was developed where *Shmt1* was removed[70]. *Shmt1-/-* mice were viable and fertile, suggesting that *Shmt1* is not essential for growth and development. SHMT1 protein activity has also been shown to be responsive to nutritional status; protein levels and activity are decreased in MCF-7 cells cultured in vitamin B6 deficient media[71].

1.1.2.2.3 Purine precursor synthesis

One-carbon units for purine synthesis come from 10-formylTHF (**Figure 1.3**), which is used to by the enzymes glycinaminde ribonucleotide transformylase and 5'-aminoimidazole-4-carboxamine ribonucleotide transformylase to produce the intermediates formylglycinamine ribonucleotide and formylaminoimidazole-4-carboxaminde ribonucleotide, respectively. These two intermediates are necessary for the formation of the purine precursor inositol monophosphate (IMP). Both AMP and GMP are produced from IMP, reduced by ribonucleotide reductase to their decarboxylated forms, and converted to dATP and dGTP by nucleoside monophosphate and diphosphate kinases[72].

1.1.2.2.4 MethyleneTHF Dehydrogenase, MethenylTHF Cyclohydrolase, FormylTHF Synthetase (MTHFD1)

As mentioned previously, the requirement of adult tissues for nucleotides is small, as sufficient nucleotides are provided by salvage of precursors from DNA degradation. Any adult tissues which do require *de novo* synthesis of purines (in the liver and kidney, for example) are thought to obtain sufficient amounts of precursors from 10-formylTHF produced by the trifunctional cytoplasmic methyleneTHF dehydrogenase, methenylTHF cyclohydrolase, 10-formylTHFsynthetase (MTHFD1) enzyme[73]. *MTHFD1* is ubiquitously expressed in adult human and mouse tissues[74; 75]. The mRNA levels of this housekeeping gene vary between tissues, due in part to the post-transcriptional regulation by insulin-like growth factor 1 which stabilizes *MTHFD1* mRNA[76]. *MTHFD1* expression is high, and remains unchanged, during development[77].

The role of MTHFD1 is to maintain the balance between 5,10-methyleneTHF and 10-formylTHF in the cytoplasm. The dehydrogenase domain catalyses the reversible conversion of 5,10-methyleneTHF to methenylTHF using NADP as a co-factor. MethenylTHF is generally a short lived intermediate which is rapidly converted to 10-formylTHF by the cyclohydrolase domain. The synthetase domain catalyses the ATP-dependent, reversible formation of 10-formylTHF from formate and THF[73]. *In vivo*, the properties of and co-factor

availability for MTHFD1 suggest that the reactions proceed towards the formation of 5,10-methyleneTHF[78].

1.1.2.2.5 Serine Hydroxymethyltransferase 2 (SHMT2)

Under normal conditions in adult cells, mitochondrial folate metabolism is important for glycine synthesis. However, when the demand for purines is high (conditions of increased growth), mitochondrial folate metabolism can be implemented in order to provide sufficient one-carbon groups for nucleotide precursor synthesis. Reduced folates cannot cross the mitochondrial membrane; therefore one-carbon units must enter the mitochondria in the form of serine, glycine and formate[13; 14; 79]. Serine hydroxymethyltransferase 2 (SHMT2) uses mitochondrial THF and serine to form 5,10-methyleneTHF and glycine in a reversible reaction. *SHMT2* shares 63% sequence identity with *SHMT1* and produces a protein of equivalent size[80]. The expression of *SHMT2* does not vary considerably between tissues or during development, although slightly higher levels can be found in adult liver, muscle and pancreas[60]. *SHMT2* expression is not influenced by nutritional status or cell cycle, although increased expression has been observed in some tumor cells[62]. Interestingly, the *SHMT2* promoter has been shown to be a direct target of c-MYC binding[62].

In the reversible SHMT2 reaction, evidence suggests that the production of glycine and 5,10-methyleneTHF is strongly favored under normal conditions. Chinese Hamster Ovary (CHO) cells lacking a functional SHMT2 are glycine auxotrophs, although cells lacking a functional SHMT1 were not. This suggests that SHMT2 is the major cellular producer of glycine[81].

1.1.2.2.6 MethyleneTHF Dehydrogenase 2 (MTHFD2) and FormylTHF Synthetase 1L (MTHFD1L)

Additional one-carbon groups necessary for *de novo* purine synthesis under growth conditions come from formate which is produced during mitochondrial folate metabolism. Formate is produced through two reactions; first 5,10-methyleneTHF produced by SHMT2 is converted to methenylTHF, which is then converted to 10-formylTHF in a two step reaction catalyzed by the bifunctional, NAD dependent, methyleneTHF dehyrogenase 2 (MTHFD2). 10-

formyl is then converted to formate through the reversible synthetase activity of the mitochondrial 10-formylTHF synthetase (MTHFD1L)[73].

This pathway is ordinarily not active in adult cells[73]; however it appears to be critical during development[77]. Cultured cells lacking *MTHFD2*, when growth stimulated, could not produce sufficient purines to support growth[82]. *MTHFD2* expression has been detected in almost all transformed cell lines[83], but while the mRNA was detectable at very low levels, no protein or activity has been detected in adult tissues[84]. *MTHFD1L*, on the other hand, is ubiquitously expressed with increased expression in thymus and low levels in kidney and liver[85]. It also has increased expression in colorectal cancer[86]. The *MTHFD2* promoter has a serum responsive element, which allows expression of the gene under conditions of cell growth[87], and, like *MTHFD1*, *MTHFD2* mRNA is stabilized by insulin-like growth factor 1[84].

The availability of the NAD cofactor suggests that, under normal conditions, the production of 10-formylTHF is favored. The increased 10-formylTHF would then drive the MTHFD1L reaction towards formate synthesis, particularly if this product is being removed by MTHFD1 in the cytoplasm for 10-formylTHF production. High levels of formate can be toxic to cells, which may explain why MTHFD2 is low to non-detectable in adult tissues. In adult tissues, formate may be used by MTHFD1L to produce 10-formylTHF which serves in the production of formylmethionyl-tRNA in the mitochondria[73].

1.1.2.3 SAM synthesis and DNA methylation reactions

SAM is the universal methyl donor for cellular methylation reactions. The majority of SAM is used in the liver for creatine synthesis; other common uses include methylation of proteins, neurotransmitters and DNA[16]. This thesis will focus on SAM as the methyl donor for DNA methylation reactions. The cycle of SAM synthesis (refer to **Figure 1.2**) begins with the production of methionine from the toxic amino acid homocysteine. Homocysteine is also the end product of the SAM dependent methylation reactions, since the by-product S-adenosylhomocysteine (SAH) is hydrolyzed by the enzyme SAH hydrolase (SAHH) to form homocysteine. A build up of homocysteine can be damaging to

cells. High plasma levels of homocysteine are associated with oxidative stress and inflammation which may increase the risk for many diseases such as cardiovascular disease, neural tube defects and cancer[88; 89]. Additionally, the SAHH reaction favors the production, instead of hydrolysis of SAH, a potent inhibitor of methyltransferases. High homocysteine can increase SAH production. Therefore, homocysteine must be efficiently removed or metabolized by the cell[16]. Removal of homocysteine can be accomplished through three mechanisms. First, homocysteine can be directed towards SAM synthesis by re-methylation to methionine. Second, homocysteine can be converted to cystathionine by the enzyme cystathionine-β-synthase (the transsulfuration pathway) which eventually produces glutathione, an important cellular redox regulator. However, only some tissues express a functional transsulfuration pathway (liver, kidney, pancreas, intestine and brain). Third, when the transsulfuration pathway is not present and/or the ability of tissues to re-methylate homocysteine to methionine is overwhelmed, excess homocysteine can be exported to the plasma[90].

1.1.2.3.1 Methionine Synthase (MTR)

Methionine synthase (MTR) catalyses the vitamin B12 dependent re-methylation of homocysteine to form the amino acid methionine. *MTR* is expressed in all tissues with the lowest expression in skeletal muscle and the highest expression in kidney and pancreas[91]. *MTR* expression is also high in fetal tissues[92]. *MTR* transcription can be influenced by cellular metabolites; for example homocysteine increases *MTR* transcription[93]. *MTR* expression and activity do not appear to correlate, however, suggesting post-transcriptional regulation[94; 95]. Two upstream open reading frames in the *MTR* promoter, which do not form functional proteins themselves, behave as negative regulators of *MTR* translation by stalling the translational machinery[96]. Vitamin B12 levels also affect the translation of *MTR* by moving *MTR* mRNA from the ribonucleoprotein pool to the polysome pool in order to increase translational efficiency[97].

The MTR reaction requires vitamin B12, or cob(I)alamin, as a co-factor in a two step reaction. The first step is donation of a methyl group from 5-

methylTHF to cob(I)alamin, forming methylcobalamin. The second step is the transfer of the methyl group from methylcobalamin to homocysteine, forming methionine[98]. Normal regulation of methionine synthesis by MTR is dependent on two competitions; between homocysteine use for re-methylation or the transsulfuration pathway and between methionine use for SAM or protein synthesis. Age and methionine supplementation to a normal diet can decrease MTR activity and instead direct homocysteine towards transsulfuration[99]. MTR activity is also decreased when vitamin B12 levels are decreased, resulting in decreased SAM and low levels of DNA methylation[95]. Furthermore, alcohol can inhibit MTR activity[100]; however low folate levels do not[95].

Deficiencies in vitamin B12 and/or in MTR lead to increased homocysteine levels and megaloblastic anemia due to a phenomenon termed "methyl trapping". This is because MTR is the only enzyme capable of metabolizing 5-methylTHF, which is necessary not only for SAM synthesis but also to regenerate methyl groups in the form of THF for the SHMT and MTHFD1 reactions[101]. Mice deficient in *Mtr* highlight the importance of the MTR reaction; the *Mtr*-/- genotype is embryonic lethal[102].

1.1.2.3.2 Methionine Synthase Reductase (MTRR)

Methionine synthase reductase (MTRR) is necessary for proper MTR function. Every few hundred MTR reaction cycles, the cob(I)alamin co-factor is oxidized to cob(II)alamin, which cannot be used by MTR. MTRR reduces cob(II)alamin to cob(I)alamin in an FAD dependent manner and then SAM is used to re-methylate cob(I)alamin back to methylcobalamin[98].

MTRR has been found to be expressed in heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas[103]. Examination of the human gene detected evidence of alternative splicing and a CG rich area in the promoter region which overlaps with the transcriptional start site and is a potential target for regulation by DNA methylation[104].

Mice deficient in *Mtrr* have helped elucidate the role of MTRR in the cell. As in *Mtr*-/- mice, the *Mtrr*-/- genotype is embryonic lethal in mice. A gene trap mouse model with some residual activity displayed increased plasma

homocysteine and decreased methionine as would be expected[98]. However the level of SAM in the liver was increased in relation to SAH. This suggests that a mechanism exists to spare SAM when methionine levels are low. The action of the enzyme glycine N-methyltransferase (GNMT) in liver could explain this; this enzyme uses SAM to convert glycine into sarcosine and SAH, and is inhibited by 5-methylTHF. When MTRR is low 5-methylTHF accumulates and may inhibit GNMT, thus sparing SAM. Additionally, liver expresses betaine homocysteine methyltransferase, which can use betaine as an alternate methyl donor for homocysteine re-methylation (see section 1.1.2.3.3)[98; 105]. Therefore cells have developed alternative mechanisms to maintain at least some SAM synthesis under conditions of low MTRR activity.

1.1.2.3.3 Betaine Homocysteine Methyltransferase (BHMT)

In some tissues, the enzyme betaine homocysteine methyltransferase (BHMT) is an alternate route for re-methylation of homocysteine using betaine as a methyl donor and producing methionine and dimethylglycine[106]. In humans, *BHMT* appears to only be expressed in liver and kidney[107]. *BHMT* expression is induced under conditions of low methionine, even more so when a low methionine diet is supplemented with methyl donors such as betaine and choline. *BHMT* mRNA expression appears to correlate with BHMT protein and activity levels[108]. *BHMT* expression has also been found to be hormone responsive and therefore may differ between males and females[109].

BHMT protein activity is regulated by dietary restrictions and cellular metabolites. BHMT activity is increased with increasing choline, betaine or cysteine, but only when combined with other dietary restrictions such as low methionine. High levels of SAM or selenium deficiency can reduce the activity of BHMT[109]. The effects of dietary folate on BHMT activity are dependent on other nutritional factors present. When folate deficiency was combined with selenium deficiency, BHMT activity was decreased; however in one animal model, folate deficiency alone increased BHMT activity but addition of ethanol to the diet blocked this effect[109].

Plasma betaine levels are inversely correlated with plasma homocysteine levels and are a strong modifier of homocysteine under conditions of low MTHFR activity, plasma folate, or plasma vitamin B12[110]. In fact, in an *in vivo* model of rat liver, BHMT was shown to process 25% of cellular homocysteine[109]. Betaine can be obtained from the diet from dietary choline, which is converted to betaine by choline oxidase and betaine aldehyde dehydrogenase[109]. Studies using the MTHFR deficient mouse model have shown that dietary betaine supplementation can rescue survival and improve the brain defects observed in *Mthfr*-/- mice; possibly by reducing homocysteine levels and increasing available methionine and SAM[111; 112]. A similar study also found that dietary betaine supplementation could correct infertility and improve sperm counts in surviving *Mthfr*-/- mice[113]. Human studies involving dietary betaine supplementation show that betaine can reduce plasma homocysteine in humans[114] and is particularly effective in individuals with low folate or vitamin B12 status and the *MTHFR* 677TT genotype[110]. One study has even suggested that dietary betaine supplementation may be associated with decreased risk for colorectal adenomas in women[115].

1.1.2.3.4 Methionine Adenosyltransferase 2 (MAT2)

SAM is synthesized from methionine in an ATP dependent manner by the enzyme methionine adenosyltransferase (MAT). Two main forms of this enzyme exist: MAT1 and MAT2. MAT1 is a liver specific form, while MAT2 is ubiquitously expressed and will therefore constitute the main focus of this section[116]. *MAT*2 expression is regulated by E2F and Sp2 and 3 transcription factor binding sites in the promoter[117]. The Sp 2 and 3 sites are thought to be critically important for differential expression of *MAT*2[118]. Increased expression of *MAT*2 is associated with increased growth and transformation[116]; high *MAT2* expression is found in human intestine and in tumors[119]. Alcohol and SAM are known to decrease the expression of *MAT2*[116; 120].

The MAT2 enzyme is composed of two subunits, a catalytic alpha and regulatory beta subunit. Expression of both subunits has been found to be increased in human colorectal cancers and stimulation of cultured cells with growth factors increases the expression of the alpha subunit[116]. The beta subunit

itself has two variant forms which are expressed in most tissues. Variant 1 of the beta subunit has been found to be protective against apoptosis in colon and liver cancer cells in culture[121].

1.1.2.3.5 DNA Methyltransferase 1 (DNMT1)

SAM is the methyl donor for DNA methylation reactions. The reaction is catalyzed by DNA methyltransferases (DNMT) which add the methyl group of SAM to carbon five of the cytosine ring forming 5-methyl-cytosine ($^{5\text{-me}}$C) and SAH. DNA methylation represents an epigenetic change, meaning it effects a heritable change in gene expression which is not resultant of a change in DNA sequence. Other modifications which represent epigenetic changes are histone modifications and microRNAs.

There are five known DNMTs: 1, 2, 3a, 3b and 3l. DNMT1 is the maintenance methyltransferase and methylates newly formed DNA when copying a strand which originally contained a $^{5\text{-me}}$C ("hemimethylated DNA"). DNMT3a and b are *de novo* methyltransferases which lay down new methylation patterns on unmethylated DNA in germ cells and during development. DNMT3l has no DNA methylation activity but is necessary to enhance the activities of DNMT3a and b (reviewed in [122]). The function of DNMT2 is to methylate the aspartic acid tRNA[123]. Since this thesis is primarily concerned with adult somatic cells, the focus will be on DNMT1.

DNMT1 is expressed in dividing adult tissues, but is particularly high in post-mitotic male and female germ cells. Increases in *DNMT1* expression are frequently observed in colorectal cancers. The DNMT1 enzyme contains a catalytic C-terminal domain and an N-terminal domain necessary for protein-protein interactions and recognition of hemi-methylated DNA[122]. DNMT1 enzyme activity is inhibited by SAH[16].

Cytosine methylation by DNMT1 occurs almost exclusively within the context of cytosines adjacent to guanines in the DNA strand (CpG motif). Between 4 and 6 % of cytosines in human DNA are methylated under normal conditions[124]. The majority of CpGs in the genome (70-80%) are methylated and are associated with transposable elements and repeat regions[125]. The remaining

20-30% of normally unmethylated CpGs are found within CpG islands, which make up about 1-2% of the human genome. CpG islands occur in or near the promoter elements of about 60% of human genes; under some conditions methylation of CpG islands can occur and is generally associated with imprinted genes, X chromosome inactivation in females[126], tissue specific gene expression, or with inappropriate silencing of tumor suppressor genes (discussed further in section 1.5.2)[127]. The main consequence of CpG methylation is a tightening of the DNA-histone structure resulting in a closed chromatin conformation and inactivation of gene transcription. In fact, high levels of DNA methylation are detected in transcriptionally inactive heterochromatin; transcriptionally active euchromatin is comparatively methylation free.

It is generally considered that DNA methylation represses transcription by blocking the access of transcriptional machinery to promoter regions[128; 129]. It is currently unknown how DNMT1 is targeted to specific sites in the genome or whether DNA methylation is the initial step in gene silencing or a consequence of it (discussed in [130]). It is known, however, that DNA methylation alone is not sufficient to alter the chromatin conformation and repress transcription. Methylated cytosines recruit methyl cytosine binding proteins (e.g. MeCP1 and 2, MBD 1-4) and DNMT1 itself recruits histone deacetylases, which together form complexes associated with altering the histone structure and a closed chromatin conformation[122; 127; 131]. The DNMT1 enzyme also has an unusual feature in that it binds with higher affinity to cytosines which are paired opposite to abnormal DNA structures than to hemimethylated DNA. This suggests that the origin of DNMT1 was as a DNA repair enzyme and provides a link between DNA methylation changes and DNA damage observed in cancer (discussed further in section 1.5.2) [132].

Besides regulation of gene expression, DNA methylation is critical for genome stability. The majority of CpG methylation in cells occurs at repetitive regions and parasitic sequences such as retrotransposons and Alu sequences[133; 134]. DNA methylation is critical to prevent these sequences from becoming reverse transcribed and re-inserted elsewhere in the genome ("jumping"). Parasitic gene

insertion at random locations in the genome can interrupt normal gene functioning, lead to increased recombination and genome instability[135; 136].

1.2 Folic acid supplementation and fortification

Folate metabolism has been shown to be involved in the etiology of many diseases and disorders. Based on a wealth of evidence that dietary folate intake is key in preventing neural tube defects[137], the Food and Drug Administration instituted mandatory fortification with 140-150μg folic acid/100g grains and cereals in the United States starting in 1996[138]. Similar legislation was adopted in Canada starting in 1998[139]. Since the institution of mandatory fortification, significant increases in plasma folate, serum folate, and red blood cell (RBC) folate as well as decreases in plasma homocysteine have been observed[140-142]. Additionally, rates of neural tube defects dropped significantly after fortification was instituted in Canada[143] and in the US[144]. Some claim that this may be misleading, however, since rates of neural tube defects in North America were on the decline pre-fortification and what is observed is only the continuation of that trend[145].

The safety of mandatory fortification is not without question. One of the major initial concerns was that increased intake of folic acid would correct the overt symptoms of vitamin B12 deficiency (pernicious anemia) without correcting the neurological abnormalities, leading to an increase in undiagnosed B12 deficiency in at risk elderly populations[76; 146-148]. Studies conducted after the institution of fortification in Canada have shown that, although the incidence of folate deficiency has dropped to an estimated 0.5%, the incidence of vitamin B12 deficiency has not changed[149]. This indicates that masking of vitamin B12 deficiency is a valid public health concern, particularly since a US study found that up to 14.5% of elderly patients were B12 deficient[147].

Additional concern is that the high amount of folic acid in fortified foods combined with consumption of larger portion sizes and increased multivitamin use would result in the daily folic acid intake of most North Americans exceeding the determined safe upper limit of folate intake (1000 μg/day). In fact, actual

folic acid consumption after fortification has so far exceeded the expected amount, up to an estimated >200μg/day [150]. Intakes of folic acid in excess of >200 μg/day exceed the body's capacity to metabolize and leads to folic acid accumulation in the serum[151]. Accumulation of un-metabolized folic acid in the blood is hypothesized to be associated with numerous potential health complications. In one study, women over 60 years of age who consumed >200 μg/day of folate had decreased natural killer cell toxicity[152]. It is hypothesized that, since folic acid is known to inhibit particular enzymes involved in folate metabolism, high blood folic acid levels could disrupt normal folate metabolism[153]. However, studies in rats fed supra-physiologic doses of folic acid have shown increased serum folic acid, decreased plasma levels of the toxic amino acid homocysteine, but no effect on the levels of key folate metabolites or on the activities of key enzymes in the folate metabolic pathway[154].

Other concerns related to fortification include increased risk for spontaneous abortion, incidence of multiple births, resistance and tolerance to anti-folate chemotherapeutics, anti-inflammatory and anti-seizure drugs[145; 155]. Studies have since shown that the incidence of spontaneous abortion and multiple births has not changed since the institution of fortification; however some evidence suggests that folic acid supplementation interferes with the efficacy of chemotherapeutics such as methotrexate as well as malarial anti-folate drugs[145].

It was hoped that fortification might reduce the risks of cardiovascular disease but little supporting evidence exists, although a reduced incidence of stroke has been observed[153]. Furthermore, it has been hypothesized that excess folic acid in the population may be reducing the phenotypic severity of the *MTHFR* 677C→T polymorphism. The polymorphic enzyme is more stable when folate levels are high. This could result in a higher survival rate for polymorphism carriers, thereby propagating a potentially harmful allele in the population[156].

The role of folate in cancer development (discussed in detail later) has been widely debated and various hypotheses have been put forward concerning the effect of folic acid supplementation on cancer risk. One study has found that

rates of pediatric neuroblastoma have decreased by 60% post-fortification, although the incidence of pediatric acute lymphoblastic leukemia has stayed the same[157]. In contrast, a hypothesis put forward by Mason[158] examining the colorectal cancer (CRC) incidence trends before and after fortification suggests that the trend towards decreasing rates of CRC observed pre-fortification is no longer observed post-fortification. This trend reversal was not explained by increased endoscopy rates. Based on this observation, it was hypothesized that folic acid fortification could be promoting the growth of pre-cancerous lesions (polyps, adenomas) which have been found to occur at high frequency in the population[158].

1.3 Colorectal cancer (CRC)

1.3.1 Development and cell types of the gastrointestinal tract

The function of the intestine is the digestion of food and the absorption of nutrients and water. It is divided into the small (duodenum, jejunum, and ileum) and large (colon and rectum) intestines.

The intestinal mucosa is composed of a connective tissue layer and the epithelial layer. The connective tissue layer, the lamina propria, is composed of mesenchyme containing fibroblast cells, blood vessels, lymph nodes, immune cells, enteric nerves and smooth muscle fibers [159]. The epithelial layer is highly folded in order to increase the absorptive area; the small intestine contains the finger-like villi and tubular crypts while the large intestine contains only large indented crypts. Crypts contain the proliferative stem cells which generate all the differentiated cell types of the epithelium [160]. The stem cells divide asymmetrically, producing one stem cell which remains in the crypt and one daughter cell which migrates up and differentiates [159]. Around 6-10 crypts support one villus. There are four types of differentiated cells in the villus: absorptive enterocytes (colonocytes in colon), mucus secreting goblet cells, hormone-secreting endocrine cells and anti-microbial Paneth cells [159]. A mature villus contains about 3500 cells, which in the progress of about 3-5 days will migrate up the length of the villus to the top where they will apoptose and be shed

into the intestinal lumen. The loss of cells in the intestine varies between species; mice shed around $2x10^8$ cells/intestine/day while humans shed around 10^{11} cells/intestine/day[161].

The intestinal tract develops from the ventral endoderm, which in mice envaginates at embryonic day 7.5-8.0 to form a complete tube by day 8.5-9.0. The splanchnic mesoderm surrounds the tube to give rise to the mesenchyme and muscle; neural crest cells also migrate in to form the enteric nervous system. From embryonic day 14.5-18.5 the first columnar epithelial cells and villi form[159]. In the first few days of life, the intervillus epithelium begins to fold, forming crypts composed of a mixed population of cells[162]. By postnatal day 7, Paneth cells begin to appear and by post-natal day 9, the maximum number of villi is reached[159; 163]. At postnatal day 14 the crypt stem cells are fully formed and the crypts become a monoclonal population of cells[162]. Finally, at postnatal day 48, all crypts are fully formed and the maximum crypt-villus height is reached[159; 163].

1.3.2 Prevalence

Colorectal cancer (CRC) is the third most common cancer in North America behind lung and breast (women) or prostate (men) cancer. However, it is second only to lung cancer in terms of cancer related deaths. The lifetime chance of developing CRC is approximately 1/20. Fortunately, mortality rates for this cancer appear to be decreasing[164]. Roughly 85% of CRC cases are due to environmental factors, while the remaining 15% are due to inherited pre-disposition[165].

1.3.3 Molecular mechanisms in spontaneous CRC

It is well known that CRC progresses from normal epithelium to adenoma to carcinoma. Transformation of the normal epithelium occurs when one cell acquires a growth advantage and continues to divide unchecked; most colorectal tumors are monoclonal in nature[166]. Common genetic events which are well established in CRC progression include inactivating mutations in retrovirus associated sequence (*RAS*; found in 50% of CRC), amplifications of c and b myelocytomatosis oncogene (c- and b-*MYC*), truncating mutations in adenomatous polyposis coli (*APC;* found in 80% of carcinomas), loss of

chromosome 5q (found in 20-50% carcinomas and 30% adenomas), loss of chromosome 17p (a region known to include *p53*, found in 75% carcinomas), and loss of chromosome 18q (a region known to include deleted in colorectal cancer, DCC, found in 70% of carcinomas and 50% of adenomas)[166]. Other hallmarks of sporadic CRC include microsatellite instability (occurs in 13% sporadic tumors), chromosomal instability (87% of sporadic tumors), decreased total DNA methylation (hypomethylation) and increases in promoter methylation of specific genes (hypermethylation)[165]. A hypothesized order of molecular events for some CRC cases is as follows: normal epithelium acquires a mutation in *APC*, the intestine becomes dysplastic and progresses to an early stage adenoma, mutations in *RAS* and *DCC* enable progression to a late stage adenoma, then acquisition of a *p53* mutation causes progression to carcinoma [165].

1.3.4 Molecular mechanisms in hereditary CRC

Two common hereditary forms of CRC exist which constitute the majority of the heritable cases. Familial adenomatous polyposis (FAP) represents ~1% of hereditary CRC with an age of onset of 39 years[165]. Patients typically present with hundreds of pre-cancerous polyps in the distal colon, which have a high probability of progressing to aggressive tumors[167]. FAP is caused by germ line mutations in the *APC* gene[168]. *APC* mutations are nearly 100% penetrant[169] and result mainly in protein truncations[170; 171]. APC is part of the Wnt signaling pathway and normally functions to repress β-catenin. The Wnt signaling pathway controls proliferation in the crypts. In this pathway receptor signaling leads to stabilized β-catenin, which increases transcription of growth promoting genes [159]. Mutations in *APC* lead to increased Wnt signaling and proliferation[165].

Hereditary non-polyposis colorectal cancer (HNPCC) accounts for 2-4% of hereditary CRCs with an average age of onset of 45 years. HNPCC patients do not develop hundreds of polyps and fewer, less aggressive, tumors are generally found in the proximal colon. This syndrome is associated with a high incidence of multiple tumors forming within a short time of each other and a high incidence of recurrence after resection. HNPCC is characterized by tumors with microsatellite instability and is caused by mutations in DNA mismatch repair

genes. Common mutations are missense mutations in or epigenetic silencing of the MutL homolog 1 (*MLH1*), MutS homolog 2 (*MSH2*) and post mitotic segregation increased 2. Mutations in these genes results in a decreased DNA repair capacity, leading to increased DNA strand breaks and chromosomal abnormalities[165].

1.3.5 Mouse models of CRC

The use of mouse models to study CRC has advantages and disadvantages. Mouse models are easily manipulated, allowing for control over environment, diet and genetic background. This control over experimental conditions allows molecular mechanisms to be examined in a detail not always possible in human studies. However, mouse and human physiology and genetics are not always comparable. In many cases, the physiologic and molecular response to a given experimental condition differs between mice and humans. In the case of CRC models, for example, rodents are coprophagic and have different populations of colonic microflora which may affect tumor development [172]. Furthermore, mutations and carcinogens which cause colonic tumors in humans often cause only intestinal tumors in mice and the molecular progression of those tumors may not be concordant with what is observed in humans[173].

1.3.5.1 The *Apc*$^{min/+}$ mouse model

In an ethylnitrosurea mutagenesis screen a line of mice was discovered which developed multiple adenomas in the intestine tract at a very early age (~3 months). This model was called the MIN model for multiple intestinal neoplasias[174]. It was later learned that the defect in MIN mice was a mutation in the *Apc* gene [175]. The *Apc*$^{min/+}$ mutation was found to be 100% penetrant and the molecular and cellular progression was found to mirror FAP [176]. This model has also been used to study aspects of sporadic CRC as well, due to the high incidence of *APC* mutations in sporadic tumors.

Other models have been created with different mutations in *Apc* than the one found in the *Apc*$^{min/+}$ mouse. The *Apc* delta 716 and *Apc* 1638n knock out mice do not have as severe a phenotype as the *Apc*$^{min/+}$ mouse and only develop a

few adenomas in the small intestine. They may therefore be a better model of sporadic tumors which acquire *APC* mutations [173].

1.3.5.2 Other mouse models of CRC and strain differences in cancer susceptibility

Based on the success of the $Apc^{min/+}$ model of FAP, mouse models of HNPCC have also been attempted. *Msh2* mutant mice die of lymphoma and only develop gastrointestinal tumors if they survive past 6 months with the help of lymphoma suppressing agents. Fifty percent of *Mlh1* mutant mice live to 6 months, but all succumb to lymphomas and gastrointestinal tumors by 13 months. Though not completely reflective of the human conditions, the *Mlh1* mouse model seems to be the better model for HNPCC in that relatively few intestinal tumors develop [177].

The use of chemical carcinogens to induce CRC in mice is often used. The two most widely used carcinogens are dimethylhydrazine (DMH) and its metabolite azoxymethane (AOM). DMH requires activation through liver metabolism and its main effect is to cause methylation of guanine residues in DNA, leading to point mutations. Tumors induced by DMH take about 6 months to appear, although that varies with dose. Often AOM is chosen because it does not need to be metabolised and has a latency of only 4 weeks. Other techniques which are used in conjunction with carcinogens include the use of bile-acids as co-carcinogens, injury to the intestine (including the use of sutures and small bowel resection) and gastric surgery. These models are widely used because they can produce colon tumors in mice, whereas genetic models mostly produce intestinal tumors. Although they may reflect human CRC cases where the underlying cause is carcinogen exposure, the molecular progression in these models does not reflect the human cases [173].

An interesting observation from studies involving mouse models of CRC is that strains of mice with different genetic backgrounds display varying susceptibilities to cancer. The $Apc^{min/+}$ mouse was originally bred on the C57Bl/6 mouse background; however when those mice were crossed with the AKR/J and MA/MyJ inbred strains it was noted that the F1 hybrids had decreased numbers of

adenomas[178]. This was linked to differences between strains in the secretory type II phospholipase A2 (*Pla2g2a*) gene; wild-type *Pla2g2a* resulted in decreased while mutated *Pla2g2a* resulted in increased adenoma formation[179]. Different responses to carcinogen injection and radiation treatment have also been observed. The C57Bl/6 strain is resistant, while the BALB/c strain is susceptible, to DMH, AOM, dextran sodium sulphate, and ethylnitrosurea induced CRC [173; 180; 181], N-methyl-N-nitrosurea induced gastric adenocarcinomas [182], and x-irradiation induced cancers[183].

1.4 Dietary folate and CRC

Dietary folate deficiency has been implicated in the development of many cancers including cancers of the lung, pancreas, esophagus, stomach, cervix, ovary, breast, and liver [145]. The strongest data, however, link folate deficiency to CRC development. This thesis will focus on the association between dietary folate and risk for colorectal cancer.

1.4.1 Human studies

Most case-control and cohort studies agree that low dietary folate is associated with an increased risk for CRC (reviewed in [184; 185; 2]). In a meta-analysis of studies conducted before 2005, a slight decrease in risk was found with increased folate intake in case-control studies. However the heterogeneity was too high for statistical significance (OR=0.76; 95%CI=0.62-1.05). In cohort studies a significant 25% decrease in risk was found to be associated with increased dietary folate (RR=0.75; 95%CI=0.64-0.89) [186].

Some cohort studies have found that increased dietary folate is associated with increased CRC risk. In a cohort study by de Vogel *et al.* [187], men with a truncating mutation in *APC* and an increased intake of dietary folate had an increased risk for CRC; although when the *APC* mutation was not present, the risk was decreased. In a prospective study, Van Guelpin *et al.* [188] found that increased plasma folate levels were associated with an increased CRC risk.

In one randomized placebo-controlled trial, folic acid supplementation (1mg/day) of patients with colorectal adenomas found a non-significant decrease

in adenoma recurrence in a follow-up exam 1 to 2 years later [189]. In another very small study (11 participants), rectal biopsies from a folate supplemented (1mg/day) group showed decreased cellular proliferation in the muscosa compared to the placebo group after 3 months. Decreased proliferation would presumably prevent formation of new adenomas[190]. In a more recent study with a 3 to 5 year follow-up period, no effect of folate supplementation (1mg/day) was observed on adenoma recurrence at the first follow-up, but in the 60% of participants who underwent a second follow-up, the supplementation was associated with an increased incidence of 3 or more adenomas and occurrence of non-colorectal cancer[191].

Several caveats must be kept in mind when interpreting the human data linking folate and CRC risk. The first is that many confounding risk factors are known which interact with dietary folate to modulate risk (e.g. smoking, alcohol and gene polymorphisms, such as *MTHFR* 677C→T). Secondly, it is difficult to accurately predict the folate status of the colon from food intake estimates and blood folate measures. Evidence suggests that CRC patients with normal blood folate levels have localized folate deficiency in the colon [2; 192]. Lastly, the timing of dietary folate deficiency is known to affect whether CRC risk is increased or decreased [2]. In a recently developed mathematical model of folic acid supplementation and CRC risk, it was shown that folic acid supplementation is only protective when initiated before 20 years of age; if initiated later cancer growth is promoted[193]. The importance of timing in the initiation of CRC by folate deficiency is particularly clear in the evidence from rodent models of folate and CRC risk.

1.4.2 Mouse and rat studies

Using the well-established *Apc*$^{min/+}$ mouse model, many studies have examined the impact of dietary folate deficiency on tumor multiplicity. In one of the earliest studies, *Apc*$^{min/+}$ mice were fed a high fat, low fiber diet with decreased calcium, vitamin D and folate designed to mimic the typical North American diet. This study found an increased incidence of intestinal tumors, as well as the appearance of colonic tumors (rare in this model)[177]. Subsequent

studies examined the effect of folate deficiency alone. In one study *Apc*$^{min/+}$ mice were placed on either a folate sufficient or deficient diet for either 3 or 6 months. Those placed on the deficient diet for 3 months developed more adenomas; those on the diet for 6 months developed fewer adenomas than those on the sufficient diet [194]. In another study, *Apc*$^{min/+}$*Msh2*-/- mice were placed on folate-sufficient or deficient diets at either 3 weeks or 6 weeks of age until reaching 11 weeks of age. The mice who were placed on the deficient diet at 3 weeks of age (presumably before tumor initiation) developed more tumors; those placed on the deficient diet at 6 weeks of age (after tumor initiation) developed fewer tumors compared to mice on the sufficient diet [195]. Following this, a study of *Apc*$^{min/+}$ mice fed folate and choline deficient diets post-weaning found similar results [196]. The authors of these studies hypothesized that while folate deficiency damages normal intestinal tissue, leading to neoplasia, tumor tissue requires adequate folate in order to sustain increased growth while deficiency results in growth arrest [194-196]. A recent study taking a more in depth approach placed wild-type female C57Bl/6 mice on a folate sufficient or deficient diet during mating with *Apc*$^{min/+}$ males as well as during pregnancy and lactation. The litters were then randomized to receive either the sufficient or deficient diets at weaning. The maternal diet appeared to have no effect on tumor incidence; however female mice placed on the sufficient diet at weaning had more, larger, tumors than those placed on the deficient diet [197].

Studies using a rat model of CRC have also provided insight into the impact of folate deficiency on CRC risk. Some studies took the approach of using carcinogen injection (DMH or AOM) induced CRC. Placing the rats on a folate-deficient diet before injection increased the appearance of aberrant crypt foci (a pre-neoplastic lesion)[198] dysplasia [199], and carcinoma [200], but did not affect the frequency of *Apc* or *p53* mutations[201] compared to the folate sufficient groups. An interesting study using a microarray approach, examined the changes in gene expression in weanling and 1 year old rats fed 0 mg or 8 mg folic acid/kg diet for 20 weeks. In the 0 mg group there were far more age related expression changes in cancer causing genes than in the 8 mg group. This suggested that folate

deficiency may aggravate age-related genetic changes which promote cancer development[202].

Of additional interest is the observation that rats fed a folate, choline and methionine (folate-methyl) deficient diet developed spontaneous liver tumors without carcinogen injection or genetic predisposition [203; 204]. This provided evidence that folate deficiency could have transformative properties when combined with other nutrient deficiencies.

1.4.3 MTHFR and CRC

Many studies examining the impact of the *MTHFR* 677C→T and 1298A→C polymorphisms have concluded that, under conditions of adequate folate, the *MTHFR* 677TT genotype is protective against CRC [205-214], as is the 1298CC genotype [211; 213; 214]. However that protection disappears or is reversed when dietary folate is low [206; 208; 215-217]. In contrast, some studies found no association [218-220] or an increased risk for CRC [216; 221-224] in *MTHFR* 677TT individuals regardless of folate intake. A meta-analysis performed on studies conducted before 2007 found that *MTHFR* 677CT and TT individuals have a decreased risk for CRC compared to *MTHFR* 677CC individuals (OR 0.95; 95% CI=0.90-1.00, P = 0.04). This decreased risk was not observed for adenomas (OR 0.97; 95% CI=0.90-1.05, P = 0.45)[225].

Alcohol and smoking further modify the risks associated with *MTHFR* polymorphisms and CRC. *MTHFR* 677TT individuals who consumed low amounts of alcohol were found to be protected against CRC [206; 212]and those who consumed moderate to high amounts of alcohol (>30g/day) had no protection [205; 206] or an increased risk for CRC [215; 226; 227]. Smoking alone was found to increase risk for colorectal adenomas, with a further increase in risk if the smoker was *MTHFR* 677TT and had low blood folate. Conversely, in smokers with high blood folate, the *MTHFR* 677CC genotype was associated with increased risk [208].

It has also been found that the presence of microsatellite instability (MSI) can influence the effect of the *MTHFR* 677TT genotype on CRC risk. *MTHFR* 677TT individuals were found to have more MSI positive (MSI+) tumors [217; 228; 229]. Although one study found that, in individuals with high folate, the *MTHFR*

677TT genotype was associated with a decreased risk for MSI+ tumors [230] and another study found no association between *MTHFR* genotype and MSI status [231]. A study by Ulrich *et al.*, while finding no association between the *MTHFR* 677TT genotype and MSI, did find that *MTHFR* 677TT individuals had a decreased risk of developing mutations in *P53* (frequently found in CRC) when adjusted for smoking, alcohol and folate intake [232].

Despite inconclusive population studies, it is clear that *MTHFR* polymorphisms have some influence, whether positive or negative, on CRC risk. In fact, studies have been underway to test the efficacy of MTHFR inhibitors as a cancer treatment and initial trials in cell culture have met with some success [233; 234]

1.4.4 Polymorphisms in other folate metabolism genes and CRC risk

In addition to *MTHFR*, variations in several genes involved in the folate metabolic pathway have been studied in association with cancer risk.

In one animal model study, *Mtr* +/- mice were mated with $Apc^{min/+}$ mice. *Mtr*+/-$Apc^{min/+}$ mice did not have significantly different tumor numbers than those that were wild-type for *Mtr* [235]. The majority of data, though, comes from studies of human populations.

The *MTR* 2756GG genotype was found to be associated with decreased risk for CRC [210; 236; 237] especially when the *MTR* 2756AG or GG genotype was combined with the *MTHFR* 677CT or TT genotype [209]. However other studies found no association [218; 238] or increased risk for CRC in MTR 2756GG individuals when dietary methionine was low or in the absence of the *TS* 3'untranslated region deletion polymorphism [239].

The *MTRR* A66G polymorphism was associated with an increased risk for CRC in one study [209] and other less common polymorphisms were also associated with increased risk [240; 241]. The consistent data for *MTRR*, as opposed to *MTR*, could be explained by the observation that the *MTRR* 66GG genotype, but not the *MTR* 2756GG genotype, is associated with low red blood cell folate and increased plasma homocysteine [242]. The increased frequency of the MTRR variant in the population is another possibility[237].

The *TS* 3rpt/3rpt genotype, which increases *TS* expression, conferred a decreased risk for CRC when dietary folate intake was high[237; 243] but not when folate or vitamin B2 intake was low; under those conditions the 2rpt/2rpt genotype was protective [243; 244]. In *TS* 3rpt/3rpt individuals who drank >30g alcohol/day or had the *MTHFR* 677CC genotype, the risk for colorectal adenomas was increased [245]. The effect of the *TS* 3rpt polymorphism may also be gender specific and influenced by post-menopausal hormone use [238].

The wild-type *SHMT1* 1420CC genotype was found to decrease risk for colorectal adenomas in vitamin B2 adequate compared to vitamin B2 inadequate individuals[244]. The BHMT 742AA genotype was associated with an increased risk for colorectal cancer [240] but a decreased risk for colorectal adenomas [241].

1.5 Folate and CRC: mechanisms

Low dietary folate has been hypothesized to initiate tumor formation through two mechanisms. First low dietary folate limits one-carbon groups available for dTMP synthesis leading to a build up of dUMP, which could be misincorporated into replicating DNA as dUTP and lead to DNA instability. Secondly, low dietary folate limits one-carbon groups for SAM synthesis and DNA methylation reactions, resulting in inappropriate changes in gene expression and chromosome instability. Both mechanisms are discussed below.

1.5.1 Nucleotide imbalances and DNA damage

Nucleotide synthesis is coordinated with cell cycle. Three points of regulation exist; ribonucleotide reductase is down-regulated by dATP and up-regulated by ATP, CTP synthase is down-regulated by CTP, and dCMP deaminase is up-regulated by dCTP and down-regulated by dTTP. Both balanced and unbalanced increases in nucleotides can be mutagenic [72]. dNTPs occur in naturally unbalanced proportions and the concentrations may vary between tissues. In general, dGTP is the least abundant while dCTP is the most abundant nucleotide[57]. Increasing the dGTP pool is mutagenic to cells [72] and some immune disorders lead to accumulations in dGTP or dATP. More commonly, decreases in dTTP lead to the condition known as "thymineless death". dTTP depletion leads

to increased single and double-strand breaks and cell death through apoptosis [57]. This is the mechanism of action of classic anti-folate chemotherapeutics, such as methotrexate[57].

1.5.1.1 Uracil misincorporation and repair

When dUMP is given in excess to cells, DNA damage and apoptosis are observed similar to what occurs in thymineless death [246]. It was discovered that depleted dTTP leads to accumulation of dUTP and, since DNA polymerase can not distinguish between the two, dUTP gets incorporated into replicating DNA [247]. The presence of uracil in DNA is also the result of cytosine deamination, catalyzed by the activation-induced cytosine deaminase. Cytosine deamination occurs more frequently in single-stranded DNA, such as in replication forks [248]. The amount of uracil from both sources is estimated to be about 400 uracils/genome/day [249].

Uracil misincorporated into DNA is removed by the action of the uracil DNA glycosylases. There are four mammalian uracil DNA glycosylases: uracil DNA glycosylase (UNG), thymine DNA glycosylase (TDG), single-strand selective monofunctional uracil DNA glycosylase (SMUG1), and methylbinding domain 4 (MBD4) [250]. UNG and SMUG1 act on single-stranded DNA substrates. UNG preferentially excises uracil from U:A mispairs that result from uracil misincorporated at replication; SMUG1 preferentially excises uracil from U:G mispairs which are a result of cytosine deamination [251]. TDG and MBD4 act on double-stranded DNA substrates. TDG repairs uracil present in double-stranded DNA and T:G mispairs which are a result of $^{5\text{-me}}$C deamination; MBD4 repairs T:G and U:G mismatches from $^{5\text{-me}}$C and cytosine deaminations [252]. Uracil DNA glycosylases remove uracil by cleaving the N-glycosidic bond, leaving an abasic site to which they remain attached, protecting the site, until the apurinic/apyrimidinic endonuclease 1(APE1) arrives. APE1 cleaves the sugar-phosphate backbone of DNA, leaving a 5'deoxyribose phosphate. The resultant DNA single-strand break (ssb) can be repaired by either the long or short patch repair pathways. Short patch repair is the presumed major pathway and involves removal of the 5'deoxyribose phosphate and insertion of the proper

complementary base pair by DNA polymerase-β. This is the rate limiting step. The remaining nick in the DNA backbone is sealed by DNA ligase 3 [249; 250]. Long patch repair occurs largely at replication foci and involves removal of the 5'deoxyribose phosphate and the insertion of several nucleotides through the action of DNA polymerases ε and δ. The removal of the additional nucleotides and nick resealing is performed by flap-endonuclease 1 and DNA ligase 1, which require the presence of proliferating cell nuclear antigen [249; 250].

1.5.1.2 Detection and repair of double-strand breaks

If two excised uracils are present within a few base pairs of each other on opposing strands, base excision repair fails and a DNA double-strand break (dsb) occurs [253]. The decision of whether the cell will repair those dsbs, or proceed to apoptosis, depends on the severity of the breaks. Studies with the DNA damaging agent bleomycin have shown that if the cell detects any more than 500 dsbs or any more than 150 000 ssbs, it will apoptose. If fewer ssbs or dsbs are detected, the cell will initiate repair pathways [254].

The presence of repairable dsbs leads to two signaling pathways, depending on the source and type of damage. Damage induced by ultraviolet light and replication stress is detected by the ataxia telangiectasia and Rad3 related (ATR) protein kinase, whose known targets include the Hus 1 homolog, RAD 1, 9, 17 and 26 homologs and, ultimately, checkpoint kinase 1 (CHK1). DNA dsbs, such as those caused by irradiation, are detected by the ataxia telangectasia mutated (ATM) protein kinase, whose known targets include γ-histone H2AX, p53, p53 binding protein 1, mediator of DNA checkpoint 1, breast cancer 1, Nijmegen breakage syndrome 1, meiotic recombination 11 homolog, RAD 50 homolog and, ultimately, checkpoint kinase 2 (CHK2) [255]. Many of these proteins form complexes with each other that facilitate tight regulation of the response system and, when activated, are often recruited to sites of DNA damage (e.g. phosphorylated histone H2AX) [256]. ATM signalling also plays a role in DNA damage induced apoptosis when repair fails, via p53-mediated increased transcription of pro-apoptotic genes. ATM signalled, p53 independent, apoptosis can also occur [257].

Activation of either CHK1 or CHK2 initiates cell cycle arrest in order for cells to repair DNA damage. The targets of CHK1 and CHK2 are the members of the cell division cycle 25 family (CDC25): CDC25A, CDC25B and CDC25C [258]. Specifically, when dsbs occur, CHK2 initiates arrest in the G2 phase of cell cycle. Arrest at this phase is preferred since the DNA has been duplicated in preparation for mitosis and this facilitates repair by homologous recombination [259]. To begin G2 arrest, CHK2 phosphorylates CDC25C on an inhibitory residue, leading to export of CDC25c from the nucleus to the cytoplasm where it remains inactive, bound to the 14-3-3 protein. Another key step in G2 arrest is the inactivation of polo-like kinase 1 (PLK1) mediated by p53 and breast cancer 1 [260]. In normal cell cycle progression, PLK1 phosphorylates CDC25c, promoting its nuclear transition and activation. It also targets for degradation the wee1 homolog (WEE1) and myt1 kinase (MYT1), whose function is to inactivate cell division cycle 2 (CDC2 or CDK1) [261]. Their inactivation allows CDC25c to remove the inactivating phosphate group from CDC2 permitting the CDC2-cyclin B complex to form and initiate mitotic entry [262]. During G2 arrest, inactive PLK1 can not phosphorylate CDC25c, nor can it inactivate WEE1 or MYT1. The end result is that CDC2 remains phosphorylated, can not complex with cyclin B, and the cell cycle is arrested in order for repair to take place[261].

After repair is complete, PLK1 and CDC25B activity are necessary for progression back into mitosis and in the continuation of the cell cycle (reviewed in [263]). In yeast, a system has developed called "adaptation" where excessive dsbs cannot be repaired and the cell cycle is continued, resulting in mitotic catastrophe and death. It is not known whether adaptation occurs in mammals but it has been shown that over-expression of PLK1 can override the G2 checkpoint [261]. Progression through cell cycle with un-repaired dsbs could lead to cancer causing mutations and indeed over-expression of PLK1 in cell culture results in cellular transformation [264]. Furthermore, PLK1 has been shown to be over-expressed in colorectal cancer [265-267] and is a current target of anti-cancer therapy [268; 269].

1.5.1.3 Folate deficiency and uracil misincorporation

Folate deficiency has been shown to disrupt the balance of nucleotide pools, cause uracil misincorporation into DNA, disrupt base excision repair, lead to DNA dsbs, and cell cycle arrest. In rats fed a folate-deficient diet, dTMP, dGTP and dATP were decreased in spleen [270]. In rat lymphocytes stimulated to grow in culture and incubated with folate-deficient media there was decreased dCMP, dTMP, dGTP and dATP, resulting in cells with decreased DNA synthesis and cell cycle arrest in S and G2 [271]. In the well known rat model of methyl-deficient diet induced hepatocarcinoma, many studies have shown that methyl group deficiency increases dUTP, dsbs, the frequency of abasic sites, breakage of the p53 gene, and apoptosis and decreases dTTP and DNA methylation in the liver and lymphocytes [272-275]. In studies of folate deficiency alone, rats fed the deficient diet showed increased breaks in p53 [276], and decreased base excision repair in the intestine [277]. In a repair deficient CHO cell line, incubation with folate-deficient media not only caused dsbs but when re-supplemented with folate sufficient media after the breaks were present, the cells developed the ability to form tumors in nude mice. Cells maintained in folate-deficient media could not form tumors in nude mice[278].

In human studies, low plasma and red blood cell folate levels were correlated with increased dUTP and micronucleus frequency (a measure of DNA damage)[279-281]. Supplementation of 700μg folic acid/day was found to decrease micronucleus frequency [282]. In support of these findings, culture of human epithelial cells or growth stimulated lymphocytes in folate-deficient media resulted in increased uracil misincorporation, dsbs, and decreased DNA repair ability and growth [283-285]

Since *MTHFR* 677C→T results in lower enzyme activity, there is the potential that more one-carbon groups could be spared for dTTP synthesis. Although in one study with humans fed a low folate diet for seven weeks, *MTHFR* 677TT individuals did have increased dTTP [286], a study with folic acid supplemented humans found only a trend towards increased dTTP in *MTHFR* 677TT compared to *MTHFR* 677CC subjects [287]. Two other studies found no

effect of *MTHFR* 677C→T on uracil misincorporation or DNA strand breaks [288; 289].

1.5.2 Alterations in DNA methylation

A hallmark of tumors is global DNA hypomethylation occurring in concert with hypermethylation in the promoter regions of certain genes. These methylation patterns are in stark contrast to those present in the cells of the surrounding normal tissue [290]. Patterns of altered methylation in cancer cells are often found in areas where age-related alterations in methylation occur, providing a potential mechanism for the link between increased age and cancer predisposition [291]. However, it is still widely debated whether changes in DNA methylation are a cause of cancer formation or merely a consequence of the altered state of transformed cells [130]. One of the main arguments for DNA methylation changes as consequence and not cause comes from the observation that mutations in the DNMTs do not predispose to cancer. Complete knock-outs of DNMT1 are embryonic lethal, but mouse models with decreased DNMT activity exist and evidence from these mice suggests that decreasing DNMT1 activity does impact tumor formation. DNMT1-deficient mice crossed with $Apc^{min/+}$ mice have decreased numbers of adenomas with more protection being conferred as levels of DNMT1 activity decrease [292-295]. There are several possible mechanisms which may explain these results including decreases CpG island methylation and decreased frequency of $^{5\text{-me}}$C related point mutations[292; 294]. In general, it is hypothesized that decreased global DNA methylation could lead to activation of oncogenes, loss of imprinting, re-activation of mobile DNA elements, and chromosome instability. Simultaneously occurring regions of increased DNA methylation could silence tumor suppressing genes and increase the rate of point mutations [126]

Genomic DNA hypomethylation observed in cancer has been shown to affect the promoters of oncogenes such as *H-RAS* and *c-MYC*, which are normally methylated and silenced [296]. Additionally, loss of imprinting of the insulin-like growth factor 2 (*IGF2*) was found in 30% of people with CRC, but only in 10% of healthy individuals [297]. Individuals with loss of imprinting show decreased

differentiation in the colonic epithelium [298] and in cancer patients the loss of imprinted *IGF2* occurred both in the tumor and adjacent normal intestine, suggesting it may pre-dispose to cancer formation [299]. A mouse model for loss of imprinting for *IGF2* was created and, when mated with $Apc^{min/+}$ mice, a decrease in tumor numbers was observed[298]. A closer examination of these mice found that there were fewer advanced adenomas and more micro-adenomas, again suggesting that loss of imprinting of *IGF2* could be involved more in tumor initiation than progression [299]. Global DNA hypomethylation has also been observed to increase the incidence of retroviral insertion into the genome. This may disrupt tumor suppressing genes and/or increase chromosome instability. In fact, the *APC* gene was observed to be disrupted by a LINE element in CRC [300]. Additionally, chromosome instability has been observed to occur around satellite sequences and result in chromosome breakage in some cancers [301; 302].

Regions of high promoter methylation (hypermethylation) have the potential to inactivate tumor suppressing genes, even in the context of global DNA hypomethylation. In human cancer cells lacking *DNMT1,* satellite sequences were demethylated, but the tumor suppressing gene p16 remained methylated and silenced [303]. Some other genes commonly known to be silenced by hypermethylation in CRC include *APC*, calcium channel alpha 1G, procalcitonin, hypermethylated in cancer 1, methylguanine methyltransferase, tissue inhibitor of metalloproteinase 3, Wilm's Tumor 1, and *MLH1*. Methylation of these genes can silence a wild-type copy when a mutated copy is already present (a "second hit" according to Knudson's two-hit hypothesis of cancer formation) [124]. Alternatively, they can occur as part of the CpG island methylator phenotype (CIMP) which characterizes some tumors. The CIMP phenotype appears when certain groups of genes are all methylated and silenced in a particular cancer. CIMP cancers most often show microsatellite instability, increased *K-RAS* mutations and decreased *p53* mutations [291]. The CIMP phenotype appears in 50% of adenomas that progress into carcinomas, but in only 25% of adenomas that regress [304]. Increased DNA methylation in gene regions has another potentially damaging side effect in that $^{5\text{-me}}C$ is easily deaminated to

thymine, particularly in replicating DNA. The rate of $^{5\text{-me}}$C deamination is 2.2x faster than for unmethylated cytosine and accounts for 30% of all point mutations. High rates of point mutations due to $^{5\text{-me}}$C deamination are frequently found in the body of the *p53* gene [305].

The apparent paradox between simultaneous global DNA hypomethylation and regional hypermethylation in cancer could be explained by the properties of DNMT1. As previously mentioned, DNMT1 binds with higher affinity to sites of DNA damage than to hemi-methylated DNA. Since DNA damage is also increased in cancer, DNMT1 may be sequestered to those sites and not available at the replication fork to methylate newly formed DNA strands. This could also explain why genes which are commonly found to be disrupted by DNA damage are also targets of regional hypermethylation (e.g. *p53*) [275].

1.5.2.1 Folate deficiency and altered DNA methylation

Folate deficiency is thought to decrease SAM and therefore decrease the methyl groups available for DNA methylation. Additionally folate deficiency is associated with increased SAH, a potent inhibitor of methyltransferases, thus exacerbating the problem. It has been observed that rats fed a folate-deficient diet had decreased SAM in the liver, but not in the colon and no resulting change in global DNA methylation or methylation in the *c-Myc* promoter after 15-24 weeks of depletion [306]. More severe forms of folate depletion and longer treatments also showed decreased SAM and increased SAH in the liver, but no change in global DNA methylation in the colon [198; 307; 308], although methylation changes have been observed in the liver and brain [125]. Decreases in global DNA methylation were only seen when folate deficiency was combined with choline and methionine deficiency; then hypomethylation of oncogenes (*H-Ras, c-Fos, c-Myc*) and increased methylation of tumor suppressing genes (*Egfr, p53*) was observed [309; 310]

In human studies folate deficiency was associated with decreased global DNA methylation in lymphocytes [280] and increased promoter methylation in 6 genes in individuals with low dietary folate, high alcohol and CRC [311]. *MTHFR* 677C→T, however, has been found to be associated with decreased $^{5\text{-me}}$C content in normal colonic mucosa [312] and in lymphocytes of people with low folate intake

1.6 Thesis Rationale

The evidence from human epidemiological studies is conflicting as to whether folate deficiency promotes or prevents CRC. Additionally, limited data exist as to the potential mechanisms behind the link between folate deficiency and CRC. This information would be important for designing and implementing effective nutritional guidelines and treatments for CRC. In particular, more information would be provided as to the efficacy and safety of general folic acid fortification. Also lacking is information pertaining to the underlying molecular events which lead to individual differences in susceptibility to environmentally induced CRC. In this thesis an animal model of long-term dietary folate deficiency will be developed and described with the goal of understanding the effect of folate deficiency on the intestine at the molecular level. Additionally, the effect of long-term dietary folate deficiency will be examined in an animal model known to be resistant to tumor formation.

In Chapter II th' ffect of long-term dietary folate deficiency on non-transgenic BALB/c mice without a carninogen induced pre-disposition to intestinal cancer will be described. The goal of this work was to determine if long-term folate deficiency could induce intestinal tumors without the presence of any pre-disposing transgene or carcinogen injection. Chapter II also describes an examination of DNA damage levels and DNA damage response genes, with the goal of attempting to determine the mechanisms behind how folate deficiency may influence tumor formation.

In Chapter III the effect of long-term dietary folate deficiency on non-transgenic C57Bl/6 mice without a carcinogen induced pre-disposition to intestinal cancer will be described and compared to the observations obtained in Chapter II. The goal of this work was to determine if dietary folate deficiency would influence the C57Bl/6 strain, known to be less susceptible to tumor formation. Chapter III also describes a further examination of the effects of the folate-deficient diet on DNA damage, as well as on DNA methylation, in the pre-neoplastic intestine of both the BALB/c and C57Bl/6 strains. The goal of this was to determine whether increased DNA damage, decreased DNA methylation, or

[313]. In another study, *MTHFR* 677TT individuals had a decreased chance of having methylation in the *APC* promoter and an increased chance of having methylation in the promoter of methylguanine methyltransferase when dietary folate was low [244].

both is more critical to tumorigenesis induced by folate deficiency. The effects of betaine supplementation to the folate-deficient diet in BALB/c mice will also be described with the goal of determining whether supplementation with this alternate methyl donor would influence DNA methylation levels and/or tumor formation in the BALB/c strain.

In Chapter IV the expression of enzymes involved in folate metabolism will be compared between the BALB/c and C57Bl/6 strains. The goal of this work was to determine whether differences in expression in folate-metabolizing enzymes could influence one-carbon distribution in a way that would explain why one strain was susceptible to tumor formation while the other was not. The effects of DNA damage on DNA copy number in BALB/c intestines will also be described, with the goal of determining whether increased DNA damage could lead to gross chromosomal abnormalities, such as amplifications or deletions, which could influence tumor formation in BALB/c mice.

CHAPTER II

Low dietary folate initiates intestinal tumors in mice, with altered expression of G_2-M checkpoint regulators *Polo-Like Kinase 1* and *Cell Division Cycle 25c*

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Cancer Research (2006) 66(21): 10349-56

2.1 Abstract

Clinical reports have suggested that low dietary folate increases risk for colorectal cancer. Animal studies for investigation of folate and tumorigenesis have used carcinogen induction or mice with germ-line mutations. We have developed a new spontaneous tumor model in which mice, with or without a null allele in a key folate-metabolizing enzyme, *Methylenetetrahydrofolate reductase* (*Mthfr*), develop intestinal tumors due to low dietary folate alone. On folate-deficient diets, 12.5% of *Mthfr*+/+ mice and 28.1% of *Mthfr*+/-mice developed tumors; mice on control diets were negative. Dietary and genotype effects on tumor development were significant. To investigate mechanisms of folate-dependent tumorigenesis, we examined levels of DNA damage and gene expression of two genes involved in DNA damage response and G_2-M checkpoint regulation, *polo-like kinase 1* (*Plk1*) and *cell division cycle 25c* (*Cdc25c*). Folate deficiency increased DNA damage and decreased expression of both genes (assessed by quantitative reverse transcription-PCR and immunofluorescence) in normal intestine compared with levels in mice on control diets. An immunofluorescence assay for CDC25c activity (phosphorylated CDC2) also found CDC25c activity to be decreased in folate-deficient normal intestine. In tumors, however, *Plk1* and *Cdc25c* mRNA were found to be higher (11- and 3-fold, respectively) compared with normal intestine from folate-deficient mice; immunofluorescence studies of PLK1, CDC25c and phosphorylated CDC2 supported these findings. Our data suggest that folate deficiency can initiate tumor development, that *Mthfr* mutation may enhance this phenomenon, and that altered expression of *Plk1* and *Cdc25c* may contribute to folate-dependent intestinal tumorigenesis.

2.2 Introduction

Colorectal cancer is estimated to develop in ~5% of the population and that figure increases to 50% if nonmalignant tumors are included. The majority of cases are sporadic and due largely to environmental factors. Hereditary forms may account for up to 15% of all colorectal cancers[165].

An insufficient dietary intake of folate has been associated with increased risk for the development of colorectal cancer. Studies suggest a 30 % to 40% reduced risk in individuals consuming high dietary folate compared with those with low dietary folate[314]. Because one-carbon units within the folate metabolic pathway are required for conversion of dUMP to dTMP and for the conversion of homocysteine to methionine, dietary folate deficiency can reduce availability of methyl groups for DNA repair/synthesis and for methylation reactions. Abnormal DNA methylation patterns are commonly observed in a wide variety of tumors and are associated with altered expression of tumor suppressor genes or oncogenes. Insufficient conversion of dUMP to dTMP can also increase uracil misincorporation into DNA, which leads to double-strand breaks. These breaks can result in the formation of chromosomal abnormalities, such as translocations and gene amplification, which can disrupt gene expression and contribute to tumorigenesis[16].

Genetic disturbances in folate metabolism can also modulate risk for colorectal cancer. Methylenetetrahydrofolate reductase (MTHFR) synthesizes 5-methyltetrahydrofolate, the folate derivative utilized in homocysteine remethylation to methionine. A common variant in *MTHFR* (a C to T transition at nucleotide 677) results in a mild MTHFR deficiency that can confer protection against colorectal cancer when folate levels are adequate[202]. However, this protection is not observed when dietary folate is low, and under these conditions, the variant may be associated with increased risk for colorectal cancer and other neoplasias[222].

To examine the mechanisms behind folate deficiency and the link to colorectal cancer, we and others have used the *Apc*$^{min/+}$ mouse model of intestinal neoplasia. These mice have a germ-line mutation in the *adenomatous polyposis coli* (*Apc*) gene, the same gene that causes a hereditary form of colorectal cancer in man (*familial adenomatous polyposis*; reviewed in [165]). Somatic mutations in the *Apc* gene have also been found in up to 80% of sporadic colorectal cancers[165]. Other genetic models for colorectal cancer include transgenic mice with a disruption in the mismatch repair genes *Mlh1* and *Msh2*[173]. The non-genetic

models that have been used to study colorectal cancer include carcinogen induction models (administration of dimethylhydrazine and azoxymethane), which can be administered alone or in conjunction with bile acid cocarcinogens, nonspecific injury, and surgical procedures known to enhance risk for colorectal cancer[173].

Several studies have reported an influence of folate levels on tumor numbers in *Apc*$^{min/+}$ mice. These studies suggest that the timing of folate deficiency (or supplementation) may be critical. At early time points, folate supplementation may decrease polyp formation[194]and folate deficiency may increase tumor numbers in these mice [196]. At later stages, folate supplementation may increase polyp number[194] and folate deficiency may decrease tumor numbers[196]. We recently examined gene expression patterns by microarray analysis in *Apc*$^{min/+}$ mice and identified 90 known genes with altered expression in tumors compared with that in normal intestine[315].

To determine whether low dietary folate alone or in combination with a genetic disturbance in folate metabolism can initiate intestinal tumor development in mice, we fed control and folate-deficient diets to mice with and without a null allele in *Mthfr (Mthfr*+/- mice). We had generated these mice in earlier work and showed that they are a good animal model for mild MTHFR deficiency in humans (the 677TT genotype) because of the similarity in residual enzyme activity and the degree of hyper-homocysteinemia. We examined the intestine in mice after ~1 year on diets and observed intestinal tumors in folate-deficient mice but not in mice fed control diets. From previous work in the *Apc*$^{min/+}$ mouse model, we had discovered that folate deficiency may induce expression changes in two genes involved in G_2-M checkpoint control: *polo-like kinase 1 (Plk1)* and *cell division cycle 25c (Cdc25c)*. We therefore examined expression of these two candidate genes for tumorigenesis in our spontaneous model to begin to address the mechanisms by which folate deficiency can lead to intestinal neoplasia.

2.3 Materials and methods

Mice. Animal experimentation was approved by the Animal Care Committee of the Montreal Children's Hospital (Montreal, Quebec, Canada). *Mthfr*+/+ and *Mthfr*+/- mice, generated in earlier work and backcrossed for at least 10 generations onto a BALB/c background[51], were housed at the Montreal Children's Hospital Research Institute animal facility. After weaning, mice were placed on amino acid-defined diets (Harlan Teklad, Madison, WI) with all the necessary components recommended by the American Institute of Nutrition[316]. A control diet contained the recommended amount of folic acid for rodents (2 mg/kg diet) and the folic acid-deficient diet contained 0.3 mg/kg diet. Both diets contained 1% succinylsulfathiazole, an antibiotic, to prevent generation of folate by intestinal bacteria. These diets have been used in our previous reports, and the folate-deficient diet was shown to be effective in lowering folate or increasing homocysteine as expected[55; 317]. Mice were fed these diets for 12 to 14 months until sacrifice. Body weight was recorded on sacrifice. Folate-deficient diet mice had a borderline significant increase in body weight compared with control diet mice by independent sample *t* test (P = 0.056).

The entire intestines were removed and examined for tumors under a dissecting microscope. Tumors were dissected and either snap frozen or fixed in 4% paraformaldehyde with surrounding normal intestine. The remaining normal intestine was either frozen or fixed. Fifty-seven mice were fed the control diet (31 *Mthfr*+/+ and 26 *Mthfr*+/-) and 80 mice were fed the folate-deficient diet (16 *Mthfr* +/+ and 64 *Mthfr* +/-).

Microarray analysis. Microarray analysis of RNA from tissues of $Apc^{min/+}$ mice was reported in our earlier publication[315], which examined changes in gene expression between the normal intestine and tumors under several conditions. The array used in that study encompassed around 34,000 mouse genes. In this study, we compared the data by grouping the normal intestine data into control diet and folate-deficient diet groups and the folate-deficient diet data into normal intestine and tumor groups. The same analytic method was used as that in our previous report[315]. This method was based on averaging probe set intensities for

probes with a "present" call for all samples within the test group and dividing it by the average intensity for "present" probe sets for the control group to give a numerical fold change. Any fold change greater than 2 with an absolute difference >200 between the average probe intensities of the two groups was considered significant. Analysis of numerical fold changes between the four pairs of mice did not reveal any interesting results; a different approach was therefore adopted. The Affymetrix Microarray Suite 5.0 (MAS5, Santa Clara, CA) analysis that had been done on these arrays provides an overall indicator of the binding ability of a probe by giving a probe set a call value of "present" if the probe set binds above a certain threshold and if the binding is significantly higher for the specific probe compared with the mismatch control probe. An "absent" call indicates one of two situations: either the specific probe binding signal is equal to that of the mismatch control or the mismatch control has no signal and the specific probe intensity is below threshold (i.e., too low to reliably detect). The latter situation can arise if the probe was indeed specific, but the transcript levels were very low/undetectable. We therefore hypothesized that if a probe set had a consistently "absent" call in all replicates of one group and a consistently "present" call in all replicates of the comparison group, then it could be assumed that the expression of the gene product was altered.

RNA extraction from normal intestine. RNA was isolated from 50 mg snap-frozen normal intestine of mice on control and folate-deficient diets using the Trizol Reagent (Invitrogen, Burlington, Ontario, Canada) according to the manufacturer's protocol. RNA was treated with 10μL DNaseI for 30 minutes before reextraction with equal volumes of a 25:24:1 mixture of phenol-chloroform-isoamyl alcohol. RNA pellets were washed in 75% ethanol and redissolved in diethylpyrocarbonate (DEPC)-treated water.

Laser capture microdissection and RNA extraction. Frozen tumors and normal intestines were sectioned from OCT (Sakura Finetek, Torrance, CA) blocks, cut into 7-μm sections, and kept at -80°C until use. A tumor section and a normal intestinal section from the same mouse were thawed in 75% ethanol for 30 seconds and in DEPC-treated water for 30 seconds and then stained with

hematoxylin for 2 minutes. Slides were immersed in bluing solution for 30 seconds, in 70% ethanol for 30 seconds, and in 95% ethanol for 30 seconds and stained for 5 seconds in eosin. Slides were then dehydrated by immersion in 95% ethanol twice for 30 seconds each, in 100% ethanol twice for 30 seconds each, and finally in xylene twice for 5 minutes each. All solutions and plasticware were treated with RNase Away (ICN Biomedicals, Irvine, CA) or DEPC-treated water to maintain RNA integrity. Laser capture microdissection was done using a PixCell II Laser Capture System (Arcturus Biosciences, Sunnyvale, CA) microscope with CapSure LCM caps (Arcturus Biosciences). RNA was extracted from dissected samples using the PicoPure RNA isolation kit (Arcturus Biosciences) according to the manufacturer's instructions and then treated with DNaseI (Invitrogen). RNA quantitation was done using the RiboGreen RNA quantitation reagent and kit (Molecular Probes, Burlington, Ontario, Canada) according to the manufacturer's instructions.

Reverse transcription-PCR. RNA (2μg) from normal intestine, extracted with Trizol, was reverse transcribed. From laser capture microdissected tissue, equal amounts of RNA (ranging from 6 to 29 ng), representing the maximum possible amount per pair, were used. Reverse transcription was done using SuperScriptII reverse transcriptase (Invitrogen) and a random hexamer for 50 minutes at 45°C. The enzyme was deactivated by incubation at 72°C for 15 minutes. Real-time PCR was done using the SyberGreen kit (Invitrogen) according to the manufacturer's protocol in an Mx3000P QPCR system (Stratagene, La Jolla, CA). For every gene, the analysis of real time data was done by using C_t values obtained across eight serial dilutions of the same sample and normalizing the values against the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*). Semiquantitative PCR was done on a Biometra T-gradient system (Montreal Biotech, Montreal, Quebec, Canada). PCR products were run on 9% acrylamide gels and stained with ethidium bromide. Gels were visualized on a Bio-Rad GelDoc 2000 imaging system (Bio-Rad, Mississauga, Ontario, Canada) and bands were quantified using Quantity One 4.0.1 software. Normalization between samples was done by comparison to *Gapdh*. Statistical significance was

assessed by independent sample *t* test for comparison of normal intestine on the two diets or by paired sample *t* test for comparison of normal intestine with tumor.

Immunofluorescence. Intestines (with and without tumors), fixed in 4% paraformaldehyde, were embedded in paraffin and cut into 6- to 7-μm sections. Sections were treated according to the protocol provided by DakoCytomation, Inc. (Mississauga, Ontario, Canada). Protein Block serum-free was used for the blocking step (DakoCytomation Inc). One section per slide was incubated only with secondary antibody (negative control) and the other sections were covered in either a 1:50 (PLK1, phosphorylated histone H2AX), 1:800 (CDC25c), or 1:200 (phosphorylated CDC2) dilution of primary antibody in antibody diluent (DakoCytomation). All primary antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA), except for phosphorylated histone H2AX, which was purchased from Upstate Biotechnology (Lake Placid, NY). For visualization, slides were incubated with a 1:5,000 dilution of AlexaFluor 488-conjugated anti-rabbit secondary antibody (Molecular Probes). Slides were then washed in PBS and counterstained with a 1:3,000 dilution of a 1μg/μL stock of propidium iodide (Molecular Probes) according to the manufacturer's protocol, rinsed again in PBS, dried, and mounted in Prolong Gold Antifade reagent (Molecular Probes). Imaging was done using the Zeiss AxioImager.Z1 with the AxioVision 40 version 4.5.0.0 imaging program (Carl Zeiss Imaging Solutions, Toronto, Ontario, Canada). For ease of viewing, Adobe Photoshop software was used to enhance the brightness of all pictures equally.

To quantify the number of double-strand breaks, the number of foci containing the phosphorylated form of histone H2AX was determined. This protein has been shown to form nuclear foci around sites of double-strand breaks; the foci are quantifiable when immunostained[318]. A focus was defined as a concentrated spot of staining inside the nucleus. Three individuals blinded to sample identity counted the number of foci and total number of nuclei in three pictures taken at random per sample. All cell types were included in the counts (e.g., cells in the villi, crypts, and underlying connective tissue). The number of

foci was averaged over the three pictures per sample and divided by total number of nuclei. The results of the three independent counts were averaged per sample to achieve the average number of foci per 100 cells. Independent sample *t* test was used to assess significance.

2.4 Results

Tumor incidence and histology. A total of 57 mice (31 *Mthfr*+/+ and 26 *Mthfr*+/-) were placed on the control diet (**Table 2.1**). None of these mice developed intestinal tumors. However, of the 80 mice (16 *Mthfr* +/+ and 64 *Mthfr*+/-) that were fed folate-deficient diets, 25% (20 of 80) of these mice developed at least 1 adenoma or adenocarcinoma in the duodenum. By visual inspection, 13 mice had a single tumor, whereas 4 mice had 2 tumors each and 3 mice had 3 tumors each. The dietary and genotype effects were highly significant ($P < 0.01$, Fisher's exact test, 2 by 2 analysis). Twelve and a half percent (2 of 16) of *Mthfr*+/+ mice developed tumors compared with 28.1% (18 of 64) in the *Mthfr*+/- group on the FD.

Paraffin-embedded sections of normal intestine and tumors from mice fed control diet and folate-deficient diets were examined by routine histology by an on-site pathologist. Seven intestines from control diet mice and 12 intestines from folate-deficient diet mice were examined (**Table 2.1** and **Fig. 2.1**).

Table 2.1 Tumor incidence in intestine of mice on control diet or folate-deficient diet, with and without a null allele in *Mthfr*

No. mice examined for tumors	Diet		Total mice	% Mice with tumor
	Control	Folate-deficient		
Mthfr +/+	31 (0)	16 (2)	47	12.5
Mthfr +/-	26 (0)	64 (18)	90	28.1*
Total mice	57 (0)	80 (20)	137	
% Mice with tumor	0	25†		
Histological examination of intestine	Total examined	Adenoma	Adenocarcinoma	Polyp
Control diet	7	0	0	1
Folate-deficient diet	12	2	3	3

NOTE: The numbers in parentheses indicate the number of mice that developed tumors in each group. None of the mice on control diet developed tumors compared with 25% of mice on folate-deficient diet. Within the folate-deficient diet group, 12.5% (2 of 16) of mice with no *Mthfr* mutation and 28.1% (18 of 64) of *Mthfr* +/- mice developed between 1 and 3 duodenal tumors. Both diet and genotype effects were significant. Histological examination of intestines revealed 1 intestinal polyp and no adenomas or adenocarcinomas in the control diet group. Of the 12 folate-deficient diet intestines, 5 tumors were observed and further examined. Of these, 2 were adenomas and 3 were adenocarcinomas, some of which showed invasion into the submucosa. Three other folate-deficient diet intestines showed a single micropolyp.

*$P < 0.01$ for comparison of *Mthfr*+/+ and *Mthfr*+/-.

†$P < 0.001$ for comparison between control diet and folate-deficient diet.

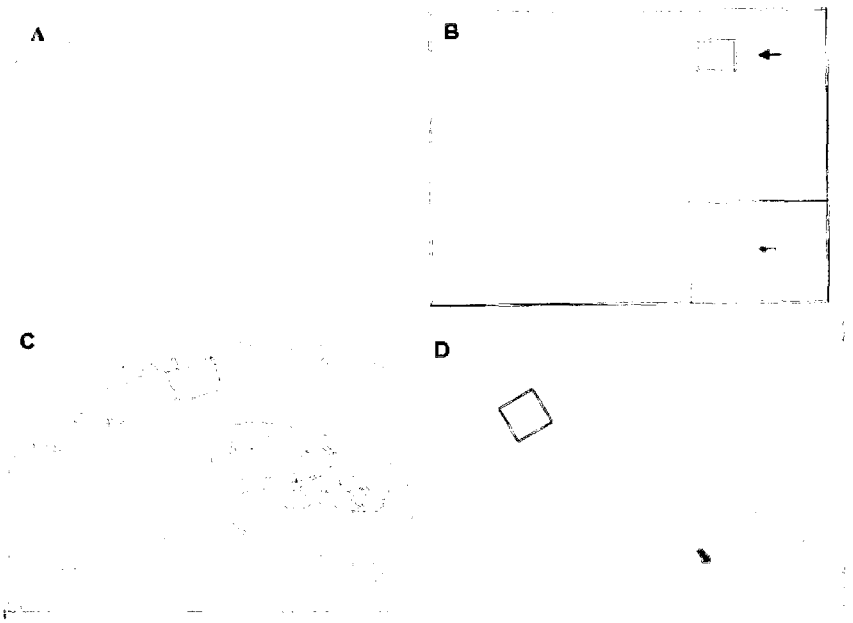


Figure 2.1 Histological characterization of intestines. A, H&E-stained normal intestine from a mouse fed control diet for 12 months. Magnification, x40. B, H&E-stained polypoid hyperplasia of the epithelium (micropolyp, *arrow*) covered by normal mucosa from a folate-deficient diet mouse. Magnification, x40. *Inset*, higher magnification of the boxed area (x400) with some dark staining proliferating cells (*green arrow*). C, H&E-stained villous adenoma from a folate-deficient diet mouse showing the long, villous, glandular fronds. Magnification x40. *Inset*, higher magnification of the boxed area (x400). D, H&E-stained adenocarcinoma from a folate-deficient diet mouse showing the lesions *in situ* without invasion into the submucosa. Magnification x40. *Inset*, higher magnification of the boxed area (x400), with intensely stained nuclei indicative of proliferating cells (*green arrow*).

In the control diet group, 1 of the 7 intestines was found to have hyperplastic polypoid tissue but no adenomas were observed; the other 6 intestines appeared normal (**Fig. 2.1A** and **2.1B**). Of the 12 mice from the folate-deficient diet group, there were 5 tumors; 2 had a single adenoma each (**Fig. 2.1C**) and 3 had a single adenocarcinoma with or without invasion into the submucosa (**Fig. 2.1D**). Hyperplastic polypoid tissue (**Fig. 2.1B**) was found in another 3 of the 12 folate-deficient diet intestines examined. Hyperplastic polypoid tissue is noteworthy since the progression of neoplastic disease in the intestine often proceeds from polyps to adenomas, adenocarcinomas and finally to metastatic disease [165].

Assessment of DNA damage. Studies have reported a link between folate deficiency and uracil misincorporation into replicating DNA[16]. Uracil misincorporation has been linked to increased incidence of double-strand breaks and chromosomal instability, which could lead to cellular transformation[16]. To assess DNA damage in our model, we stained control diet and folate-deficient diet normal intestines with an antibody against the phosphorylated form of histone H2AX, which is known form nuclear foci around the sites of double-strand breaks[318]. Using this method, we found that folate-deficient diet normal intestines had an average of 4.25 (±0.44, SE) foci per 100 cells, whereas control diet normal intestines had an average of 2.53 (±0.69, SE) foci per 100 cells. This increase in double-strand breaks in the folate-deficient diet normal intestine was found to be borderline significant (P = 0.069) by independent sample *t* test.

Identification of candidates by gene expression in *Apc*$^{min/+}$ mice on control diet and folate-deficient diet. In an earlier report[315], we had done microarray analysis of RNA from normal intestine and tumors of *Apc*$^{min/+}$ mice; the goal of that study was to identify candidates that could contribute to tumor growth in the *Apc*$^{min/+}$ model[315]. These mice had been fed the same control and folate-deficient diets that were used in this study. We therefore compared the data from the normal intestine of *Apc*$^{min/+}$ mice fed control diet with data from the normal intestine of *Apc*$^{min/+}$ mice fed folate-deficient diet. The goal of this comparison was to identify selected candidates altered in expression by the folate-deficient diet that could then be examined in the tissues of our new spontaneous model of

tumorigenesis described in this report. Application of the numerical fold change analysis did not yield readily interpretable results. We then included as a significant result all probe sets that displayed a consistent call value of "present" or "absent" for the control diet group and a consistent opposite call for the folate-deficient diet group. Using this approach, we found several interesting candidate genes that met these variables (**Table 2.2**). Although 12 genes were found to be consistently "present" in control diet normal intestines and "absent" in folate-deficient diet normal intestines, no genes were found for the reverse situation. Both *Plk1* and *Cdc25c* were found to be "present" in the normal intestine of $Apc^{min/+}$ mice fed control diet and consistently "absent" in the normal intestine of $Apc^{min/+}$ mice fed folate-deficient diet (**Table 2.2**). Semiquantiative reverse-transcription PCR (RT-PCR) was used to confirm the expression changes of *Plk1* in $Apc^{min/+}$ normal intestine of the two dietary groups. In 4 pairs of RNA, done in duplicate, *Plk1* showed decreased expression with an average fold change of -1.77 ± 0.45, and *Cdc25c* showed decreased expression with an average fold change of -1.87 ± 0.15, but the changes were not statistically significant. *Plk1* and *Cdc25c* are of interest because *Plk1* is a regulator of *Cdc25c* in the DNA damage response pathway and the aforementioned results with phosphorylated histone H2AX suggested that the folate-deficient diet was increasing DNA damage in the normal intestine of our mice. *Plk1* is inactivated during the DNA damage response whereas *Plk1* activation is required for reentry into mitosis once DNA damage has been repaired[261]. In undamaged cells, PLK1 activates CDC25c, which can proceed into the nucleus to dephosphorylate CDC2; CDC2 can then complex with Cyclin B to initiate mitosis. Since folate deficiency seemed to have increased DNA damage, one might expect *Plk1* inactivation to allow cells to repair DNA; the decrease in *Plk1* mRNA by microarray and RT-PCR analyses was consistent with this hypothesis.

Table 2.2. Microarray results in normal intestine and tumors from *Apc*$^{min/+}$ mice on control and folate-deficient diets

Gene	Accession No.	Probe set*	Gene product	CD NI call	FD NI call	FD T call or fold change (FD T/FD NI)
Capg	X54511	160106	Gelsolin, actin capping	P	A	P
Igg3	D14625	102721	Immunoglobulin gamma 3	P	A	No change
Ent	L17324	100120	Entactin	P	A	No change
Tacc3	AW209238	97238	Transforming acid coiled coil 3	P	A	P
Elp3	AI851229	95717	Elongation protein 3	P	A	No change
Oprs1	AF004927	94828	Sigma 1 receptor	P	A	P
Plk1	U01063	93099	Polo-like kinase 1	P	A	P
Tomm40	AF043249	160653	Mitochondrial outer membrane protein	P	A	No change
Cdc23	AA657164	104090	Cell division cycle 23	P	A	No change
Th9	AI845815	103343	Alpha catenin binding protein	P	A	No change
Cdc25c	U15562	102935	Cell division cycle 25c	P	A	2.13
Tpd52l1	AF004428	101446	Tumor protein D53	P	A	P

NOTE: Four pairs of RNA were used to compare expression in normal intestine of *Apc*$^{min/+}$ mice fed control diet or folate-deficient diet and two pairs for the folate-deficient diet mice to compare expression in normal intestine with that in tumor. Numerical fold changes were calculated from the average probe set intensities of one condition (diet or tissue type) compared with the other. Nonnumerical fold changes are defined as a consistent call of "absent" (A) or "present" (P) for the probe set for all repeats of one condition and the opposite call consistently noted for the probe set for all repeats of the opposite condition. Abbreviations: CD NI, control diet, normal intestine; FD NI, folate-deficient diet, normal intestine; FD T, folate-deficient diet, tumor.

*Description of probe sets can be obtained at http://www.affymetrix.com by adding "_at" to the end of the numbers presented in this table.

Based on these results, we were interested in determining whether folate deficiency altered the expression of *Plk1* and *Cdc25c* in tumors of the $Apc^{min/+}$ model. Our original report[315] did not show any differences in expression between normal intestine and tumors for these genes; however, this comparison grouped tissues of both diets together. We therefore did a new comparison separating the control diet normal intestine and tumors from the folate-deficient diet normal intestine and tumors. On the control diet, there was no change in gene expression between normal intestine and tumor. However, it was interesting to note that *Plk1* was found to be consistently "present" in folate-deficient diet tumors and consistently "absent" in folate-deficient diet normal intestine. In addition, *Cdc25c* was 2.13-fold increased in folate-deficient diet tumors compared to folate-deficient diet normal intestine (**Table 2.2**).

Expression of *Plk1* and *Cdc25c* RNA in spontaneous model of folate-dependent tumorigenesis. Our results in the $Apc^{min/+}$ model led us to believe that folate deficiency altered expression of *Plk1* and *Cdc25c,* two important mitotic regulators. Furthermore, the *Plk* family of kinases is upregulated in several cancers[260; 268; 319], and human studies have shown an increase in *Plk1* mRNA and protein levels in colorectal carcinoma[265-267; 320]. We therefore examined expression of these genes in the normal intestines and tumors of our new mouse model.

Using quantitative RT-PCR, *Plk1* and *Cdc25c* expression were assessed in 12 normal intestines from mice on folate-deficient diet and mice on control diet; 6 were *Mthfr*+/+ and 6 were *Mthfr*+/- mice. *Plk1* and *Cdc25c* both showed decreases in expression in folate-deficient diet normal intestine; the changes were significant for *Plk1* (-1.41 ± 0.12, P = 0.045) and there was borderline significance for *Cdc25c* (-1.61 ± 0.20, P = 0.06). These findings are consistent with those seen in $Apc^{min/+}$ mice.

Laser capture microdissection was used to isolate tumor tissue and normal intestine for RNA isolation and quantitative RT-PCR from 3 *Mthfr*+/- mice. *Plk1* was found to be increased 11.6- fold (±5.1, SE) in tumors compared to normal intestine, whereas *Cdc25c* was found to be increased 3.1-

fold (± 1.8; **Figure 2.2**). The changes in *Plk1* RNA were significant ($P < 0.02$) whereas the changes in *Cdc25c* were borderline significant ($P = 0.066$) by paired *t* test.

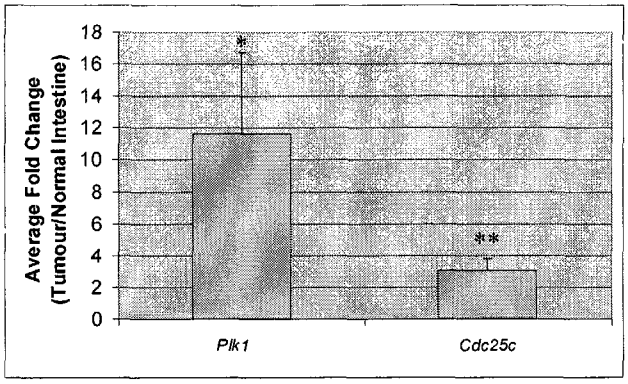


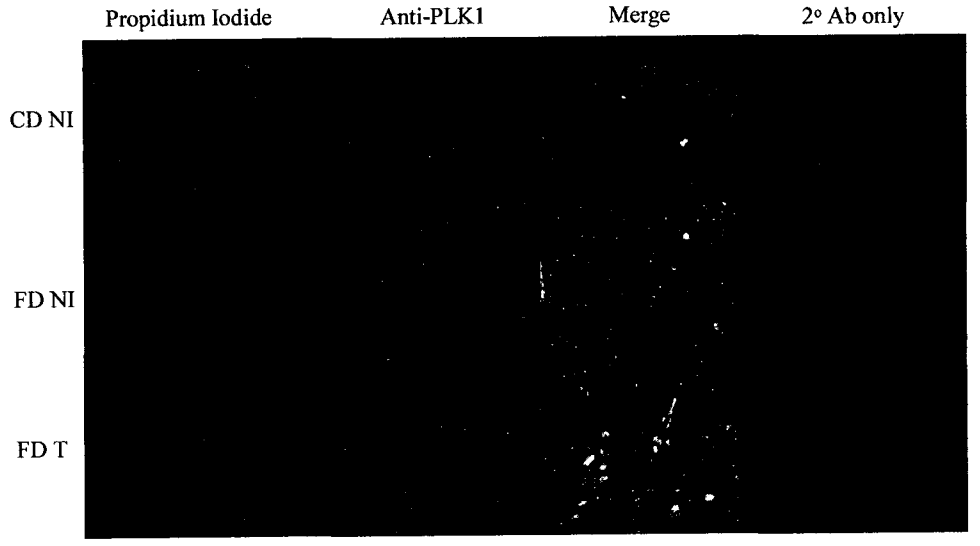
Figure 2.2. Quantitative RT-PCR for *Plk1* and *Cdc25c*. Comparison of laser capture microdissected tumors with normal intestine from the same folate-deficient diet *Mthfr*+/- mice. *Columns*, mean of 3 pairs of RNA for each gene; *bars*, SE. *Plk1* shows an 11-fold (± 5.1) increase in expression in tumors. *Cdc25c* shows a 3-fold (± 1.8) increase in expression. *, $P = 0.019$; **, $P = 0.066$ by paired sample *t* test.

Expression of PLK1, CDC25c and phosphorylated CDC2 immunoreactive proteins in normal intestine and tumors. To confirm the expression changes observed by RT-PCR, we examined the protein levels of PLK1 and CDC25c in normal intestine from control diet and folate-deficient diet mice as well as in tumors from folate-deficient diet mice. We also assessed CDC25c activity by examining phosphorylated CDC2 levels. Immunofluorescence was used to measure the 3 immunoreactive proteins on serial sections of tissues from 3 mice per group.

When comparing the immunostaining of the control diet (**Fig. 2.3A** and **2.3B**, *top row*) and folate-deficient diet (**Fig. 2.3A** and **2.3B**, *middle row*) normal intestines, there appears to be a decrease in protein levels for both PLK1 and CDC25c corresponding to the RT-PCR results. The examination of immunoreactive proteins in tumor sections (**Fig. 2.3A** and **2.3B**, *bottom row*) suggests that both proteins seem to have increased expression in tumor tissue

compared with corresponding normal intestine (**Fig. 2.3A** and **2.3B**, *middle row*).

A PLK1



B CDC25c

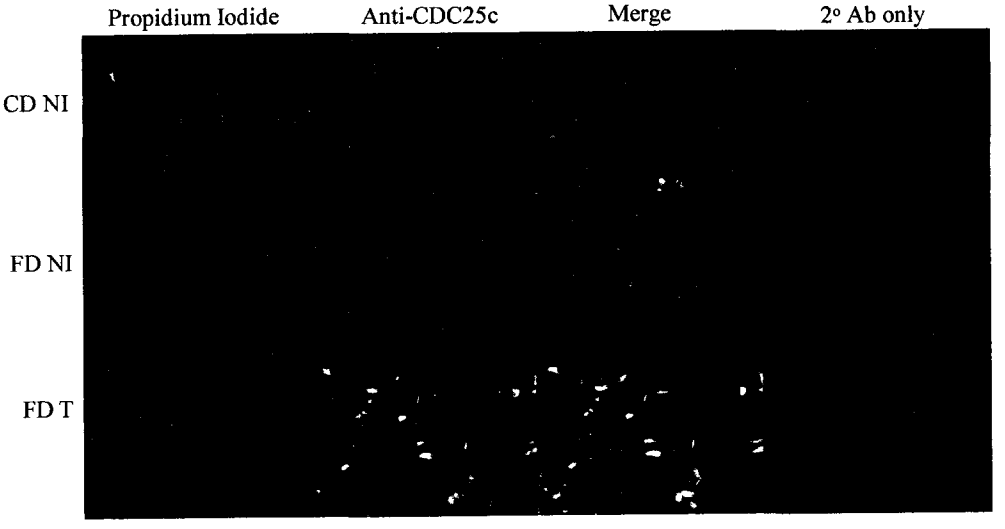


Figure 2.3 Immunofluorescence staining of PLK1 and CDC25c in normal intestine and tumors. A representative experiment (from 3 mice per group) is shown at x40 magnification. Anti-PLK1 (A; *column 2*) and anti-CDC25c (B; *column 2*) were used to stain paraffin-embedded sections of normal intestine from a control diet mouse (*top row*), normal intestine from a folate-deficient diet mouse (*middle row*) and a tumor from a folate-deficient diet mouse (*bottom row*). *Column 1*, propidium iodide was used as a nuclear counterstain. AxioVision 40 software was used to merge the propidium iodide signal with the immunofluorescent signal. Both PLK1 and CDC25c seem to decrease in folate-deficient diet normal intestine compared with control diet normal intestine and then increase in tumors from folate-deficient diet mice. *Column 4*, negative controls containing secondary antibody, but no primary antibody, for each sample. CD NI, control diet, normal intestine; FD NI, folate-deficient diet, normal intestine; FD T, folate-deficient diet, tumor.

To obtain independent confirmation of CDC25c activity, we used an immunofluorescent stain for phosphorylated CDC2. The function of CDC25c in checkpoint control is to de-phosphorylate and activate CDC2 to allow cell cycle progression[262]. Therefore, cells progressing into mitosis would be expected to have lower levels of phosphorylated CDC2 and cells that are arrested in G_2 would have higher levels of phosphorylated CDC2. **Figure 2.4** shows phosphorylated CDC2 immunofluorescence in sections of normal intestine from control diet mice (top row), normal intestine from folate-deficient diet mice (middle row) and corresponding tumors from folate-deficient diet mice (bottom row).

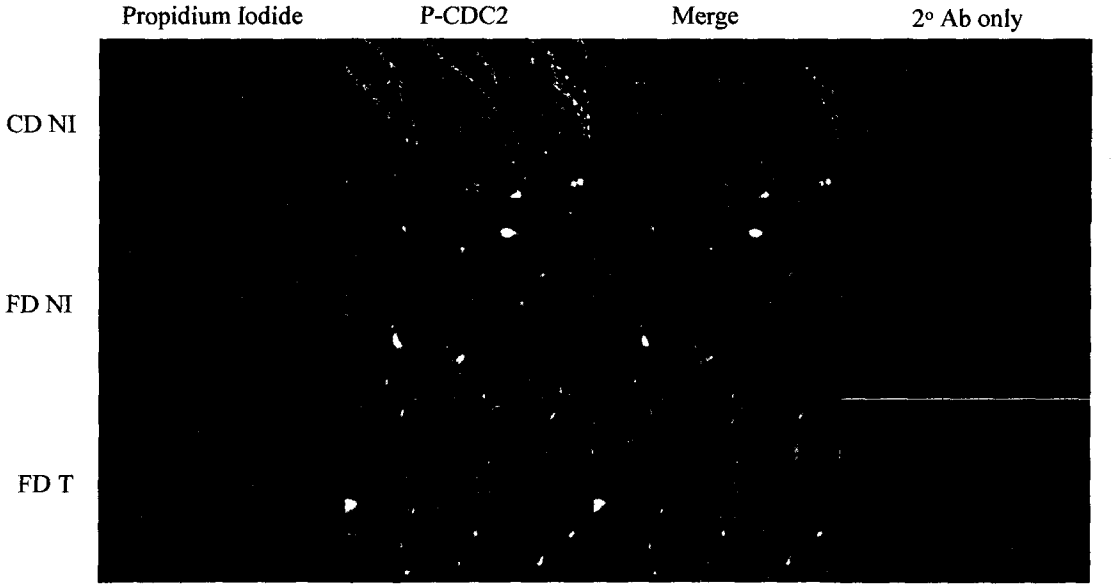


Figure 2.4 Phosphorylated CDC2 (p-CDC2) immunofluorescence as a measure of CDC25c activity in normal intestine and tumors. A representative experiment (from 3 mice per group) is shown at x40 magnification. As in **Fig. 2.3**, paraffin-embedded sections of normal intestines and tumors were stained with anti-phosphorylated CDC2 and counterstained with propidium iodide . The two images were then merged. Phosphorylated CDC2 seems to be increased in folate-deficient diet normal intestine compared with control diet normal intestine or folate-deficient diet tumors. *Column 4*, negative controls containing secondary antibody, but no primary antibody, for each sample.

It is clear that the levels of phosphorylated CDC2 are low in the undamaged normal intestine from control diet mice, where normal proliferation is occurring, and that they increase in the folate-deficient diet normal intestine where we have observed increased DNA damage and the cells are likely arrested in G_2. This finding is consistent with the decreases in *Cdc25c* mRNA (reported above) and protein levels shown in **Fig. 2.3**. In tumor tissue, there is a decrease in phosphorylated CDC2 staining (bottom row) compared to the normal intestine of folate-deficient diet mice (middle row). This observation suggests that the tumors have managed to bypass the G_2 checkpoint and continue into mitosis. Again, this finding is supported by the increases in *Cdc25c* mRNA and protein levels in tumors, as discussed for **Fig. 2.3**.

2.5 Discussion

Our model of environmentally induced colorectal cancer may be more physiologically relevant than previous animal models for examining dietary effects on neoplasia. Carcinogen induction models of colorectal cancer use doses of carcinogens that are much higher than those that would be applicable to human populations. Furthermore, the series of molecular events set in motion by carcinogen exposure do not necessarily reflect the events that occur in the progression of human cancer[173]. Although transgenetic models (such as the $Apc^{min/+}$ mouse) can mirror human hereditary cancer disorders, these cancers occur in only a minority (~15%) of cancer cases, with the majority due largely to environmental factors[165].

We have developed a new model of intestinal tumorigenesis due to a deficiency of dietary folate. Mice, with or without a mutation in *Mthfr*, can develop duodenal tumors after ~1 year on the diet. We are not aware of other animal models that develop spontaneous intestinal tumors due to a dietary deficiency alone, without radiation/carcinogen induction or a transgene. Our findings provide a clear link between folate deficiency and tumor initiation. Although epidemiologic studies have shown an inverse relationship between folate intake and risk for colorectal cancer, there has been no definitive biological

evidence to link folate deficiency and colon cancer[321].

In beginning to address the mechanisms of folate-induced tumorigenesis, we identified altered expression of two important G_2-M checkpoint control genes, *Plk1* and *Cdc25c*, in the normal intestine as well as in the tumors of folate-deficient diet mice. In normal intestine, *Plk1* had decreased expression and *Cdc25c* showed a trend towards decreased expression in folate-deficient diet tissue. We hypothesize that decreased folate leads to an accumulation of uracil due to decreased availability of 5,10-methyleneTHF, which is required for dUMP conversion to dTMP. The accumulation leads to uracil misincorporation into DNA and double-strand breaks, which increases risk for chromosomal abnormalities[279]. DNA damage, such as double-strand breaks, will arrest the cell at the G_2-M boundary by activating the ataxia-telangiectasia (ATM)/ATM and Rad3-related (ATR) pathway. Indeed, our results show that the folate-deficient diet seems to increase the amount of double-strand breaks in the normal intestine as shown by increased formation of phosphorylated histone H2AX foci. Downstream targets of the ATM/ATR pathway include checkpoint kinases 1 and 2, both of which function to phosphorylate CDC25c on inhibitory residues, keeping it in an inactive conformation[262]. PLK1 has also been shown to be inactivated by checkpoint kinase 1 during the ATM/ATR DNA damage response pathway; once the DNA damage has been repaired, PLK1 is necessary for re-entry into mitosis[261]. Because both *Plk1* and *Cdc25c* are down-regulated in response to DNA damage [261; 262], it is reasonable to conclude that the observed decrease in their mRNA and protein levels in folate-deficient diet normal intestine is due to the normal response of the cells to the increase in DNA damage. Decreasing both *Plk1* and *Cdc25c* results in cell cycle arrest, presumably to allow cells to repair their DNA before continuing into mitosis[261].

More interesting, however, is the large increase in mRNA and protein expression for both *Plk1* and *Cdc25c* in tumor tissue compared with the surrounding normal intestine. The *Plk* family of kinases and *Cdc25c* are up-regulated in several cancers and are targets of several antisense and small molecule inhibitors currently under investigation as antitumor agents[260; 268; 319].

Two studies have examined *Plk1* mRNA[265; 320] or PLK1 protein[266; 267] levels in human colorectal carcinoma. *Cdc25c* levels (mRNA or protein) have not previously been examined in colorectal tumors. Our study links the overexpression of these two critical proteins in the initiation of intestinal carcinogenesis. In the folate-deficient diet normal intestine, *Plk1* and *Cdc25c* are under tight regulation and appear to be responding in the expected way to DNA damage. However, under the extended chronic stress of folate deficiency and DNA damage, the tight regulatory mechanisms controlling their expression seem to break down, allowing increased expression and abnormal progression into mitosis.

The mechanisms that lead to increased expression of these genes require elucidation. One possibility is that the deregulation of *Cdc25c* expression is due to a decrease in p53 expression and/or function. The *Cdc25c* promoter has been shown to have consensus sequences that are responsive to p53, and increased p53 expression has been shown to down-regulate *Cdc25c*, as would occur in response to DNA damage[322]. It is possible that folate deficiency leads to decreased expression or increased mutation of p53, which would increase expression of *Cdc25c* and allow cell cycle progression. The corresponding increase in *Plk1* expression could be explained by feedback mechanisms in the G_2-M checkpoint[323]. Another possible explanation for the aberrant expression of *Cdc25c* and *Plk1* (or of p53) is abnormal DNA methylation. Many studies have shown that folate deficiency can alter methylation patterns in DNA, resulting in global DNA hypomethylation and regional promoter hypermethylation[324]. In tumors, altered methylation patterns have been shown to affect expression of key growth control genes[309]. For example, studies of hepatocarcinogenesis have revealed methylation changes within the p53 promoter region in livers of methyl-deficient rats[325].

In summary, dietary folate deficiency can initiate tumors in the intestine. The deficiency leads to increased DNA damage and decreased expression of *Plk1* and *Cdc25c* in normal intestine, presumably to repair the damage. However, the chronic stress leads to deregulation of these genes in the tumor and increases their

expression, allowing cells to proceed to mitosis without cell cycle control. The establishment of a nutritionally deficient animal model for intestinal tumorigenesis provides the biologic evidence for the important role of folate in prevention of colorectal cancer. The elucidation of the tumorigenic mechanisms in this more physiologically relevant model may lead to the design of novel therapies for this common cancer.

2.6 Acknowledgments

Grant support: Canadian Institutes of Health Research. E. Knock was the recipient of a Montreal Children's Hospital Research Institute Studentship. We thank Dr. Nada Jabado and Damien Faury for their assistance.

CONNECTING TEXT- Chapter II-III

The results of Chapter II demonstrate that long-term dietary folate deficiency is sufficient to induce intestinal tumor formation in BALB/c mice without a pre-disposing transgene or carcinogen injection. Under conditions of dietary folate deficiency, *Mthfr* deficiency may also increase tumor formation. The results of the p-H2AX staining led us to suggest that DNA damage may be the underlying mechanism behind folate deficiency induced intestinal tumor formation. Further evidence of DNA damage is provided by decreasing PLK1 and CDC25c and increasing p-CDC2 staining in the intestines of FD compared to CD mice. This staining pattern is representative of molecular events which occur during the G_2-M arrest in response to DNA damage. The increased staining of PLK1, CDC25c and decreased staining of p-CDC2 in the tumors is evidence that some cells of the intestine may be escaping G_2-M arrest and progressing to mitosis despite persisting DNA damage. This provides evidence that defects in DNA damage response may play a role in tumor promotion. In Chapter III we will determine if long-term dietary folate deficiency has the same effects on the C57Bl/6 mouse strain, onto which the *Mthfr* null allele had been previously backcrossed. The C57Bl/6 strain is known to be more resistant to tumor formation. To further asses the role of increased DNA damage, we will examine the dUTP/dTTP ratio and the percent p-H2AX nuclear foci in the intestines of CD and FD BALB/c and C57Bl/6 mice. The level of G_2-M checkpoint control proteins will also be examined in C57Bl/6 intestines and compared to the results obtained in chapter II. Additionally, the effects on global DNA methylation will be examined in the intestine and compared between the strains to determine if this could be a mechanism behind tumorigenesis induced by low folate. Finally, it will be determined if supplementation of the folate-deficient diet with an alternate methyl donor, betaine, will correct any DNA methylation defects observed and/or influence intestinal tumor formation in BALB/c mice. The effects of betaine supplementation on dUTP/dTTP ratio, percent p-H2AX foci and *Plk1* and *Cdc25c* expression will also be examined.

CHAPTER III

Strain differences in mice highlight the role of DNA damage in neoplasia induced by low dietary folate

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Journal of Nutrition (2008) 138(4): 653-8

3.1 Abstract

In earlier work, we showed that low dietary folate induced intestinal tumors in BALB/c mice. In this study, our goal was to examine the effect of the same diets on a strain that is more resistant to tumorigenesis (C57Bl/6). We also questioned whether supplementation of the folate-deficient diet (FD) with betaine, an alternate methyl donor, would influence tumor formation. C57Bl/6 mice were fed the same diets [control (CD) with 2mg folate/kg diet and FD with 0.3mg folate/kg diet] as those in our previous study for 1 y, but they did not develop tumors. We also fed BALB/c mice the FD or FD supplemented with betaine for 1 y, but there was no change in tumor incidence. To determine the relative contributions of DNA damage and altered methylation patterns, we measured intestinal dUTP:dTTP ratios, phosphorylated histone H2AX (p-H2AX) staining, and global DNA methylation in both strains. Only BALB/c mice showed changes due to diet in dUTP:dTTP (from 2.19 ± 0.20 in CD to 2.77 ± 0.18 in FD; P = 0.05) and in p-H2AX staining (from 14.10 ± 3.59% in CD to 22.40 ± 2.65% in FD; P = 0.054). In BALB/c mice only, FD tended to have less (P = 0.06) global DNA methylation than CD. Although the FD increased plasma homocysteine and the betaine-supplemented FD lowered plasma homocysteine, the latter diet did not reduce tumor incidence. We conclude that plasma homocysteine is not likely to be associated with tumorigenesis in our model. However, DNA damage may play a critical role in initiating tumorigenesis when dietary folate is low and methylation changes may also be contributory.

3.2 Introduction

Epidemiologic studies have suggested that low dietary folate is associated with increased risk for colorectal cancer. To demonstrate a direct causative role of folate deficiency in tumor development, we fed low-folate diets to mice for 1 y and showed that dietary folate deficiency alone was sufficient to induce intestinal tumors in these mice[326]. The percentage of mice that develop tumors may also increase if a null allele in *Methylenetetrahydrofolate reductase (Mthfr)*, an important enzyme of folate metabolism, is present. Insight into the mechanisms

by which folate deficiency transforms the cells of the intestine is limited, although there are several theories. Folates are necessary for the conversion of dUMP to dTMP and for the conversion of homocysteine to methionine, a precursor for methylation reactions through S-adenosylmethionine (SAM). Consequently, dietary folate deficiency can reduce availability of thymine for DNA synthesis/repair and the supply of methyl donors for methylation reactions. Disruptions in folate metabolism can therefore result in DNA double-strand breaks, abnormal DNA methylation patterns, and increased levels of homocysteine, all of which have been associated with cancer development[16; 89; 279].

In our earlier study, we used BALB/c mice with and without a null allele in the *Mthfr* gene. The common 677C→T variant of *MTHFR* in human populations has been proposed as a risk factor for certain cancers, particularly when dietary folate is low[30; 222]. A single null allele in our mice (*Mthfr*+/-) reduces the activity of MTHFR and moderately increases plasma homocysteine levels. Consequently, the *Mthfr*+/- mice are a good model for mild MTHFR deficiency in humans[51].

Different mouse strains have varying susceptibilities to cancer[183; 327]. In particular, BALB/c strains have been shown to be sensitive to carcinogen-induced colorectal tumors, whereas C57Bl/6 strains are more resistant[180]. We therefore chose to examine the effects of our folate-deficient diet (FD) on the C57Bl/6 strain by feeding them the same diets for the same length of time as those in our previous study. Because we have recently crossed the *Mthfr* null allele from the BALB/c onto the C57Bl/6 background[55], we also examined the effects of low folate in combination with MTHFR deficiency on this strain.

Our previous study found that folate deficiency increased the number of DNA double-strand breaks in the normal intestine of the BALB/c mice. We also found altered expression at the mRNA and protein levels of two DNA damage response genes: polo-like kinase 1 *(Plk1)* and cell division cycle 25c *(Cdc25c)*[326]. We therefore elected to examine the incidence of DNA double-strand breaks and expression of *Plk1* and *Cdc25c* in this study to determine whether the same

mechanisms might be operating in the C57Bl/6 background. We also measured the levels of dUTP compared with dTTP to determine whether folate deficiency resulted in imbalances of nucleotide pools that could lead to uracil misincorporation and DNA damage.

Betaine is an alternate methyl donor in the remethylation of homocysteine to methionine[16], through the action of the enzyme betaine homocysteine methyltransferase (BHMT). In other work, we supplemented *Mthfr*+/- and *Mthfr*-/- mice with betaine and showed that betaine supplementation significantly decreased plasma homocysteine[111]. Subsequently, we showed that betaine supplementation increased methionine levels and slightly reduced DNA hypomethylation in mutant mice[112]. We therefore questioned whether betaine supplementation might reduce tumorigenesis due to low dietary folate if hyperhomocysteinemia and/or disruption of methylation reactions were critical to the formation of tumors in our mouse model.

3.3 Materials and methods

Mice. Animal experimentation was approved by the Animal Care Committee of the Montreal Children's Hospital. Male and female *Mthfr*+/+ and *Mthfr*+/- mice, generated in earlier work and backcrossed for at least 10 generations onto C57Bl/6 and BALB/c backgrounds[51; 55], were housed at the Montreal Children's Hospital Research Institute animal facility. After weaning, mice were fed amino acid-defined diets (Harlan Teklad) with all the necessary components recommended by the American Institute of Nutrition[316]. The control diet (CD) contained the recommended amount of folic acid for rodents (2 mg/kg diet)[316], and the FD contained 0.3 mg/kg diet. These diets have been used and described in our previous reports and the FD was shown to be effective in lowering folate or increasing homocysteine as expected[55; 317]. The data for the CD and FD BALB/c mice were previously published[326]. For this report, BALB/c mice were given the FD or the FD supplemented with betaine (FDB), which was folate-deficient (0.3 mg/kg diet) but contained 2.93 g/kg anhydrous betaine, as described in our previous studies[111; 112]. All diets contained 1% succinylsulfathiazole, an

antibiotic, to prevent generation of folate by intestinal bacteria. Mice were fed these diets for 12 to 14 mo until they were killed by suffocation in a CO_2 chamber. Body weight was recorded after killing. Neither betaine supplementation (in BALB/c) nor folate deficiency (in C57Bl/6) affected body weight; body weight tended to be greater in FD than in CD BALB/c mice (P = 0.056) in the original study[326]. The entire intestines were removed and tumors were counted and measured under a dissecting microscope. If identified, tumors were dissected and either snap-frozen or fixed in 4% paraformaldehyde with surrounding normal intestine. The remaining normal intestine was either frozen or fixed.

mRNA and protein expression. RNA extraction from normal intestines, laser capture microdissection, and RNA extraction from tumors were performed as previously described[326]. Quantitative RT-PCR for *Plk1* and *Cdc25c* and immunofluorescence for PLK1, CDC25c, phosphorylated-cell division cycle 2 (p-CDC2) were also performed as previously described[326]. Phosphorylated-histone H2AX (p-H2AX) immunofluorescent staining was performed as previously described[326] but with a method for taking pictures using the Apotome feature of the Zeiss AxioImager.Z1, which improved resolution (Carl Zeiss Imaging Solutions). Due to the higher resolution of the pictures, it was possible to identify more foci than in the previous study. We therefore repeated and requantified the staining in the BALB/c CD and FD groups.

Plasma homocysteine. Plasma was collected and homocysteine measured as previously described[317; 328].

Global DNA methylation. We used TLC to measure the global amount of DNA methylation at CCGG sites, as previously described[295].

dUTP:dTTP ratio. Free deoxyribonucleotides were separated using an HPLC method previously described[329] with slight modifications. Briefly, frozen preneoplastic intestine was ground to powder, treated with 0.6 mol/L trichloroacetic acid, neutralized with trioctylamine, and injected onto an Econosphere C18 column (particle size 5μm, length 250 x 4.6mm, Waters instrument Part No. 70071). Separation of dUTP and dTTP was achieved by

isocratic elution with 100% Buffer A (0.1 mol/L $NH_4H_2PO_4$, 0.33 mol/L KCl, 0.25% methanol, pH 5.35) for 12 min followed by a linear gradient to 25% Buffer B (0.1 mol/L $NH_4H_2PO_4$, 0.4 mol/L KCl, 20% methanol, pH 5.0) for 18 min followed by a linear gradient to 80% Buffer B for 10 min, then 10 min of 80% Buffer B, followed by re-equilibration with 100% Buffer A for 20 min. The flow rate for the entire run was 0.8 mL/min. A UV detector monitored peaks at wavelengths of 254 nm and 280 nm. Peaks were assigned by coelution with known standards (Sigma-Aldrich).

Statistical methods. Differences in tumor incidence between groups were assessed with Fisher's exact test. Within a strain, mice fed the CD and FD were compared using independent sample *t* tests, and paired sample *t* tests were used when tumors and normal tissue within a mouse were compared. We used 2-way ANOVA to evaluate the effects of strain, diet, genotype and their interactions, followed by Tukey's post hoc test. SPSS for Windows (release 10.0.1) was used for analyses. Values are means ± SEM. Differences were considered significant at $P < 0.05$.

3.4 Results

3.4.1 Strain differences

Tumor incidence. We examined 39 CD (24 *Mthfr* +/+ and 15 *Mthfr* +/-) and 59 FD (32 *Mthfr* +/+ and 27 *Mthfr* +/-) C57Bl/6 mice of both *Mthfr* genotypes for intestinal tumors. None of the C57Bl/6 mice had intestinal adenocarcinomas, adenomas, or polyps, by gross and histological examination, in contrast to what had been observed in the BALB/c strain[326]. Previously, we observed that none of the 31 *Mthfr*+/+ or 26 *Mthfr*+/- CD BALB/c mice developed any tumors while 2/16 *Mthfr*+/+ and 18/64 *Mthfr*+/- FD BALB/c mice developed intestinal tumors. Tumor incidence differed between the BALB/c and C57Bl/6 strains ($P < 0.05$).

Plasma homocysteine. Plasma homocysteine concentrations (an indicator of nutritional folate deficiency[330; 331]) were measured in 10 samples per diet group in C57Bl/6 and BALB/c strains, 5 samples for each *Mthfr* genotype (+/+ and +/-). We observed the expected increases in plasma homocysteine due to the FD and to

the *Mthfr* +/- genotype in both the BALB/c and C57Bl/6 strains (**Table 3.1**).

Table 3.1 Effect of CD, FD, and *Mthfr* genotype on plasma homocysteine, dUTP:dTTP ratio and percent p-H2AX foci in BALB/c and C57Bl/6 mice fed the diets for 1y[1]

Strain	Diet	*Mthfr* genotype	Plasma homocysteine[2] (μmol/L)	dUTP:dTTP[1]	p-H2AX foci[1] (%)
BALB/c	CD	+/+	8.55 ± 1.19	2.19 ± 0.20[b]	14.06 ± 3.59[b]
		+/-	13.97 ± 3.29		
	FD	+/+	18.93 ± 3.58	2.77 ± 0.18[c]	22.43 ± 2.65[c]
		+/-	38.87 ± 5.42		
C57Bl/6	CD	+/+	4.99 ± 0.14[a]	1.21 ± 0.08[a]	8.95 ± 5.62[a]
		+/-	10.64 ± 0.82[a]		
	FD	+/+	9.90 ± 1.18[a]	1.27 ± 0.15[a]	11.18 ± 8.97[a]
		+/-	34.56 ± 2.56[b]		

[1]Values are means ± SEM, n=5-10, except for percent p-H2AX foci in C57Bl/6 mice, where n=2. Means in a column without a common letter differ, $P < 0.05$

[2]Strain, diet, and genotype, $P < 0.01$ by ANOVA. For the C57Bl/6 strain, diet/genotype interaction $P < 0.05$ by post hoc test. There was no diet/genotype interaction in the BALB/c strain. Means in a column with superscripts without a common letter differ.

Strain, diet and genotype affected the plasma homocysteine concentration ($P < 0.01$). It is unclear if the strain differences are biologically relevant, particularly since we do not observe consistent strain differences in homocysteine levels in other related work (our unpublished data).

Global DNA methylation. Based on the increased plasma homocysteine levels in mice fed FD, we hypothesized that the remethylation of homocysteine to methionine was impaired in FD mice of both strains. We therefore examined global DNA methylation levels in 6 CD and FD normal intestines (3 *Mthfr* +/+, 3

Mthfr +/- per group) of the two strains. In the BALB/c strain, global DNA methylation tended to be greater in CD (77.8 ± 1.1%) than in FD (72.4 ± 1.8%, P = 0.06). In the C57Bl/6 strain, global DNA methylation did not differ between mice fed the CD (60.3 ± 1.2%) and FD (60.9 ± 1.0%).

dUTP:dTTP ratios. We examined dUTP:dTTP ratios in the normal intestine of 10 CD and FD BALB/c and C57Bl/6 mice (5 *Mthfr*+/+ and 5 *Mthfr*+/- per diet group; **Table 3.1**). dUTP:dTTP ratios tended (P = 0.05) to be greater in FD mice than CD BALB/c mice. However, the dUTP:dTTP ratio did not differ between C57Bl/6 mice fed the CD or FD. Both CD and FD C57Bl/6 mice had lower dUTP:dTTP ratios than CD and FD BALB/c mice (P < 0.05). dUTP:dTTP ratios did not differ between *Mthfr* +/+ and *Mthfr*+/− mice in either the BALB/c or C57Bl/6 strains.

DNA damage. Based on the results of the dUTP:dTTP ratios, we questioned whether the increase in dUTP levels could lead to double-strand breaks and DNA damage. We examined the crypts of 5 *Mthfr*+/+ CD and FD BALB/c and 2 *Mthfr*+/+ CD and FD C57Bl/6 mice which were processed at the same time (**Table 3.1**). As in our previous study, percent p-H2AX staining tended (P = 0.054) to be greater in FD than CD BALB/c mice. In contrast, the percent p-H2AX foci did not differ between C57Bl/6 mice fed the CD or FD. These findings are consistent with the results on dUTP:dTTP ratios for these strains.

DNA damage response genes. We examined the expression of *Plk1* and *Cdc25c* in 3 CD and 3 FD (all *Mthfr* +/-) C57Bl/6 mice. mRNA levels analyzed by QRT-PCR did not differ in expression between CD and FD normal intestine in C57Bl/6 mice (ratio FD:CD normal intestine: Plk1, 1.02 ± 0.10; Cdc25c, 0.92 ± 0.10). This is in contrast to the differences observed in BALB/c mice in our previous study. Protein levels of PLK1 and CDC25c, as examined by immunofluorescence, were highly variable; therefore, we concluded that there was no consistent change due to diet (**Figure 3.1A, B**).

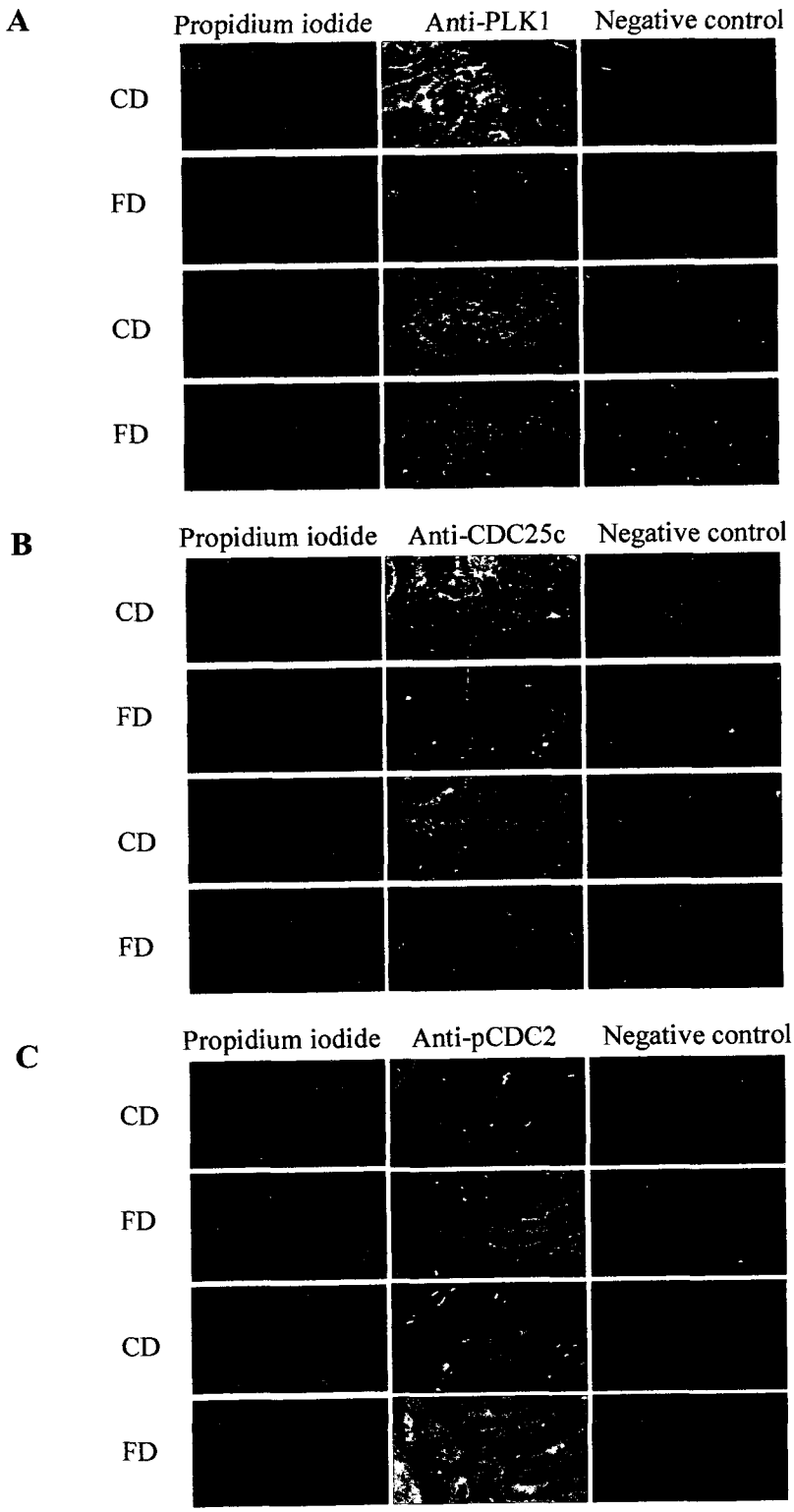

A
Propidium iodide
Anti-PLK1
Negative control
CD
FD
CD
FD
B
Propidium iodide
Anti-CDC25c
Negative control
CD
FD
CD
FD
C
Propidium iodide
Anti-pCDC2
Negative control
CD
FD
CD
FD

Figure 3.1 Effect of diet on PLK-1 (A), CDC25c (B) and p-CDC2 (C) protein levels in normal intestines from 1-y-old C57Bl/6 mice fed CD or FD (400x magnification). (A-C), Immunofluorescent staining using anti-PLK-1, anti-CDC25c, or anti-p-CDC2 antibodies (2^{nd} column), respectively. None of the 3 proteins showed a consistent change in staining intensity between CD and FD intestines. Propidium iodide was used as a nuclear counter-stain (first column). The final column represents negative controls containing secondary antibody, but no primary antibody, for each sample. n=2 samples per group.

In addition to the CDC25c protein levels, we also examined levels of the phosphorylated form of CDC2, an indicator of CDC25c activity. Increased levels of phosphorylated CDC2 are indicative of inactive CDC25c and cell cycle arrest[262]. Our earlier results on phosphorylated CDC2 levels were consistent with the change in expression of CDC25c[326]. Findings for the C57Bl/6 mice in this study showed p-CDC2 levels (CDC25c activity) did not differ between CD and FD normal intestine (**Figure 3.1C**). These results are consistent with the absence of diet-induced mRNA expression changes for *Cdc25c* and *Plk1* in this strain.

3.4.2 Betaine supplementation

Tumor incidence. We used the tumor-susceptible strain, BALB/c, and fed 42 mice the FD (24 *Mthfr* +/+ and 18 *Mthfr* +/-) and fed 44 mice the FDB (all *Mthfr* +/-). There was an 8.3% (2 mice with tumors/24) incidence of tumors in FD *Mthfr* +/+ mice and a 22.2% (4 mice with tumors/18) incidence of tumors in the FD *Mthfr* +/- mice. There was an 18.2% (8 mice with tumors/44) tumor incidence in FDB mice, which did not differ from the FD *Mthfr* +/- incidence. In the FDB mice, we observed polypoid-like hyperplasias, adenomas, and adenocarcinomas; these histologic changes are similar to those observed in the FD mice[326] (data not shown).

Plasma homocysteine. We measured plasma homocysteine from 7 FD and 7 FDB *Mthfr* +/- mice. Plasma homocysteine concentrations did not differ between FD BALB/c mice in this study compared with the concentration in FD BALB/c mice from the original study. However, the plasma homocysteine concentrations in the FDB mice (12.40 ± 1.05μmol/L) were lower than those in the FD mice (36.90 ± 4.76 μmol/L; $P < 0.001$) and did not differ from the concentrations in CD BALB/c *Mthfr*+/- mice (10.64±0.82 μmol/L, **Table 3.1**).

Global DNA methylation. We examined global DNA methylation levels in 6 FD and 6 FDB normal intestines (all *Mthfr* +/-). Global DNA methylation in FDB mice (66.3% ± 1.3) did not differ from that of FD mice (67.1% ± 1.4) in normal intestine.

dUTP/dTTP ratios. We examined potential changes in nucleotide pools in the mice supplemented with betaine in 7 FD and 7 FDB normal intestines (all *Mthfr*

+/-). dUTP:dTTP ratios did not differ between FD mice (2.00 ± 0.28) and FDB mice (2.32 ± 0.23).

DNA damage. To further assess any possible effects of betaine supplementation on DNA damage, we quantified p-H2AX foci in 3 *Mthfr* +/- mice per group fed the FD (25.49 ± 9.77) and FDB (20.22 ± 4.57). The groups did not differ, consistent with the similar dUTP:dTTP ratios in the two groups.

DNA damage response genes. We also examined expression of the DNA damage response genes *Plk1* and *Cdc25c* in 6 FD and 6 FDB normal intestines (all *Mthfr* +/-). Expression at the mRNA level did not differ between the groups for either *Plk1* (ratio FDB:FD normal intestine: 0.96 ± 0.01) or for *Cdc25c* (ratio FDB:FD intestine: 0.92 ± 0.00). In contrast, in 3 pairs of *Mthfr* +/- normal intestines and tumors, we observed increased expression of both genes in tumors, as observed in the original report for the FD[326]. However, due to small sample size, differences were not significant (FDB tumor:FDB normal intestine for *Plk1* = 2.55 ± 0.96, P = 0.167; for *Cdc25c* = 9.63 ± 6.34, P = 0.171).

3.5 Discussion

This study confirms our earlier observation that low dietary folate can induce intestinal tumors and highlights an important mechanism in this process. We observed that the C57Bl/6 strain was resistant to tumorigenesis induced by low folate, in contrast to the BALB/c strain. This conclusion is consistent with reports in the literature which indicate that C57Bl mice are more resistant to carcinogen-induced tumorigenesis[327]. The effectiveness of the FD in creating a functional folate deficiency in C57Bl/6 mice could be a concern. However, the expected increases in plasma homocysteine concentration, a classic measure of folate deficiency, were observed in both strains on the FD. Nonetheless, because we were unable to measure homocysteine concentrations in the intestine, we cannot exclude the possibility that the C57Bl/6 mice were resistant to localized folate deficiency in intestinal tissue. Betaine successfully lowered plasma homocysteine in BALB/c mice but did not affect tumor incidence, leading us to conclude that increased plasma homocysteine concentrations are not likely to be

associated with tumorigenesis in this model, although we cannot exclude tissue-specific homocysteine differences.

In our initial report, we hypothesized that the FD might lead to uracil misincorporation and DNA double-strand breaks. The constant pressure put on the DNA damage response pathway due to the long-term folate-deficient state could potentially cause an error in the complex regulation of G_2-M checkpoint control, where PLK1 and CDC25c function[326]. In this study, we obtained more direct evidence for increased DNA damage from the increased dUTP:dTTP ratios in the normal intestine of FD BALB/c mice. This observation is in concordance with the increased DNA damage found in the normal intestine of the FD BALB/c mice. As previously reported, the expression of *Plk1* and *Cdc25c* in tumors increased compared with normal intestine, suggesting a deregulation of these important cell cycle control genes. In contrast, we did not observe any indication of altered nucleotide ratios or an increase in DNA damage in the normal intestines of the FD C57Bl/6 mice. These conclusions would be strengthened by replication with increased sample numbers. Gene expression changes for *Plk1* and *Cdc25c* were also not evident in normal intestine in this strain. In further support of our argument that DNA damage is critical to folate-related tumorigenesis, the FD and FDB BALB/c mice had similar tumor incidence, similar dUTP:dTTP ratios, DNA damage, and *Plk1* and *Cdc25c* expression levels in the normal intestine. We observed overexpression of *Plk1* and *Cdc25c* in tumors compared with normal intestine in both dietary groups. These results are consistent with the proposed mechanism of DNA damage -induced transformation due to deregulation of damage response genes. The findings also indicate that betaine does not affect DNA damage levels or tumor incidence in this model.

Based on the increased plasma homocysteine concentrations in FD mice of both strains, we might expect changes in methionine and SAM concentrations and, therefore, in DNA methylation. In the BALB/c strain, global DNA methylation tended to decrease in the FD compared to CD normal intestines. However, there were no changes in DNA methylation due to diet in the C57Bl/6 strain. Our data are consistent with the hypothesis that DNA damage may occur

as an initiating event in tumorigenesis, with altered DNA methylation patterns being a secondary event[130]. On the other hand, we cannot exclude the possibility that this strain is also resistant to DNA methylation changes through an unidentified mechanism that is independent of DNA damage. Further study is warranted, particularly pertaining to the methylation state of individual genes (i.e. tumor suppressor and oncogenes), which may be contributing to tumorigenesis in our model.

Because betaine is an alternate methyl donor for remethylation of homocysteine to methionine, betaine supplementation might be associated with increased global DNA methylation[112]. However, we did not see any differences in global DNA methylation between the FD and FDB intestines. Because BHMT, the enzyme that catalyzes betaine-dependent homocysteine remethylation, is expressed primarily in liver and kidney, it is possible that betaine has some local effects in those tissues but no effect in intestine. In a previous study, DNA methylation increased in the brain of *Mthfr*-deficient mice supplemented with betaine; however, because those mice had sufficient dietary folate, it is difficult to compare these studies[112].

In conclusion, we have created a reproducible model for studying dietary influences on sporadic colorectal cancer. Our data suggest that the mechanism behind low folate-induced tumorigenesis may involve an imbalance in nucleotide pools, an increase in DNA damage, and a disruption of G_2-M checkpoint control. We hypothesize that there is a tumor-promoting environment in the normal intestine with DNA damage and altered methylation patterns in BALB/c mice. In particular, DNA damage could be a crucial step in promoting tumor growth as evidenced by the inability of the FD to increase DNA damage in the C57Bl/6 strain and the subsequent lack of tumor formation in that strain. This study highlights how innate genetic variation, such as that between the BALB/c and C57Bl/6 strains, can affect risk for spontaneous tumorigenesis and can serve to elucidate the mechanisms that contribute to the process.

3.6 Acknowledgments

This work was supported by a grant from the Canadian Institutes of Health Research (MOP-77596) and by a Scholarship to E.K. from the Fonds de la recherche en santé du Québec.

CONNECTING TEXT-Chapter III-IV

In Chapter III it was observed that C57Bl/6 mice do not develop intestinal tumors when placed on a folate-deficient diet for 1 year. Chapter III also describes increased DNA damage and decreased global DNA methylation in intestines from FD compared to CD BALB/c, but not C57Bl/6, mice. Consequently, C57Bl/6 mice did not show any changes in PLK1, CDC25c or p-CDC2 indicating no G_2-M arrest. These data led to the conclusion that, while showing a classic sign of folate deficiency (increased plasma homocysteine), C57Bl/6 mice did not develop altered DNA damage or global DNA hypomethylation in response to long-term dietary folate deficiency. Based on these data it was hypothesized that C57Bl/6 mice might regulate the enzymes of the folate metabolic pathway such that, even when folate supply is low, sufficient one-carbon units are provided for dTMP synthesis or methylation reactions. The increase in the dUTP/dTTP ratio observed in the intestines of FD BALB/c mice led to the hypothesis that these mice preferentially direct more one-carbon units towards DNA methylation reactions at the expense of dTMP synthesis. This has been previously hypothesized to be the response of most cells when faced with folate deficiency[15; 101]. In order to test these hypotheses, the expression of genes involved in folate metabolism was examined in intestines from CD and FD BALB/c and C57Bl/6 mice. Chapter IV describes the results of the gene expression analysis and the confirmation of some changes by activity assay or western blot when possible. In order to further support the gene expression data, intestinal free amino acid measurements are compared between the two mouse strains. It was also of interest to determine if the increase in DNA damage in intestines from FD BALB/c mice would lead to gross chromosomal abnormalities, such as DNA copy number changes, which might contribute to tumor formation. Chapter IV therefore also describes the examination of possible DNA copy number variation in intestines from FD BALB/c mice by aCGH. The verification of a region of potential amplification is described. Lastly, expression changes in genes involved in DNA damage repair are examined in the intestines of CD and FD BALB/c and C57Bl/6 mice.

CHAPTER IV

Differences in strain susceptibility to tumorigenesis induced by low folate may be influenced by variations in one-carbon metabolism and/or DNA repair

Erin Knock, Liyuan Deng, Qing Wu, Sapna Gupta, C. Lee Elmore, Warren Kruger, Rowena Matthews, Rima Rozen

4.1 Abstract

We previously reported that low dietary folate alone induces intestinal tumors in BALB/c, but not C57Bl/6 mice. Folate-deficient diet (FD) BALB/c, but not C57Bl/6 mice, showed some evidence of increased DNA damage in normal intestines compared to control diet (CD) mice. To determine the mechanism behind this observation, we examined several enzymes that might affect methyl supply for nucleotide synthesis, including methylenetetrahydrofolate reductase (MTHFR). Among other expression changes, we observed increased *Mthfr* expression and MTHFR activity in pre-neoplastic intestines from BALB/c compared to C57Bl/6 mice. This finding suggests that the increase in dUTP levels observed in FD BALB/c mice could be due to a lack of substrate for the thymidylate synthase reaction. To determine if the increased incidence of DNA double-strand breaks (increased p-H2AX staining) could lead to chromosomal abnormalities in pre-neoplastic intestines from FD BALB/c mice, we performed array comparative genomic hybridization (aCGH). We observed possible DNA amplification of a region with homology to exon 1 of *Thymine DNA glycosylase* (*Tdg*, involved in base excision repair) in the intestine of FD compared to CD BALB/c and C57Bl/6 mice. We then examined the levels of the full-length *Tdg* mRNA and TDG protein levels, along with other DNA repair genes; there tended to be more a prominent TDG increase in intestines from FD compared to CD C57BL/6 mice than that observed in intestines from FD compared to CD BALB/c mice. We suggest that C57Bl/6 mice may increase the expression of TDG and therefore increase the efficiency of DNA damage repair, resulting in resistance to intestinal tumor formation.

4.2 Introduction

Dietary folate metabolism is necessary for providing one-carbon units for the synthesis of S-adenosylmethionine (SAM, the universal methyl donor), purines, pyrimidines and amino acids (e.g. serine, glycine, methionine) (**Figure 4.1**).

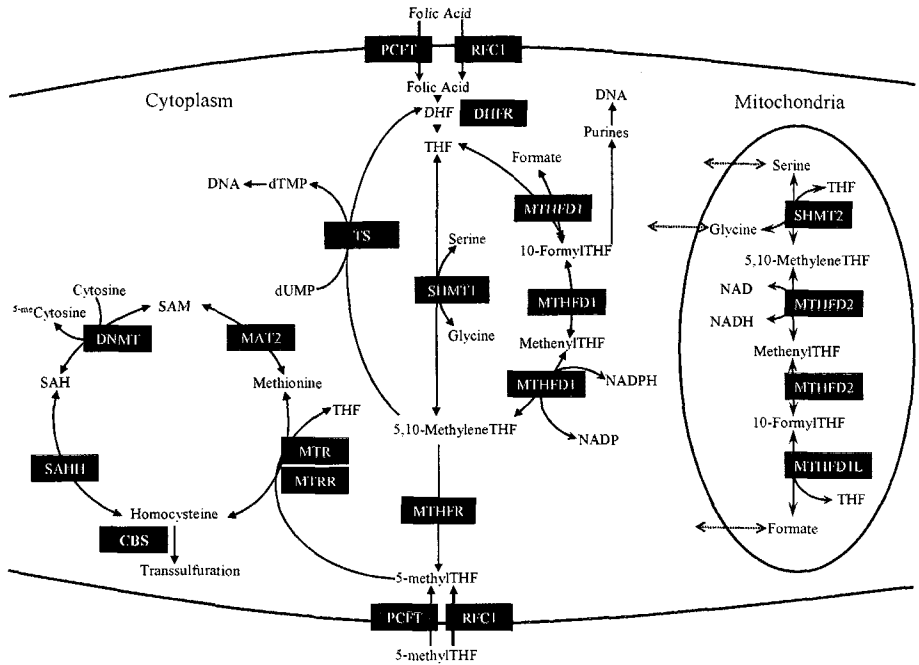


Figure 4.1 The folate metabolic pathway. The thick lines represent the plasma and mitochondrial membranes. Dashed arrows represent one-carbon units that cross the mitochondrial membrane. Enzymes appear in boxes. Abbreviations: CBS: cystathionine-β-synthase; DNMT: DNA methyltransferase; DHF: dihydrofolate; DHFR: dihydrofolate reductase; dTMP: deoxythymidylate monophosphate; dUMP: deoxyuridylate monophosphate; MAT: methionine adenosyltransferase; MTHFD1: trifunctional methylenetetrahydrofolate dehydrogenase; MTHFD1L: monofunctional formyltetrahydrofolate synthetase; MTHFD2: bifunctional methylenetetrahydrofolate dehydrogenase; MTHFR: methylenetetrahydrofolate reductase; MTR: methionine synthase; MTRR methionine synthase reductase; PCFT: proton coupled folate transporter; RFC: reduced folate carrier; SAH: S-adenosylhomocysteine; SAHH: S-adenosylhomocysteine hydrolase; SAM: S-adenosylmethionine; SHMT: serine hydroxymethyltransferase; THF: tetrahydrofolate; TS: thymidylate synthase.

Dietary folic acid is absorbed by the intestine mainly through the action of the proton coupled folate transporter (PCFT)[11; 12], although the reduced folate carrier (RFC1) may also be involved[8]. Dietary folate may enter the cell in either

the oxidized (folic acid) or reduced form (5-methyltetrahydrofolate, 5-methylTHF). Oxidized forms are converted to dihydrofolate (DHF) and then tetrahydrofolate (THF) by the enzyme dihydrofolate reductase (DHFR).

In the cytoplasm, THF and serine are used in the reversible serine hydroxymethyltransferase (SHMT1) reaction to produce glycine and 5,10-methyleneTHF[58; 59]. Serine is the major one-carbon donor for cellular reactions[332]. THF is also used by the tri-functional methyleneTHF dehydrogenase, methenylTHF cyclohydrolase, formylTHF synthetase (MTHFD1) enzyme to form 10-formylTHF for purine synthesis and in subsequent reactions to form 5,10-methyleneTHF[333]. THF and serine are also used in the mitochondria by the mitochondrial serine hydroxymethyltransferase (SHMT2) enzyme to form glycine and 5,10-methyleneTHF. This 5,10-methyleneTHF is used by the bifunctional mitochondrial methyleneTHF dehydrogenase methenylTHF cyclohydrolase (MTHFD2) enzyme to form 10-formylTHF which is the substrate for the separate mitochondrial formylTHF synthetase (MTHFD1L) reaction, forming THF and formate. Formate can cross the mitochondrial membrane and provide one-carbon units for cytoplasmic folate-dependent reactions[73; 81].

There are two enzymes which use 5,10-methyleneTHF. Thymidylate synthase (TS) requires one-carbons from 5,10-methyleneTHF to produce dTMP from dUMP for DNA synthesis. Methylenetetrahydrofolate reductase (MTHFR) catalyses the irreversible conversion of 5,10-methyleneTHF to 5-methylTHF *in vivo*[15]. The irreversible nature of the MTHFR reaction *in vivo* means that one-carbons in the form of 5-methylTHF are directed away from the TS reaction and towards methionine synthesis. One-carbons can re-enter the pool used by TS through the action of the methionine synthase (MTR) and methionine synthase reductase (MTRR) enzymes. MTR catalyses the addition of a one-carbon unit from 5-methylTHF to the toxic amino acid homocysteine, forming methionine and re-forming THF[91]. MTRR is necessary for MTR function by reducing cob(II)alamin (unusable by MTR) to cob(I)alamin (a co-factor necessary for MTR) after every few hundred MTR reactions[98].

The amino acid methionine serves as a pre-cursor for the formation of SAM, the methyl donor for DNA methylation reactions[16]. SAM formation is catalyzed by methionine adenosyltransferases (MAT); MAT1 in the liver and MAT2 in most other tissues[116]. Maintaining sufficient SAM availability for DNA methylation reactions is particularly relevant to cancer prevention since alterations in DNA methylation, including global DNA hypomethylation and regional promoter hypermethylation, are a hallmark of cancer cells[290]. Maintenance DNA methylation reactions are carried out by the DNA methyltransferase 1 (DNMT1) enzyme, producing as a by-product S-adenosylhomocysteine (SAH), which must be metabolized to homocysteine by the enzyme SAH hydrolase (SAHH). Homocysteine can be toxic to cells at high levels and can be detoxified through the transsulfuration pathway which is initiated by the enzyme cystathionine-beta-synthase (CBS)[16].

Folate deficiency has been hypothesized to increase the risk for colorectal cancer (CRC)[2; 184; 185]. Several mechanisms have been proposed. As the source of one-carbon units for DNA methylation reactions and dTMP synthesis, dietary folate deficiency may result in insufficient one-carbon unit availability for these important reactions. Insufficient one-carbons for DNA methylation reactions can lead to global DNA hypomethylation, resulting in chromosomal instability and inappropriate changes in gene expression. Insufficient one-carbons for dTMP synthesis can lead to a build up of dUMP, which can be misincorporated into DNA as dUTP and result in DNA double-strand breaks[16]. Uracil misincorporated into DNA is removed by the action of the uracil DNA glycosylases[250]. Thymine DNA glycosylase (TDG) repairs uracil in double-stranded DNA and T:G mispairs which are a result of $^{5\text{-me}}$C deamination [252]. When TDG removes a base an abasic site is left to which it will remain attached, protecting the site, until the apurinic/apyrimidinic endonuclease 1(APE1) arrives. APE1 cleaves the sugar-phosphate backbone of DNA, leaving a 5'deoxyribose phosphate. The resultant DNA single strand break (ssb) can be repaired by either the long or short patch repair pathways[249; 250]. One human case-control study has shown germline

variants in *TDG* in familial CRC cases, but not controls indicating TDG may be involved in the development of CRC[334].

Disruptions of folate metabolic enzymes can also affect CRC risk. A common variant in *MTHFR* (a C to T transition at nucleotide 677) reduces MTHFR activity and has been associated with a decreased risk for CRC when folate intake is adequate[202]. When there is inadequate folate intake, however, this variant has been associated with an increased risk for CRC[222].

In previous work we created a novel model of spontaneous intestinal tumorigenesis by feeding BALB/c mice a folate-deficient diet (FD) for 1 year. These mice developed an overall 25% incidence of intestinal tumors without a predisposing transgene or use of carcinogen induction. BALB/c mice fed an amino acid controlled diet (CD) did not develop any tumors. In a further study we fed another common mouse strain, C57Bl/6 the same CD and FD as the previous study. No C57Bl/6 mice on either diet developed any tumors [335]. Also in that study, we observed increased dUTP/dTTP ratio, and increased percentage of p-H2AX foci and decreased global DNA methylation in intestines from FD compared to CD BALB/c, but not C57Bl/6, mice.

Based on these data we were interested to determine if there were expression differences in folate metabolizing enzymes between the susceptible BALB/c and resistant C57Bl/6 strains which could begin to explain why one strain was so strongly affected by the folate-deficiency while the other was not. We hypothesize that BALB/c mice preferentially direct one-carbon units in the form of 5-methylTHF towards methionine synthesis at the expense of 5,10-methyleneTHF and the TS reaction. We hypothesize that this leads to the buildup of dUTP observed in this strain, resulting in DNA double-strand breaks and tumor formation. Alternatively, the C57Bl/6 strain regulates one-carbon metabolism such that sufficient one-carbon units are maintained for both DNA methylation and dTMP synthesis reactions, resulting in resistance to folate-deficiency induced tumorigenesis in this strain.

Based on the incidence of DNA double-strand breaks observed in intestines from FD BALB/c mice, we hypothesize that gross chromosomal

abnormalities may occur in the pre-neoplastic intestine which further promote tumor formation. We therefore pursued array comparative genomic hybridization (aCGH) in order to search for possible copy number variations in the intestines of FD compared to CD BALB/c mice.

4.3 Materials and methods

Mice. Animal experimentation was approved by the Animal Care Committee of the Montreal Children's Hospital. Animal killing and tissue collection was performed as previously described[326; 335].

mRNA and protein expression. RNA was isolated from whole normal duodenums and first-strand cDNA synthesis was performed as previously described[326]. Quantitative PCR (QPCR) was performed as previously described[326] using primers specific for *Apex1, Cbs, Foxo3, Mat2a, Mlh1, Msh2, Mthfd1, Mthfd2, Mthfr, Mtr, Mtrr, Pcft, Rfc1, Shmt1, Shmt2, Tdg, and Ts*. Primer sequences and conditions are described in **Supplementary Table 4.1** (see Appendix I). Primers and conditions for the amplification of *Dnmt1* have been previously published[336]. Target gene expression was normalized to *Gapdh*. Protein levels were determined from 50μg of whole cell lysates derived from whole normal duodenums. Whole cell lysates were prepared by homogenizing intestine in lysis buffer containing 50mmol/L KH_2PO_4, 1mmol/L EDTA, and protease inhibitors followed by incubation on ice for 5 min and centrifugation for 15 min at 20,000g. The supernatant was then removed and the protein quantified using the Bio-Rad protein assay (Bio-Rad, Mississauga, Ontario, Canada) and stored at -80°C. Protein separation was performed by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane for 2 hours at 70V. The membrane was blocked in 2-5% skim milk powder in TBS containing 2% Tween. Protein detection was performed using antibodies directed against MTHFD1 (1:4,000, a gift from R. McKenzie, McGill University), MTHFD2 (1:5,000, also a gift from R. McKenzie), MTHFR (1:4,000, previously prepared in our lab[30]), MTRR (1:5,000, previously prepared in our lab[98]), SHMT2 (1:5,000, a gift from P. Stover, Cornell University), or TDG (1:5,000, a gift from M. Tini, University of

Western Ontario). For blots examining proteins which were sufficiently different in size from β-ACTIN (50Kda) the membranes were cut and a portion of the membrane was incubated with the [illegible]body of interest while the other portion was incubated with a 1:5,000 dilution of anti-β-ACTIN antibody (Sigma, Oakville, Ontario, Canada) as a loading control. Otherwise, the membrane was stripped using a 1x strip solution (Chemicon, Temecula, California, USA) according to the manufacturer's instructions and then re-probed with the anti-β-ACTIN. After washing, the membrane was incubated with the appropriate secondary antibody (1:5,000 anti-rabbit, GE Healthcare, Baie d'Urfé, Quebec, Canada, or 1:5000 anti-sheep, Santa Cruz Biotechnologies, Santa Cruz, California, USA), then washed again. Visualization was performed using the ECL kit (Amersham Biosciences, Baie d'Urfé, Quebec, Canada) and exposure to X-ray film (InterScience, Markham, Ontario, Canada). Quantity-One v.4.0.1 software (Bio-Rad, Mississauga, Ontario, Canada) was used to quantify the appropriate bands. All QRT-PCR and western blot results were normalized relative to the value for the control group: BALB/c CD *Mthfr*+/+.

Enzyme activity assays. The MTHFR activity assay was performed as previously described[27]. The MTR activity assay was performed in the lab of Rowena Matthews, also as previously described[337]. The TS activity assay was performed as described[338] with the following changes. Briefly, following total protein extraction as described[338]; each sample was diluted to a concentration of 300μg/100μL. The final concentration of protein was determined by running serial dilutions of protein samples and determining the concentration needed for optimal enzyme efficiency. The substrate 5,10-methyleneTHF was synthesized as described[338], but 75μL of the substrate was used instead of the recommended 50μL. The negative control sample was an equal mix of the protein samples totalling 300μg of protein/100μL mix including all components of the reaction mix minus the substrate (no folate control). All samples were run in duplicate. Tritiated dUMP was added in order to detect TS activity; active enzyme releases tritium into the reaction mix, which is then incorporated into water. Unreacted dUMP is absorbed by 48 hour incubation with activated charcoal. Detection of

tritiated water formed from the reaction was performed in a scintillation counter in duplicate. The readout was given in decays per minute, which was converted to becquerels/reaction volume and each experiment normalized to the average value for the BALB/c CD *Mthfr*+/+ samples.

Intestinal amino acids. An aliquot of total cell lysates, prepared as described above (mRNA and protein expression) was sent to the lab of Warren Kruger for measurements of total free amino acids as described [339; 340].

Thin layer chromatography. Thin layer chromatography (TLC) was performed as previously described [335].

Array comparative genomic hybridization (aCGH) and verification. High quality DNA which had been previously isolated[335] was sent to Empire Genomics at the Roswell Park Cancer Institute in Buffalo, New York. The DNA was hybridized to the mouse ACCUarray™ tiled BAC array with 500Kb median spacing for copy number analysis as described [341]. The results were visualized using the arrayCGH Viewer program, provided by Empire Genomics[342] along with the February 2006 version of the mouse genome in UCSC genome browser. A copy number change was defined as a region where there was more than one contiguous BAC with a fold change greater than 1.2 fold or less than -1.2 fold (FD/CD sample). Confirmation of DNA amplification or deletion was done using QPCR on DNA samples previously isolated from whole normal duodenums of mice which were from different mice than those used for aCGH [335]. Average DNA amplification of each gene was normalized to a non-amplified region (*Gapdh*).

Statistical analysis. Two-way ANOVA was used to evaluate the effects of strain, diet, genotype, and their interactions, followed by Tukey's post hoc test. For individual group comparisons, independent sample t-test was used. SPSS for Windows (release 10.0.1) was used for analyses. Values are means ± SEM. Differences were considered significant at $P < 0.05$.

4.4 Results

Strain, diet and genotype differences in expression of genes involved in folate metabolism. To test the hypothesis that BALB/c and C57Bl/6 mice have different distributions of one-carbon units, we examined the expression of genes related to folate metabolism using mRNA isolated from the normal intestine of 6 CD and 6 FD (3 *Mthfr*+/+ and 3 *Mthfr*+/-) BALB/c and C57Bl/6 mice (**Table 4.1**).

Table 4.1 Summary of gene expression changes observed in normal intestines of CD and FD, *Mthfr*+/+ and +/-, BALB/c and C57Bl/6 mice[1].

Gene	Strain difference?	Diet difference?	Genotype difference?
Cbs	No	↓ in FD vs. CD (C57Bl/6 only)	No
Dnmt1	↑ in BALB/c vs. C57Bl/6	No	No
Mat2a	↑ in BALB/c vs. C57Bl/6	No	↓ in *Mthfr*+/- vs. *Mthfr*+/+
Mthfd1	↑ in BALB/c vs. C57Bl/6	↓ in FD vs. CD	↑ in *Mthfr*+/- vs. *Mthfr*+/+
Mthfd2	↑ in BALB/c vs. C57Bl/6	No	No
Mthfr	↑ in BALB/c vs. C57Bl/6	No	No
Mtr	↓ in BALB/c vs. C57Bl/6 (when comparing within diet groups only)[2]	No	No
Mtrr	↑ in BALB/c vs. C57Bl/6	↓ in FD vs. CD	No
Pcft	No	↑ in FD vs. CD (BALB/c only)	↓ in *Mthfr*+/- vs. *Mthfr*+/+ (C57Bl/6 only)
Rfc1	↑ in BALB/c vs. C57Bl/6	No	No
Shmt1	↑ in BALB/c vs. C57Bl/6[2]	No	No
Shmt2	↑ in BALB/c vs. C57Bl/6	No	No
Ts	No	No	No

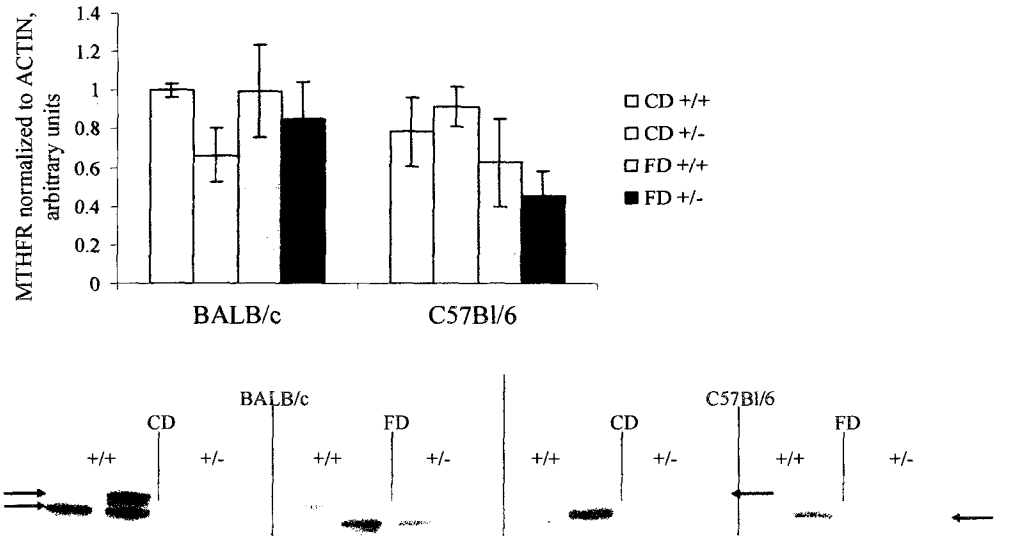
[1]-n= 3-4 per strain/diet/genotype group. Arrows indicate a significant ($p<0.05$) increase or decrease in expression for the comparison described by ANOVA.

[2]-$p<0.05$ by t-test.

BALB/c mice had increased expression of *Dnmt1, Mat2a* (the main MAT isoform in the intestine [119]), *Mthfd1, Mthfd2, Mthfr, Mtrr, Rfc1, Shmt1* and *Shmt2* compared to C57Bl/6 mice, regardless of diet or genotype. Expression of *Mtr* was decreased in BALB/c compared to C57Bl/6 mice, but only when comparing within diet groups (CD or FD). No strain differences in expression were observed for *Cbs*, *Pcft* (the main intestinal folate transporter[11; 12]) and *Ts*. FD mice had decreased expression of *Mthfd1* and *Mtrr* compared to CD mice, regardless of strain or genotype. Expression of *Cbs* was decreased in FD compared to CD C57Bl/6 mice only. Expression of *Pcft* was increased in FD compared to CD BALB/c mice only. *Mthfr*+/- mice had decreased expression of *Mat2a* and increased expression of *Mthfd1* compared to *Mthfr*+/+ mice, regardless of diet or strain. *Pcft* expression was decreased in *Mthfr*+/- compared to *Mthfr*+/+ C57Bl/6 mice only. No changes in *Mthfr* expression due to genotype were observed due to the production of a non-functional transcript in the *Mthfr*+/- mice.

Strain differences in MTHFR protein and activity. The observation that BALB/c mice had increased expression of *Mthfr* compared to C57Bl/6 mice (**Table 4.1**) led us to hypothesize that BALB/c mice may favor 5-methylTHF synthesis at the expense of 5,10-methyleneTHF. Based on this, we examined the levels of MTHFR protein and enzyme activity in the normal intestine of 12 to 14 CD (6 to 8 *Mthfr*+/+ and 6 *Mthfr*+/-) and 11 to 13 FD (6 to 7 *Mthfr*+/+ and 5 to 6 *Mthfr*+/-) BALB/c and C57Bl/6 mice (**Figure 4.2**).

A) MTHFR protein levels



B) MTHFR enzyme activity

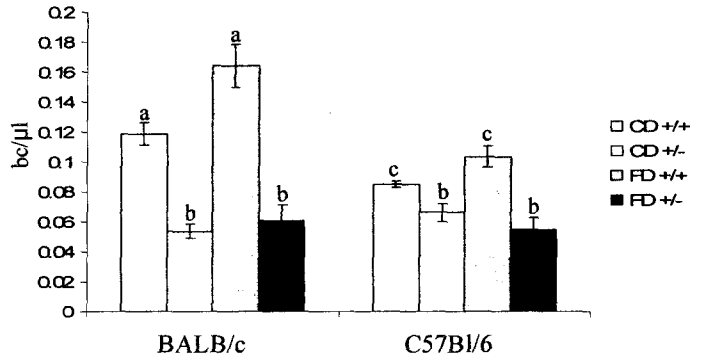
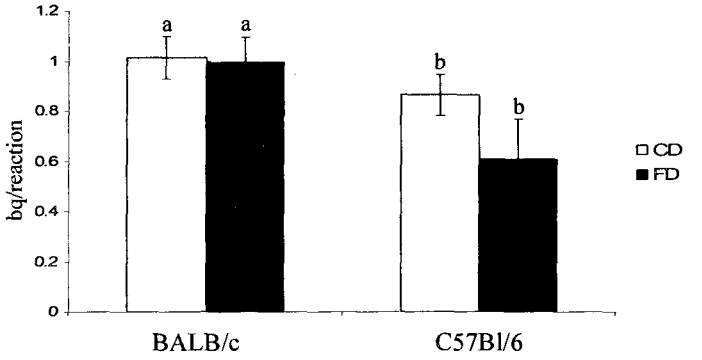


Figure 4.2 MTHFR protein and activity levels in normal intestines from CD and FD, *Mthfr*+/+ and +/-, BALB/c and C57Bl/6 mice. A) Average MTHFR protein levels relative to the CD *Mthfr*+/+ BALB/c group (top panel) and a representative western blot (bottom panel); the red arrow indicates the 70kDa isoform, the black arrow the 77kDa isoform. n=5-8 per group. B) Average MTHFR enzyme activity. n=3-4 per group, columns without a common letter differ ($p<0.05$).

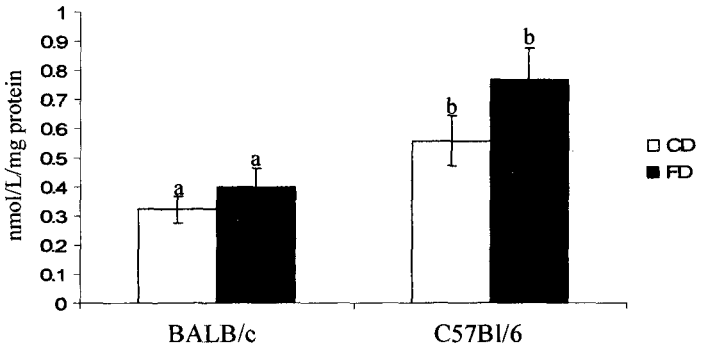
FD BALB/c mice tended to have more total MTHFR protein than FD C57Bl/6 mice (P = 0.084; **Figure 4.2A**, top panel). No other differences were found due to the high variability within each group. MTHFR has two different isoforms, which show tissue specific expression patterns [20]. The expression of the 77kDa isoform tended to differ between BALB/c and C57Bl/6 mice and between CD and FD mice (**Figure 4.2A**, bottom panel). Eighty-nine percent (17/19) of CD BALB/c mice expressed both isoforms versus 63% (12/19) of FD BALB/c mice; 21 % (4/19 per diet) of CD and FD C57Bl/6 mice expressed both isoforms. The identity of the 70 and 77kDa isoforms was confirmed by running CD and FD BALB/c and C57Bl/6 extracts next to a sample mix spiked with extract from bacterial cultures expressing either the 70kDa form alone, the 77kDa form alone, or both isoforms equally (data not shown). *Mthfr*+/+ BALB/c mice had increased MTHFR activity compared to *Mthfr*+/+ C57Bl/6 mice. *Mthfr*+/- mice had decreased MTHFR activity compared to *Mthfr*+/+ mice, regardless of strain or diet, as expected (**Figure 4.2B**). The MTHFR activity results are consistent with the *Mthfr* mRNA strain difference results.

Strain differences in one-carbon metabolism which influence 5,10-methyleneTHF availability. The observation that BALB/c mice had increased MTHFR activity compared to C57Bl/6 mice (**Figure 4.2**) supports the hypothesis that BALB/c mice favor 5-methylTHF synthesis at the expense of 5,10-methyleneTHF. Based on this, we examined the enzyme activities, substrate/product ratios or protein levels of several enzymes which utilize or synthesize 5,10-methyleneTHF in the normal intestines of CD and FD *Mthfr*+/+ and +/- BALB/c and C57Bl/6 mice (**Figure 4.3**).

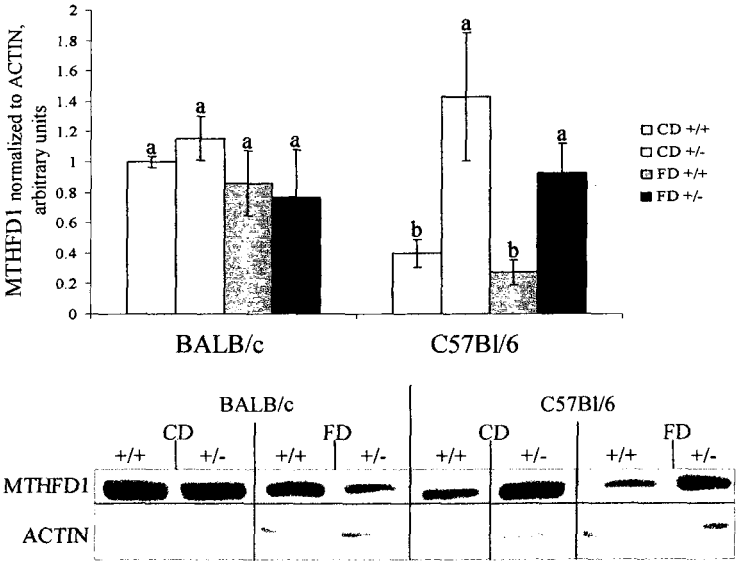
A) TS enzyme activity



B) Ratio of free intestinal serine/glycine



C) MTHFD1 protein levels



D) MTHFD2 protein levels

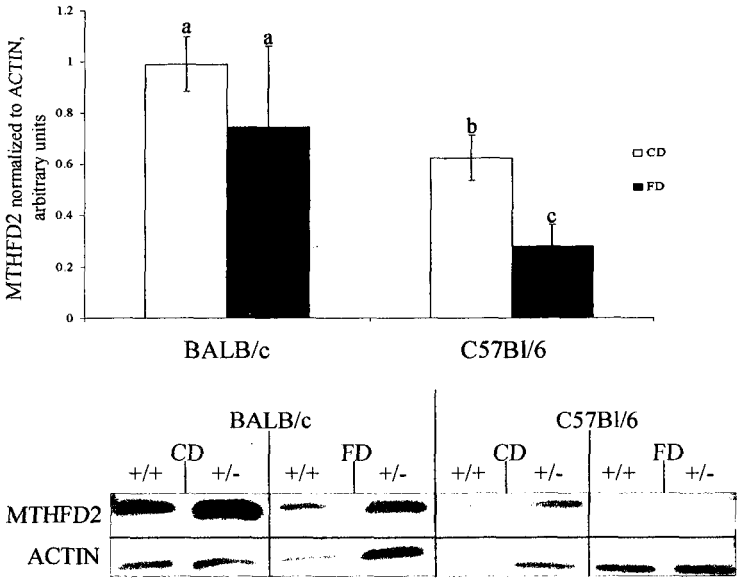


Figure 4.3 Examination of enzymes which influence 5,10-methyleneTHF availability in normal intestines of CD and FD, *Mthfr*+/+ and +/-, BALB/c and C57Bl/6 mice. A) Average TS enzyme activity normalized to the CD *Mthfr*+/+ BALB/c group, n=8 per group B) Average ratio of free intestinal serine to glycine, n=6-8 per group C) Average MTHFD1 protein levels relative to the CD *Mthfr*+/+ BALB/c group (top panel) and a representative western blot (bottom panel), n=4-8 per group D) Average MTHFD2 protein levels relative to the CD *Mthfr*+/+ BALB/c group (top panel) and a representative western blot (bottom panel), n=9-12 per group. Columns without a common letter differ ($p<0.05$).

BALB/c mice had increased TS enzyme activity compared to C57Bl/6 mice, regardless of diet or genotype (**Figure 4.3A**, 8 CD and 8 FD mice per strain, 4 *Mthfr*+/+, 4 *Mthfr*+/- per diet group). This is in contrast to what was observed for *Ts* mRNA expression (**Table 4.1**).

The SHMT1 reaction is reversible *in vivo* based on the relative abundance of its substrates (serine and THF or glycine and 5,10-methyleneTHF) [64]. Serine is the major one-carbon donor for cellular reactions[332], therefore it could be hypothesized that the SHMT1 reaction favors glycine and 5,10-methyleneTHF synthesis. Although we observed increased expression of *Shmt1* in BALB/c compared to C57Bl/6 mice (**Table 4.1**), we could not predict from the expression data whether this reaction may be using or producing 5,10-methyleneTHF. We therefore examined the ratios of free intestinal serine/glycine in the normal intestines of 8 CD (4 *Mthfr*+/+ and 4 *Mthfr*+/-) and 6 FD (3 *Mthfr*+/+ and 3 *Mthfr*+/-) BALB/c and C57Bl/6 mice. BALB/c mice had a significantly lower serine/glycine ratio than C57Bl/6 mice, regardless of diet or genotype (**Figure 4.3B**). The SHMT1 reaction may therefore favor glycine production over serine in BALB/c mice. Since SHMT2 has been shown to be the main determinant of cellular glycine [81], we examined SHMT2 protein levels in intestines from 6 to 8 CD (2 to 4 *Mthfr*+/+ and 4 *Mthfr*+/-) and 7 to 8 FD (4 *Mthfr*+/+ and 3 to 4 *Mthfr*+/- per diet group) BALB/c and C57Bl/6 mice. There was no change in average SHMT2 protein levels due to strain or diet (data not shown). We did, however, observe increased SHMT2 levels in CD *Mthfr*+/– mice compared to CD *Mthfr*+/+ mice, regardless of strain by western blot (from 0.8±0.1 in CD *Mthfr*+/+ to 3.1±0.5 in CD *Mthfr*+/– mice; $P < 0.05$).

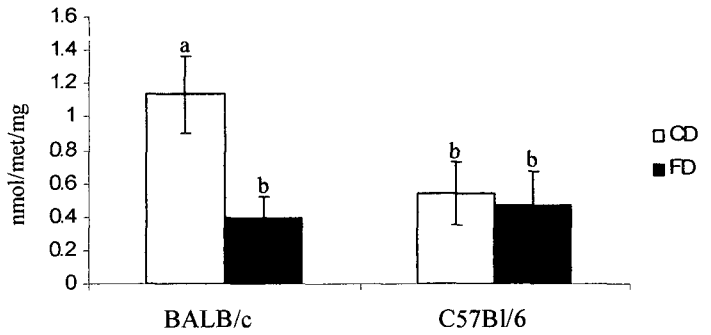
Since enzyme activity assays were not possible in our samples, we examined the protein levels of MTHFD1 in intestines from 8 CD (4 *Mthfr*+/+ and 4 *Mthfr*+/-) and 12 FD (6 *Mthfr*+/+ and 6 *Mthfr*+/-) BALB/c and C57Bl/6 mice. *Mthfr*+/+ BALB/c mice had increased MTHFD1 protein compared to *Mthfr*+/+ C57Bl/6 mice, regardless of diet (**Figure 4.3C**). *Mthfr*+/- mice had increased MTHFD1 protein compared to *Mthfr*+/+ in C57Bl/6 mice only. We did not

observe any diet differences in MTHFD1, contrary to the *Mthfd1* expression results; this is likely due to the high variability with each group (**Table 4.1**).

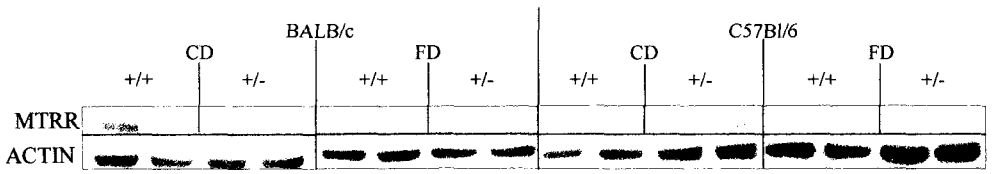
Increased MTHFD1 may favor purine synthesis in adult tissues [73]. In embryos and tumor cells, when the demand for purines is high, additional one-carbon units are provided in the form of formate from mitochondrial folate metabolism [73; 77; 82]. Based on this we examined the expression of MTHFD2 protein levels in intestines from 9 to 12 CD (4 to 5 *Mthfr*+/+ and 5 to 6 *Mthfr*+/-) and 9 to 10 FD (4 *Mthfr*+/+ and 5 to 6 *Mthfr*+/-) BALB/c and C57Bl/6 mice. BALB/c mice had increased MTHFD2 protein compared to C57Bl/6 mice, regardless of diet (**Figure 4.3D**). This is consistent with the *Mthfd2* mRNA strain difference results (**Table 4.1**). We also observed a decrease in MTHFD2 protein in FD compared to CD C57Bl/6 mice, which was not observed in the *Mthfd2* expression results.

Strain differences in methionine production. We examined the enzyme activity of MTR, the protein levels of MTRR and the free intestinal methionine levels in the normal intestine of CD and FD, *Mthfr*+/+ and +/-, BALB/c and C57Bl/6 mice (**Figure 4.4**).

A) MTR enzyme activity



B) MTRR protein levels



C) Free intestinal methionine

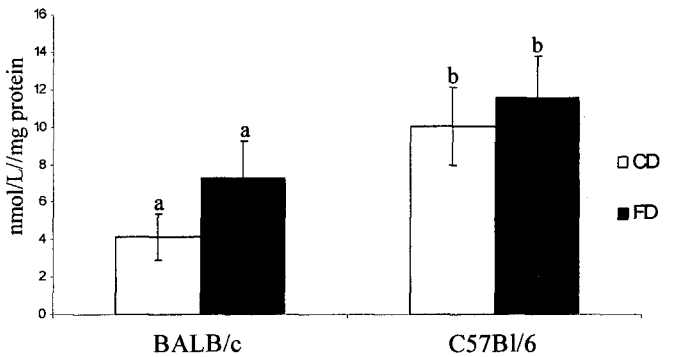


Figure 4.4 Examination of enzymes involved in methionine synthesis and tissue methionine levels in normal intestines of CD and FD, *Mthfr*+/+ and +/-, BALB/c and C57Bl/6 mice. A) Average MTR enzyme activity, n=4 per group B) Representative MTRR western blot, 8/8 CD and 2/8 FD BALB/c mice and 2/7 CD and 0/8 FD C57Bl/6 mice showed a detectable band for MTRR, even when films were over-exposed C) Average free intestinal methionine, n=6-8 per group. Columns without a common letter differ ($p<0.05$).

CD BALB/c mice had increased MTR activity compared to CD C57Bl/6 mice, regardless of genotype (4 CD and 4 FD mice per strain, 2 *Mthfr*+/+ and 2 *Mthfr*+/- per diet group). FD BALB/c mice had significantly decreased MTR activity compared to CD BALB/c mice, regardless of genotype (**Figure 4.4A**). This is in contrast to the *Mtr* mRNA results, where CD BALB/c mice had decreased *Mtr* expression compared to CD C57Bl/6 mice (**Table 4.1**). MTR may therefore be post-transcriptionally regulated.

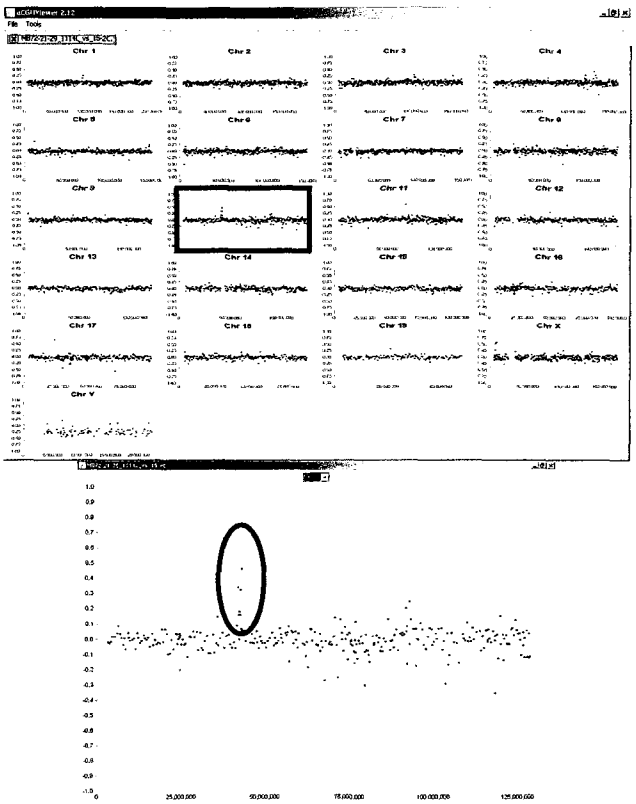
The MTRR protein was detected in intestines from 8/8 CD and 2/8 FD BALB/c mice and 2/7 CD and 0/8 FD C57Bl/6 mice (**Figure 4.4B**, 3 to 4 *Mthfr*+/+ and 4 *Mthfr*+/- per diet group). This result held even when the blots were exposed to film for 1 hour or more and is consistent with the strain and diet differences observed in the *Mtrr* mRNA results (**Table 4.1**). We then examined free intestinal methionine in intestines from 8 CD (4 *Mthfr*+/+ and 4 *Mthfr*+/-) and 6 FD (3 *Mthfr*+/+ and 3 *Mthfr*+/-) BALB/c and C57Bl/6 mice. BALB/c mice had significantly decreased free intestinal methionine compared to C57Bl/6 mice, regardless of diet or genotype (**Figure 4.4C**). Free intestinal homocysteine was under the levels detectable by the method used. Plasma homocysteine levels have been previously published[335].

We questioned whether intestinal methionine levels might correspond with global DNA methylation levels. In our previous study[335], TLC was used to examine diet differences within the BALB/c and C57Bl/6 strains. Due to the limitations of the technique, however, the two strains could not be directly compared. We therefore repeated the TLC comparing intestines from 6 CD BALB/c to 6 CD C57Bl/6 mice and 6 FD BALB/c mice to 6 FD C57Bl/6 mice (3 *Mthfr*+/+ and 3 *Mthfr*+/- per diet group). No changes were observed between CD BALB/c mice compared to CD C57Bl/6 mice (from 58.1±2.4% in C57Bl/6 to 55.9±3.9% for BALB/c) or between FD BALB/c compared to FD C57Bl/6 mice (from 64.6±2.4% in C57Bl/6 to 64.2±5.0% in BALB/c). This does not appear to correspond with the observed intestinal methionine levels.

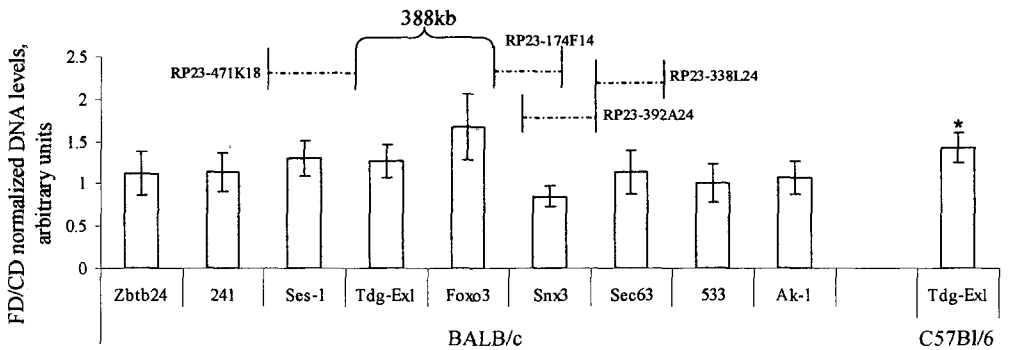
Strain differences in DNA damage and repair. To test the hypothesis that increased DNA damage in intestines from FD BALB/c mice results in gross

chromosomal abnormalities, aCGH was performed on DNA isolated from intestines of 4 FD *Mthfr*+/- BALB/c mice compared to 1 CD *Mthfr*+/- BALB/c mouse (**Figure 4.5**).

A) aCGH results

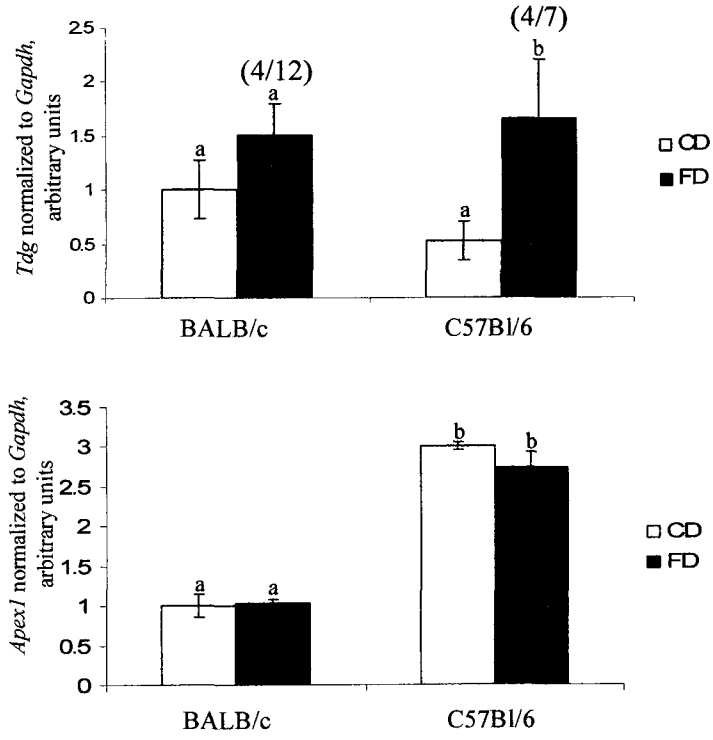


B) Verification of DNA amplification



BALB/c											C57Bl/6
zbtb24	241	Ses-1	Tdg-Ex1 (1)	Tdg-Ex1 (2)	Foxo3 (1)	Foxo3 (2)	Snx3	Sec63	533	Ak-1	Tdg-Ex1
1/8	1/7	2/7	4/8	0/8	7/8	3/8	0/7	2/8	1/8	1/8	2/8

C) *Tdg* and *Apex1* mRNA levels



D) TDG protein levels

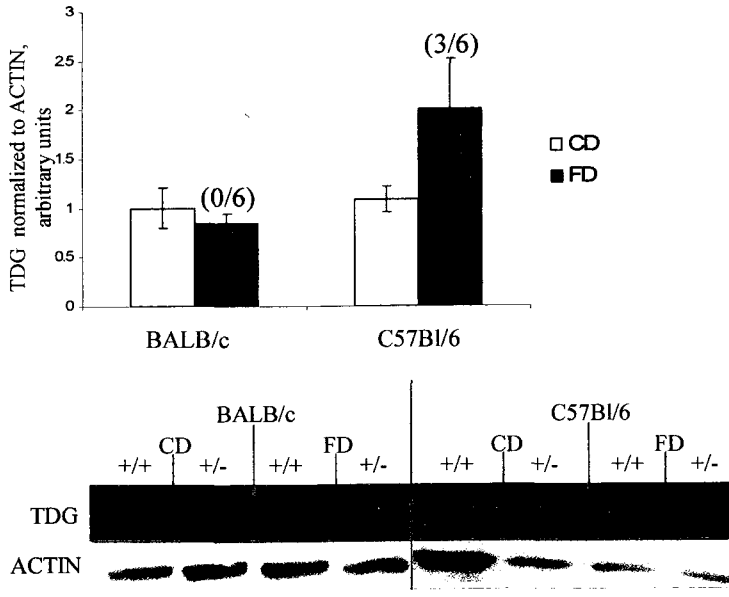


Figure 4.5 Array comparative genomic hybridization (aCGH) results and verification from FD compared to CD *Mthfr*+/- BALB/c mice A) arrayCGH Viewer output of a representative FD BALB/c sample normalized to the CD BALB/c sample. The x-axis denotes the chromosomal position in base pairs; the y-axis denotes the log-transformed fold change of the FD/CD sample. Chromosome 10 (boxed) is enlarged in the bottom panel, circled are the 4 BACs found to be amplified in all FD compared to CD samples. n=4 FD compared to 1 CD B) Average DNA amplification in FD/CD samples for regions of interest (top panel). The samples used for verification were different than the samples sent for aCGH analysis. The position of the 4 candidate BACs (pictured as dashed lines with the associated name beginning with RP) are pictured at top. The DNA levels of the *Tdg*-exon1 predicted transcript (*Tdg*-Ex1) were also examined in CD and FD C57Bl/6 mice (at right). The bottom panel represents the numbers of individual mice with a >1.8 fold increase in FD samples compared to the CD average. Verification of *Tdg*-Ex1 and *Foxo3* was performed in two separate experiments (averaged in top panel, labeled (1) and (2) in bottom panel). The *Tdg*-Ex1 DNA levels were also examined in individual FD C57Bl/6 mice (at right). n=8-12 per diet group C) The top panel represents average full-length *Tdg* mRNA expression normalized to the CD *Mthfr*+/+ BALB/c group. Numbers of individual mice with a >1.8 fold increase in FD samples compared to the CD average is indicated in brackets above the FD column. The bottom panel represents average *Apex 1* mRNA expression normalized to the CD *Mthfr*+/+ BALB/c group. All 8 C57Bl/6 mice had a >1.8 fold increase in expression compared to the average *Apex1* expression of all 8 BALB/c mice. The mice used were different than those used for the aCGH or DNA verification. n=4-12 per diet group, columns without a common letter differ D) Average TDG protein levels normalized to the CD *Mthfr*+/+ BALB/c group (top panel) and a representative TDG western blot (bottom panel). Numbers of individual mice with a >1.8 fold increase in FD samples compared to the CD average is indicated in brackets above the FD column. n=6 per diet group. Different mice were used than those used in the previous experiments. *=$p<0.05$

One region on chromosome 10 (41562397-42673019) was found where 3 contiguous BACs (RP23-174F14, RP23-392A24 and RP23-388L24) and 1 other BAC 388Kb upstream (RP23-471K18) were amplified 1.2 fold or higher in all FD, but not the CD sample (**Figure 4.5A**). UCSC genome browser identified several RIKEN cDNAs within the candidate region which we chose not to pursue in this study, but several known genes did fall within the candidate region including: *Sestrin 1* (*Ses1*), *Armadillo repeat containing 2* (*Arc2*), *Forkhead boxO3* (*Foxo3*), *Lactation elevated 1* (*Lace1*), *Sorting nexin 3* (*Snx3*), *Nuclear receptor subfamily 2 group E member 1* (*Nr2e1*), *Osteoporosis associated transmembrane protein* (*Ostm1*), *Translocation protein SEC63 homolog* (*Sec63*), and *Sex comb on mid-leg like 4* (*Scmll4*). We also noted a predicted transcript for *Thymine DNA glycosylase* (*Tdg*) exon 1, which appears in the February 2006, but not the recently updated June 2007, version of the mouse genome on UCSC genome browser. This predicted transcript was of interest to us due to its relation to the *Tdg* gene, which lies downstream on chromosome 10 and is involved in DNA repair.

In order to verify the amplification, one gene was chosen within each BAC (*Sec63, Snx3, Ses1*), one gene (*Foxo3*) and the predicted transcript (*Tdg*) within the gap region, two cDNAs which lay just outside the amplified region (2410017P07-RIKEN cDNA, designated "241" and 5330439J01-RIKEN cDNA, designated "533") and two cDNAs which were more than 1Kb outside the amplified region (a Zinc finger, *Zbtb24*, and AK158018-RIKEN, designated "AK-1"). Verification was performed on DNA isolated from intestines of 7 to 8 CD and 7 to 8 FD BALB/c mice (3 to 4 *Mthfr*+/+ and 3 to 4 *Mthfr*+/- per diet group). The mice examined were not the same mice from which DNA was sent for aCGH. None of the genes showed significant average DNA amplification in FD compared to CD BALB/c mice. A significant increase in average DNA levels was observed for the predicted *Tdg*-exon1 transcript in 8 FD compared to 8 CD C57Bl/6 mice (**Figure 4.5B**, top panel, 4 *Mthfr*+/+ and 4 *Mthfr*+/- per diet group). We then looked at the DNA level values for individual FD mice and compared them to the average DNA level value for the CD group of the same strain. We

chose an arbitrary cut-off of a >1.8 fold increase in an FD sample compared to the CD average as being amplified. When examined in this manner, 0 to 2 out of 7 or 8 FD mice examined had a >1.8 fold increase compared to the CD average for all of the genes located outside the amplified region or within the regions covered by the BACs. In the BALB/c strain 4/8 FD mice examined had a >1.8 fold increase for the predicted *Tdg*-exon1 transcript and 7/8 FD mice examined had a >1.8 fold increase for *Foxo3* compared to the CD group average (**Figure 4.5B**, bottom panel, indicated with (1)). We repeated the experiment on different mice and this time found no FD mice with a >1.8 fold increase in the predicted *Tdg*-exon1 transcript and 3/8 FD mice with a >1.8 fold increase in *Foxo3* compared to the CD group average (**Figure 4.5B**, bottom panel, indicated with (2)). In the C57Bl/6 strain, 2/8 FD mice had a >1.8 fold increase in *Tdg*-exon1 compared to the CD group average (**Figure 4.5B**, bottom panel).

We then wished to determine if the potential DNA amplification of *Foxo3* resulted in increased gene expression. We also wondered what the expression of the full-length *Tdg* gene might be in both mouse strains. TDG enzyme functions to excise uracil from double-stranded DNA [250] and may therefore directly influence the amount of DNA damage observed in our model. We examined *Tdg* and *Foxo3* expression using mRNA isolated from intestines of 12 CD and 12 FD (6 *Mthfr*+/+ and 6 *Mthfr*+/- per diet group) BALB/c mice and 4 CD (2 *Mthfr*+/+ and 2 *Mthfr*+/-) and 7 FD (4 *Mthfr*+/+ and 3 *Mthfr*+/-) C57Bl/6 mice. These mice were not the same mice from which DNA was isolated for aCGH or QPCR verification of amplification. Average *Foxo3* mRNA expression did not differ between FD and CD BALB/c or C57Bl/6 mice (1.1±0.4 fold increase FD/CD for BALB/c and 0.7±0.2 fold increase FD/CD for C57Bl/6). When mice were examined on an individual basis, as was done for the DNA levels, 4/12 FD BALB/c mice, but no FD C57Bl/6 mice, had a >1.8 fold increase in *Foxo3* expression compared to the CD group average for that strain.

FD C57Bl/6, but not BALB/c, mice had significantly increased average *Tdg* mRNA expression compared to CD mice (**Figure 4.5C**). When examined individually 4/12 FD BALB/c and 4/7 FD C57Bl/6 mice had a >1.8 fold increase

in *Tdg* mRNA compared to the CD group average for that strain. Additionally, FD C57Bl/6, but not BALB/c mice, tended to have increased average TDG protein levels compared to CD mice (**Figure 4.5D**). This was in agreement with the average *Tdg* mRNA results. When examined individually 3/6 FD C57Bl/6, but no FD BALB/c mice, had a >1.8 fold increase in TDG protein compared to the CD group average for that strain. These data suggest that DNA repair capacity might differ between the BALB/c and C57Bl/6 strains. We therefore examined mRNA expression of *Apex1* (the mouse ortholog of APE1, the next step after TDG in base excision repair) and two genes involved in mismatch repair which are commonly known to be involved in CRC, *mut L homolog 1* (*Mlh1*) and *mut S homolog 2* (*Mlh2*) in the normal intestines of 6 CD and 6 FD (3 *Mthfr*+/+ and 3 *Mthfr*+/- per diet group) BALB/c and C57Bl/6 mice. The expression of *Apex1* was significantly increased in C57Bl/6 intestines compared to BALB/c mice, regardless of diet or genotype (**Figure 4.5C**, bottom panel). No expression differences for *Mlh1* and *Msh2* were found due to strain, diet or genotype (data not shown).

4.5 Discussion.

The expression of key genes involved in folate metabolism has never been compared between the BALB/c and C57Bl/6 strains. Based on our observation of the differential response of these strains to long-term dietary folate deficiency[335], it was therefore of interest to perform this comparison. Our observation that C57Bl/6 mice are resistant to folate deficiency induced tumorigenesis might suggest that they are able to transport more folate into the intestine and therefore, while showing signs of global folate deficiency (high plasma homocysteine), may not have localized folate deficiency in the intestine. C57Bl/6 mice, however, did not show increased expression of either *Pcft* or *Rfc1*. In fact we observed that the BALB/c strain had increased expression of both of these genes and that *Pcft* was increased in expression in FD compared to CD BALB/c, but not C57Bl/6, mice. We therefore suggest that C57Bl/6 mice do not transport more folate into the intestine than BALB/c mice. However we cannot exclude the possibility that

C57Bl/6 mice are more resistant to other factors which lead to localized folate depletion [192].

Other gene expression changes include increased expression of *Dnmt1* in BALB/c compared to C57Bl/6 mice; increased *Dnmt1* expression is frequently observed in CRC[122]. *Mat2a* expression was increased in BALB/c compared to C57Bl/6 mice. Increased *Mat2a* expression could lead to increased SAM synthesis and therefore increased DNA methylation, or it could be a consequence of decreased SAM availability and is up-regulated in order to increase SAM synthesis. Increased *Mat2a* expression in the liver, however, has been associated with increased growth and transformation[116] and *Mat2a* expression is increased in tumors compared to normal tissue[119]. *Mat2a* expression was decreased in *Mthfr*+/- compared to *Mthfr*+/+ mice. This is consistent with the hypothesis that decreased *Mthfr* expression could result in decreased DNA methylation[16] and with our recent observation of more DNA methylation decreases at specific loci in *Mthfr*+/- compared to *Mthfr*+/+ intestines by restriction landmark genomic scanning (Garcia-Crespo *et al.*, unpublished data). The decrease in *Cbs* expression in FD compared to CD C57Bl/6, but not BALB/c, mice could reflect a decrease in SAM availability under FD conditions. It is well known that SAM increases CBS activity[343; 344]. Although the effect of SAM on *CBS* transcription has not been examined, it is possible that transcriptional control could be involved in the regulation of CBS activity by SAM. We were unable to measure CBS activity in the intestine, also the amount of homocysteine in the intestine was below the threshold of detection for the method used; therefore it is difficult to interpret the *Cbs* expression results. It is intriguing, however, to suggest that there may be differences in homocysteine metabolism between the strains. This warrants further investigation.

Based on the data from our previous study, we were interested in the expression of genes involved in influencing the amount of 5,10-methyleneTHF for dTMP synthesis. Since the irreversible MTHFR reaction *in vivo* directs one-carbons exclusively towards 5-methylTHF production at the expense of 5,10-methyleneTHF, it is notable that we observed increased *Mthfr* mRNA expression

and enzyme activity in BALB/c compared to C57Bl/6 mice. Although the relative activities of the two MTHFR isoforms is unknown, it is intriguing to hypothesize that the increased activity in BALB/c mice compared to C57Bl/6 mice may be due to the more frequent expression of the 77kDa isoform in that strain. Based on these data our hypothesis that BALB/c mice may preferentially direct one-carbons towards methionine synthesis at the expense of the TS reaction is plausible. In agreement with this, a recently developed mathematical model of folate metabolism predicts that low MTHFR levels (such as in C57Bl/6 mice) increase pyrimidine synthesis[345].

There was no strain difference in *Ts* mRNA expression; TS activity was increased in BALB/c compared to C57Bl/6 mice. We suggest that increased TS activity may be a way for BALB/c mice to increase the competition of TS with MTHFR for 5,10-methyleneTHF. The increased dUTP/dTTP ratio observed in FD BALB/c mice in our previous study[335] indicates that there is insufficient substrate available for the TS reaction to form sufficient dTMP. Therefore, although the TS activity is increased, the lack of 5,10-methyleneTHF availability due to increased MTHFR activity can result in decreased dTMP synthesis.

We found increased mRNA expression of *Shmt1* and *Shmt2* in BALB/c compared to C57Bl/6 mice, but no change in SHMT2 protein levels. Although others have been able to detect SHMT1 in colon[65], we were unable to detect sufficient amounts of SHMT1 protein by western blot in the duodenum (where tumors are mostly observed in our model). The decreased serine/glycine ratio in intestines from BALB/c compared to C57Bl/6 mice could be evidence that the SHMT1 reaction may be proceeding towards the formation of 5,10-methyleneTHF, perhaps to try to provide more one-carbon groups for dTMP synthesis. Previous studies indicate that serine may be the major one-carbon donor for dTMP and methionine synthesis, but that dTMP synthesis is favored over methionine synthesis under folate-deficient conditions[346]. Recently, however, a SHMT1 knock out mouse was created. This mouse was viable and fertile, which indicates that SHMT1 may not be an essential source for one-carbon units[65]. The authors conclude that decreasing SHMT1 may increase SAM

levels. They also suggest that the effect of SHMT1 on uracil misincorporation into DNA may be dependent on the amount of SHMT1 present and on the presence of folate and choline. [65]. We are therefore only able to speculate as to the effect of *Shmt1* expression on 5,10-methyleneTHF levels, SAM levels and uracil misincorporation in our study.

We observed increased *Mthfd1* mRNA and MTHFD1 protein expression (*Mthfr*+/+ mice only) in BALB/c compared to C57Bl/6 mice. Although the MTHFD1 reaction is reversible, it is generally considered that the reaction proceeds towards the formation of 5,10-methyleneTHF in mammalian cells[78]. The MTHFD1 reaction also provides one-carbon units in the form of 10-formylTHF for *de novo* purine synthesis in adult cells which require more purines than can be synthesized by the salvage pathway alone [73]. It could therefore be hypothesized that increased MTHFD1 levels (as found in *Mthfr*+/+ BALB/c mice) could lead to increased purine synthesis in the intestine. This could provide the cells of the BALB/c intestine with a growth advantage by increasing the amount of purines for DNA synthesis. Furthermore, if the 10-formylTHF produced by the MTHFD1 reaction is being used for increased purine synthesis, there would be less of this compound available to re-form 5,10-methyleneTHF. This would further deplete the supply of that metabolite for the TS reaction. Further support for increased purine synthesis in the BALB/c intestine comes from our observation of increased *Mthfd2* mRNA and MTHFD2 protein levels in that strain compared to C57Bl/6 mice. To date, MTHFD2 has only been detected during development and in transformed cell lines, where it is necessary to maintain sufficient purine synthesis [77; 83; 84]. This is the first report to observe detectable amounts of MTHFD2 in adult tissues. The increased levels of this protein in BALB/c mice could be associated with the need for increased purine synthesis, thereby priming the intestine to support the increased growth of tumor cells.

Mtr expression was decreased (when comparing within diet groups) and *Mtrr* was increased (regardless of diet) in BALB/c compared to C57Bl/6 mice. *Mtrr* was also decreased expression in FD compared to CD normal intestines.

The MTRR protein data were consistent with the QRT-PCR results; however the MTR activity data were not. MTR activity was increased in BALB/c compared to C57Bl/6 mice; however it has been previously reported that MTR is post-transcriptionally regulated[94; 95]. MTR activity and MTRR protein levels were decreased in FD compared to CD BALB/c mice. Since MTR activity is necessary to re-form THF for 5,10-methyleneTHF synthesis, we suggest that FD BALB/c mice have further depletion of this metabolite for dTMP synthesis.

Based on the intestinal methionine data, we would suggest that BALB/c mice could have decreased DNA methylation capacity compared to C57Bl/6 mice. The TLC data, however, is evidence that, at least on a global scale, there is no difference in global DNA methylation capacity between the two strains. This does not preclude the possibility that there are biologically important differences in regional DNA methylation between the strains. This possibility is currently being investigated using restriction landmark genomic scanning results produced in the BALB/c strain. Interestingly, data from one study indicate that BALB/c mice may have a decreased SAM/SAH ratio compared to C57BL/6 mice[65]. It is therefore intriguing to suggest that BALB/c mice may have decreased DNA methylation capacity.

Most applications of the aCGH technology are employed to examine DNA copy number variations in tumors compared to adjacent normal tissue. We have employed this technology to examine the possibility that folate-deficiency induced DNA damage may be creating DNA copy number variations in pre-neoplastic intestine. We anticipated that these variations may be undetectable or at low frequency since only a few cells within the entire population of the intestine may acquire these changes. It was for this reason that we re-analyzed the data by examining the individual FD values compared to the CD group average value. Using this approach, we observed DNA amplification of the predicted *Tdg*-exon 1 transcript and *Foxo3* gene in FD compared to CD BALB/c intestines and of the predicted *Tdg*-exon 1 transcript in FD compared to CD C57Bl/6 intestines. Increased amplification potential has been previously observed in DNA repair deficient Chinese hamster ovary cells cultured in folate-

deficient media[278]. However this is the first study to show DNA amplification of a particular region due to folate deficiency alone.

FOXO3 is a transcription factor which plays various roles within the cell including oxidative stress response [347] and control of immune cell proliferation[348]. It is generally thought to be a tumor suppressor gene due to its role in increasing apoptosis[349-351] but no association has been reported between *Foxo3* and CRC. A link between FOXO3 and inflammation has been reported[348] and it is therefore interesting to hypothesize that FOXO3 may be involved in CRC growth, since inflammation is a known risk factor for CRC. Although the average DNA amplification of *Foxo3* was the largest fold increase in BALB/c mice, this did not appear to result in an increase in average mRNA expression. The observation that 4/12 FD BALB/c mice had a > 1.8 fold increase in *Foxo3* does correlate with the DNA amplification; we therefore suggest that the increased *Foxo3* expression may not be detectable in the intestine due to the small proportion of cells which may be over-expressing that gene.

We observed possible DNA amplification of the predicted *Tdg*-exon 1 transcript in FD compared to CD BALB/c and C57Bl/6 mice. Although the full-length *Tdg* gene is ~40Mb downstream of the potentially amplified region, we were interested in the possibility of increased expression of a repair gene under FD conditions. FD C57Bl/6 mice tended to have increased *Tdg* mRNA and protein levels compared to CD mice. C57Bl/6 mice also had significantly increased expression of *Apex1* compared to BALB/c mice, which cleaves the sugar-phosphate backbone at the abasic site left by TDG, so that a new base may be inserted[250]. Based on this we suggest that C57Bl/6 mice have increased ability to repair misincorporated uracil and any double-strand breaks resulting from uracil excision by increasing base excision repair. The lack of increased *Tdg* mRNA or protein levels in most FD BALB/c mice and decreased expression of *Apex1* compared to C57Bl/6 mice leads us to suggest that, by some mechanism, BALB/c mice are unable to increase base excision repair. This is similar to observations made in studies using folate and methyl deficient rats. In this model, the folate and methyl deficient diet caused increased expression of base excision

repair genes, but high levels of uracil in DNA and DNA double-strand breaks persisted. The authors suggest that folate and methyl deficiency may exceed the cell's base excision repair ability[273].

Folate deficiency may have overwhelmed the repair capacity of the BALB/c intestine, even in the 4 BALB/c mice which did have a >1.8 fold increase in expression of *Tdg*. Decreased DNA repair capacity in BALB/c mice is consistent with reports in the literature which show that BALB/c mice have reduced ability to re-join radiation induced double-strand breaks compared to C57Bl/6 mice, possibly due to decreased expression of DNA protein kinase C (necessary for V(D)J recombination repair)[352]. The repair deficiency in BALB/c mice could increase the amount of DNA double-strand breaks; as observed in FD mice[335] and may be the underlying cause of the tumor formation in this strain. Based on data where increased promoter methylation of *Tdg* and subsequent decreased *Tdg* expression was observed in multiple myeloma cell lines compared to normal plasma cells[353], we hypothesize that regional DNA promoter hypermethylation may be the cause behind the lack of *Tdg* increase in most FD BALB/c mice. We are currently investigating this possibility using bisulfite sequencing.

In conclusion, increased MTHFR activity in BALB/c compared to C57Bl/6 mice may be depleting the supply of 5,10-methyleneTHF for dTMP synthesis in that strain, resulting in the increased dUTP/dTTP ratio previously observed. Increased MTHFD1 and MTHFD2 levels in BALB/c compared to C57Bl/6 mice may also provide the intestine with a growth advantage by increasing purine synthesis. Increased DNA damage due to folate deficiency in both strains of mice may lead to chromosomal abnormalities such as DNA amplification. C57Bl/6 mice, however, may be able to increase the expression of a repair genes in order to repair that damage. This may be the mechanism behind the resistance to tumor formation in FD mice of this strain.

4.6 Acknowledgements.

This work was supported by the Canadian Institutes of Health Research (CIHR) to R.R. E.K. is the recipient of a studentship from the Fonds de Recherche en Santé du Québec. The authors would like to thank the following people for the generous gift of antibodies: Dr. Robert Mackenzie (McGill University), Dr. Patrick Stover (Cornell University) and Dr. Marc Tini (University of Western Ontario).

CHAPTER V

General Discussion

5.1 Model of spontaneous intestinal tumors

This thesis describes the discovery and characterization of a novel model of environmentally induced intestinal tumorigenesis. BALB/c mice fed a folate-deficient diet for 1 year developed intestinal tumors displaying a progression which mirrors that of human CRCs with the formation of polyps, then adenomas, adenocarcinomas and finally invasion of the submucosa with potential for metastasis[165]. There are two noteworthy aspects of this model. First, it is the first model to show that folate deficiency alone in non-transgenic mice or through the use of carcinogen injection, is sufficient to induce intestinal tumors. In this respect this model is more applicable than the rat model of folate and methyl group deficiency induced liver tumor formation, since intestinal tumors are formed in this case. Secondly, this model is applicable to the estimated 85% of environmentally induced cases of CRC, as opposed to the estimated 15% of cases due to genetic predispositions, to which genetic animal models of intestinal cancer apply.

Evidence from epidemiological studies in humans has often been conflicting as to whether folate deficiency promotes or prevents CRC (reviewed in [2]). The human data can be difficult to interpret given the large number of confounding factors which can influence CRC and which may not all necessarily be taken into account in the published studies. Using the model of folate-deficiency induced intestinal tumorigenesis presented here, we had the advantage of being able to control for genetic background by the use of inbred mouse strains, folate intake, and, to some extent, environmental conditions. All mice were housed in the same conditions throughout the study. Tissues from each group in an experiment were processed together and run together whenever possible to minimize experimental variation. This model is therefore free of the confounding factors present in the human studies and enables us to make a direct link between folate deficiency and intestinal tumor formation.

Information from our model can also be gained when compared to data obtained from other animal models. In a recent study from our lab, *Apc*$^{min/+}$ mouse mothers were placed on an amino acid controlled, folate-deficient, or folic

acid-enriched diet during pregnancy and nursing and the offspring placed on the same diet as the mother until killed. The offspring of folate-deficient diet fed mothers had fewer tumors than offspring of control diet fed mothers. There was no difference in tumor number between the control and folic acid-enriched diet groups. In a further study, the three diets were given to $Apc^{min/+}$ mice at weaning whose mothers had previously been fed standard mouse chow. The mice fed the folic acid-enriched diet developed significantly more tumors than mice fed a control diet at weaning. There was no difference in tumor number between the control and folate-deficient diet groups[354]. From this study it can be concluded that folate deficiency may be protective while folate supplementation may promote tumor formation in cases where a pre-disposing genetic mutation is present. The studies presented in this thesis provide evidence to suggest that in cases where a hereditary pre-disposition is not present folate deficiency promotes tumor formation. Therefore when considering the effect of folate deficiency on tumor development, genetic background must be taken into account.

Other studies using the $Apc^{min/+}$ mouse or carcinogen injection models have shown that folate deficiency initiated after the formation of pre-neoplastic lesions (polyps, adenomas) reduces tumor formation but if initiated before the formation of pre-neoplastic lesions, tumor formation is increased[194-196; 200]. These data appear to coincide with the hypothesis that folic acid fortification is correlated with increasing CRC rates in North America due to the high proportion of the population with pre-existing colorectal polyps or adenomas[158]. The model presented in this thesis represents the effect of folate deficiency initiated before pre-neoplastic lesions appear and reflects human cases where lifetime intake of dietary folate is low. This model is in agreement with a recent mathematical model of the effects of folate supplementation on the intestine which predicts that folate supplementation may only be protective in people under the age of 20 (presumed to have no pre-disposing lesions)[193].

There are several limitations of this model. First, it is difficult to directly compare data from a mouse model to humans. Notably, most mouse models of CRC develop intestinal and not colonic tumors. This may be due to several

factors including, but not limited to, rodent coprophagy[172], differences in the populations of colonic microflora in rodents and humans [172], and the different proliferation rates of rodent and human intestinal epithelia[161]. We also chose a diet with a severe degree of folate-deficiency which may not reflect the levels of folate-deficiency observed in humans. We chose this level of folate-deficiency in order to maximize our chances of observing any effects. It is still plausible, however, that similar changes could be seen using a more moderate form of folate-deficiency.

Secondly, this model requires at least 8 months (our earliest observed adenoma) on the diet before tumors are observed, which may not be cost or space effective. Additionally, only 1 to 3 tumors will develop per mouse ranging in size from 0.5mm-2mm in diameter. Therefore limited tumor sample is available and experiments must be planned accordingly. Thirdly, due to space considerations and technical limitations, we were only able to obtain limited numbers of mice per group. Finally, it would be expected, given our control of environmental conditions, that if folate deficiency causes intestinal tumor formation we would observe tumors in all of our FD mice. This was not the case and may be reflective of the importance of other environmental factors in CRC progression which we could not control. For example, although all mice were housed in similar conditions some small variations existed such as the presence of plastic toys, of which pieces were sometimes found in the intestines of dissected mice. We could also not control for the level of physical activity of the mice, some of which may have been more active than others; physical activity being well known to reduce the risk for CRC (recently reviewed in[355]). Additionally, we had periodic outbreaks of infection, followed by periods of treatment, which may or may not have impacted the intestinal health of our mice. All of these factors, however, affected both CD and FD mice equally since they were housed in the animal facility at the same period of time and were of the same age, often being littermates. Therefore we conclude that, while the above mentioned factors may have affected the incidence of tumors, folate deficiency alone remained the primary variable which induced tumor formation.

One aspect of our model which we did not investigate was the potential involvement of genes known to be mutated in CRC. While we did not find any expression differences in *Mlh1* and *Msh2*, two genes known to be involved in HNPCC, we did not examine enzyme activity or look for possible mutations. We also examined the expression of *Trp53* and *c-Myc* in BALB/c intestines (Garcia-Crespo *et al.*, unpublished data), but found no expression changes due to diet or genotype. The commonly mutated exons (5, 6, 7 and 8) of the *Trp53* gene were successfully sequenced but no variations found in intestines from FD compared to CD BALB/c mice. Sequencing of *Trp53* in laser capture microdissected tumors was unsuccessful. Examination of the levels of *Apc*, *Igf 2*, *β-catenin*, *Dcc* and other genes known to be involved in CRC is warranted.

5.2 Mechanisms

In this thesis several mechanisms are proposed to explain how dietary folate deficiency induces intestinal cells to acquire a growth advantage and initiate tumor formation: uracil misincorporation, DNA damage checkpoint control, DNA damage repair and alterations in DNA methylation.

FD BALB/c mice had increased dUTP/dTTP ratios in the pre-neoplastic intestine compared to CD mice, while this change was not observed in the intestines of the tumor resistant C57Bl/6 mice. It is well known that increased uracil levels can cause DNA dsbs[250], therefore it was not surprising to observe a trend towards an increased percentage of p-H2AX nuclear foci in intestines from FD compared to CD BALB/c mice, but no change in the intestines of tumor resistant C57Bl/6 mice. It could therefore be concluded that increased uracil levels may promote DNA damage which could initiate tumor formation in the susceptible BALB/c strain. More direct and quantitative measures of uracil misincorporation and DNA double-strand breaks are available which do not have the problems associated with immunofluorescent staining (non-specific antibodies, autofluorescence etc), the comet assay, for example. Using this assay it would have been possible to digest isolated DNA with uracil DNA glycosylase and directly measure the amount of DNA dsbs (represented by the "comet" tail

length) caused by uracil in DNA. This technique, however, requires the use of live, dividing, cultured cells, which we were not able to obtain from the intestines of our mice. Alternatively, it would be of interest to develop a method for scraping of intestinal epithelial cells for study. The intestine is a multi-layered organ and perhaps more marked changes would be observed if only the proliferating epithelial layer was examined without contaminating effects from other tissues.

We attempted to use a cell culture model of folate deficiency induced tumor formation by incubating Caco-2 human intestinal carcinoma cells in control and folate-deficient media. Given that these cells were already transformed, however, the effects of folate deficiency on this line were only applicable to cancer cells and did not give any information as to the effects of folate deficiency on untransformed intestinal cells. It would be of interest in the future to create primary cultures of intestinal epithelia from the BALB/c and C57Bl/6 mouse strains or to use existing minimally transformed rat intestinal epithelial cell lines.

The DNA damage detected in the intestines of FD BALB/c mice appears to lead to G_2-M checkpoint arrest, as evidenced by decreased immunostaining and expression of PLK1 and CDC25c and increased immunostaining for p-CDC2. Ideally flow cytometry methods would be used to detect the proportion of cells arrested at each stage of the cell cycle. Again this technique requires live, dividing, cell cultures which we could not obtain. Cells will not proceed to mitosis, however, when CDC remains phosphorylated and inactive[261], therefore p-CDC staining, along with the PLK1 and CDC25c expression, provides sufficient evidence of G_2-M arrest.

The increase in PLK1, CDC25c and p-CDC observed in tumor tissue led us to hypothesize that there is pressure put on the intestinal cells of the FD BALB/c mice by the increased DNA damage. This creates the opportunity for some cells to escape G_2-M checkpoint arrest and proceed to mitosis with persisting DNA damage. This would give those cells a growth advantage and possibly lead to tumor formation. As of yet, we have not determined the mechanism behind the decrease in PLK1 and CDC25c observed in the intestine

and subsequent increase in tumor tissue from FD BALB/c mice. A cursory examination of DNA methylation in the promoter regions of both genes was undertaken using quantitative analysis of methylation by PCR (QAMP)[356], but no evidence of DNA methylation differences was found. It therefore remains unanswered whether the changes observed in Plk1 and Cdc25c are a cause of tumor formation or a consequence of some other transforming event. Further studies of proteins known to influence *Plk1* and *Cdc25c* expression are warranted.

Further evidence as to the role of DNA damage in intestinal tumor formation comes from our observation that the tumor resistant C57Bl/6 strain was able to increase the expression of *Tdg* in intestines of FD compared to CD mice while the tumor susceptible BALB/c strain was not. This led us to hypothesize that, while the FD diet may cause some uracil misincorporation into DNA, the cells of the C57Bl/6 intestine are able to mount a sufficient base excision repair response to remove the uracil and prevent DNA dsbs. This might explain why no evidence of G_2-M checkpoint arrest was observed in the intestines of FD C57Bl/6 mice; there was no damage to repair. In BALB/c mice however, the decreased expression of the G_2-M checkpoint regulators may have been sufficient to arrest the cells in order for repair to occur, but subsequent initiation of that repair may have failed. It is of interest to determine the activity of the TDG protein in the intestines of our mouse models. It would also be of interest to examine the expression of other key proteins involved in base excision repair such as DNA polymerase beta (the rate limiting step)[250] and uracil DNA glycosylase, which is thought to be the enzyme responsible for excising uracil misincorporated during DNA replication[250]. Finally, it would be of interest to determine the overall base excision repair capacity in extracts from BALB/c and C57Bl/6 intestines.

Global DNA methylation tended to be decreased in intestines from FD compared to CD BALB/c, but not C57Bl/6 mice. Decreased intestinal methionine was also observed in BALB/c compared to C57Bl/6 mice. We hypothesized that this creates an environment favorable to tumor formation in the BALB/c intestine by providing fewer methyl groups for DNA methylation reactions. This hypothesis, however, was not supported by the TLC data directly comparing the

BALB/c and C57Bl/6 strains and leaves the question as to whether localized changes in DNA methylation contribute to the strain differences in tumor formation. To address this question, restriction landmark genomic scanning (RLGS) has been employed in our lab in order to compare localized DNA methylation changes between intestines from FD compared to CD BALB/c mice. Some loci were found which were differentially methylated in some samples due to diet, genotype or both and which resulted in gene expression changes. We checked the methylation status at the same Not1 sites from the RLGS profile that were found to be changed by methylation in intestines of FD and CD C57Bl/6 mice by QAMP. Although QAMP results generally correlate well with RLGS results, it was difficult to compare the percent methylation at a particular locus between the strains due to the high variability of methylation between samples (i.e. some samples may display 0%, some 50% and some 100% methylation at the same locus). We therefore cannot currently conclude whether these loci display differential methylation between the strains. RLGS comparing intestines from CD and FD C57Bl/6 mice is currently underway to address this.

The issue of DNA methylation as a cause or consequence of tumor formation is a hotly debated issue[130]. In our model of folate deficiency induced tumor formation, we hypothesize that a DNA methylation change may be a consequence of tumor formation and that the changes observed in FD compared to CD BALB/c mice are a consequence of the increased DNA damage. Support for this hypothesis comes from studies in the literature which show that, in constructs made to contain unusual DNA structures paired with a cytosine, DNMT1 was more likely to methylate that cytosine if the matched base was something other than guanine such as inosine, *O6*-methylguanine, cytosine, adenosine, an abasic site, or a hanging 3'OH group[132]. This has led some to suggest that DNMT1 may have originally functioned as a repair enzyme and that, under conditions of increased DNA damage, the affinity of DNMT1 for the damaged sites prevents it from maintaining global DNA methylation patterns while targeting damaged sites for hypermethylation[132]. In BALB/c mice we observed increased expression of *Dnmt1* and a trend towards increased

dUTP/dTTP and DNA dsbs compared to C57Bl/6 mice. It could therefore be hypothesized that, in BALB/c mice, DNMT1 is sequestered to sites of DNA damage, leading to global DNA hypomethylation and areas of regional hypermethylation. This may be supported by our observation that there were more RLGS spot changes due to hypermethylation (26) than due to hypomethylation (18) (Garcia-Crespo et al., unpublished data). It would be experimentally difficult to prove, however, that DNMT1 is bound more often to damaged DNA in BALB/c intestines than in C57Bl/6 intestines. Therefore this hypothesis remains purely in the realm of speculation.

5.3 MTHFR and tumor formation.

One of the objectives of this thesis was to determine the impact of the *Mthfr* null allele in conjunction with folate deficiency on tumor formation. We observed an overall increased incidence of tumors in *Mthfr*+/- mice compared to +/+ mice. It is hypothesized that, under conditions of folate deficiency, decreased MTHFR levels would lead to decreased 5-methylTHF and therefore adversely affect DNA methylation[16]. We did not observe any changes in global DNA methylation due to *Mthfr* genotype; however some loci from the RLGS profile were found to be changed due to *Mthfr* genotype (Garcia-Crespo *et al.*, unpublished data). Therefore it may be that DNA methylation plays a role in the modulatory effects of *Mthfr* genotype on tumor number. Decreased MTHFR levels are also hypothesized to increase the synthesis of dTMP by sparing more 5,10-methyleneTHF for the TS reaction, a mechanism which may be protective against tumor formation under conditions of sufficient folate[16]. After running additional samples and adding the results to the data in chapter III (**Table 3.1**), we were able to detect a significant decrease in dUTP/dTTP ratio in the intestines of FD BALB/c mice only due to *Mthfr* genotype (from 2.9±0.2 in *Mthfr*+/+ to 2.1±0.1 in *Mthfr*+/- mice, n=10 per diet/genotype group $p<0.05$ by t-test). In one study from our lab using an MTHFR overexpressing transgenic model, increased dUTP/dTTP ratios were seen in the spleens of MTHFR transgenic versus wild-type mice after methotrexate injection[357]. Based on this, we believe there is

evidence to suggest that *Mthfr* genotype does affect dTMP synthesis; however such a mechanism should be protective against tumor formation in our model. Unfortunately due to the limited availability of fixed tissue for staining, we were not able to examine sufficient numbers of *Mthfr*+/- mice to make any determination as to the effect of genotype on p-H2AX, PLK1, CDC25c or p-CDC2 staining. Further examination of the effects of *Mthfr* genotype on DNA damage is therefore needed. It is clear, however, that even if the *Mthfr*+/- genotype does result in reduced DNA damage it also results in increased tumor formation in FD mice. We suggest that DNA damage is still the critical tumor initiator in *Mthfr*+/+ and +/- mice, but as yet undefined alterations in regional DNA methylation in *Mthfr*+/- mice promote increased tumor formation over *Mthfr*+/+ mice.

5.4 Betaine supplementation.

One objective of this thesis was to determine if betaine supplementation to the folate-deficient diet would provide sufficient one-carbon units to correct the decreased global DNA methylation and influence tumor formation in FD BALB/c mice. We can conclude from our observation that betaine supplementation lowered plasma homocysteine but did not influence tumor number and that high plasma homocysteine may not play a tumor promoting role in our model. The levels of intestinal homocysteine were below the level of detecting for the method used, therefore we cannot conclude as to the effect of the betaine supplementation on intestinal homocysteine levels. High intestinal homocysteine may therefore still play a role in tumor promotion in the intestine. High plasma homocysteine, however, cannot be discounted as a risk factor for tumor formation in all CRCs; it is still well known to be associated with increased inflammation and oxidative stress which can promote tumor formation[88; 89]. Although dietary betaine supplementation may be effective for patients with cancers in tissues where BHMT is expressed (liver, kidney), sufficient 5-methylTHF may not be produced for effects of supplementation to be seen in other tissues (i.e. intestine). We are confident that the dose of betaine used in our study (2.93 g/Kg diet) is sufficient

to induce physiological changes based on previous data from our lab demonstrating that this dose was sufficient to correct defects observed in *Mthfr*-/- mice [112] and that increasing the dose beyond this point did not produce any additional benefits[111].

5.5 Strain differences in folate metabolism.

We found many differences in the levels and activities of enzymes involved in folate metabolism between the susceptible BALB/c and resistant C57Bl/6 strain. One limit of this comparison was the lack of available tissue folate measurements, which might have provided further evidence to support our claim of decreased 5,10-methyleneTHF availability in BALB/c compared to C57Bl/6 mice. We are also unable, with currently available methods, to accurately determine whether SHMT1 is favoring 5,10-methyleneTHF or THF synthesis and therefore whether increased *Shmt1* expression has any effect on one-carbon distribution in our model. Unfortunately, due to the high similarity between SHMT1 and 2 activities, it would be impossible to measure SHMT1 activity alone. The decreased tissue methionine levels in BALB/c mice, despite increased MTR activity, can be explained by the fact that methionine is used for many cellular reactions, including protein synthesis; therefore free intestinal methionine may not be the best indicator of methylation capacity. Intestinal SAM/SAH ratios would give a better measurement of methylation capacity, although these measurements could not be obtained.

It is interesting to compare the results of this study to those obtained in a study by Hayashi *et al.* (2007)[358] using two human colorectal cancer cell models. HCT116 cells are known to be repair-deficient and are *MTHFR* 677TT, Caco-2 cells are known to be repair-sufficient and are *MTHFR* 677CC. Both HCT116 and Caco2 cells cultured in folate-deficient media had increased expression of *Dnmt1* and *Fpgs* and decreased expression of *Dhfr*, *Mtr*, *Mtrr* and *Dnmt3a* and *b* compared to cells cultured in folate-sufficient media. Changes unique to HCT116 cells cultured in folate-deficient media included increased expression of *Folate Receptor alpha*, and *Mthfr* and decreased expression of *Rfc1*, *Gamma-glutamyl*

Hydrolase, *Ts*, and *Shmt1*. Changes unique to Caco-2 cells cultured in folate-deficient media included increased expression of *Shmt1* and decreased expression of *Folate Receptor alpha*, and *Mthfr*. The authors concluded that, due to the increased *Mthfr* and decreased *Shmt1* and *Ts* expression, HCT116 cells preferentially shuttle one-carbon units towards SAM synthesis at the expense of dTMP synthesis. Caco-2 cells, based on the decreased *Mthfr* and increased *Shmt1* expression, preferentially shuttle one-carbon units towards nucleotide synthesis at the expense of DNA methylation reactions [358]. If we compare our expression results to those mentioned above, it is interesting to note that we draw similar conclusions; BALB/c mice (like HCT116 cells) are repair deficient with increased *Mthfr* levels and therefore preferentially shuttle one-carbon units towards DNA methylation reactions. C57Bl/6 mice (like Caco-2 cells) are repair sufficient with decreased *Mthfr* levels and therefore preferentially shuttle one-carbon units towards dTMP synthesis. A notable difference is the *Shmt1* expression results, which were opposite of the *Mthfr* expression results in the study by Hayashi *et al.*, (2007)[358], but were in the same direction as the *Mthfr* expression results in our study. Also, Hayashi *et al.* (2007) found that in the cell line with increased *Mthfr* expression, *Ts* expression was decreased[358] while we found no change in *Ts* expression between the strains but increased TS activity in BALB/c mice. Very direct comparisons of our results to those in Hayashi *et al.*, (2007)[358] are difficult, since that study involved cancer cells in culture and we are examining untransformed intestinal tissue, but the comparison is intriguing nonetheless.

We can not exclude the possibility that genetic differences outside of the folate metabolic pathway are responsible for the strain differences observed in tumor formation. It would therefore be of interest to further examine the genetic differences between the BALB/c and C57Bl/6 strains starting with global gene expression measurements, such as microarray analysis. Further investigation into the differences between these two commonly used mouse strains would be of great benefit as strain differences have been reported for susceptibility to other complex diseases, such as arteriosclerosis[359].

5.6 Folate and CRC- Model.

The data presented in this thesis provide further insight into the role of folate deficiency in influencing cancer risk and the underlying mechanisms behind it. Based on our observations, we can propose the following model.

In humans with no hereditary predisposition to CRC and no pre-disposing lesions (such as polyps or adenomas), dietary folate deficiency can initiate intestinal tumor formation, as is the case in BALB/c mice. If hereditary predispositions (such as *APC* mutations) or pre-disposing lesions are present, dietary folate deficiency may be protective against CRC progression. Dietary folate deficiency initiates tumor formation in untransformed cells through a lack of one-carbon units for dTMP synthesis, leading to increased dUMP levels which may be misincorporated into DNA as dUTP resulting in DNA dsbs. The increased DNA damage coincides with decreases in global DNA methylation with potential increases in regional DNA methylation. The level of MTHFR activity may influence an individual's susceptibility to CRC. Decreased MTHFR activity may increase susceptibility when dietary folate intake is low (such as in FD BALB/c *Mthfr*+/- mice), possibly due to decreased availability of one-carbons for DNA methylation reactions. Conversely, a greater than average MTHFR activity may also increase susceptibility (such as in BALB/c compared to C57Bl/6 mice), possibly due to decreased availability of one-carbons for dTMP synthesis. Additionally, the ability of the cells of the intestine to mount a DNA repair response may also affect susceptibility to folate deficiency induced CRC. Those whose cells can increase base excision repair (like C57Bl/6 mice) may be less susceptible than those whose cells cannot (like BALB/c mice).

5.7 Folate and CRC-Implications.

The effects of folic acid supplementation on reducing the rate of folate insufficiency in adults and in reducing the incidence of neural tube defects is well established[143; 144; 149]. The incidence of neural tube defects is now estimated to be at around 1 for every 1000 births in North America[360], however the incidence of CRC is estimated to be about 1 in 20 [164]. Furthermore, about 50% of the

population is estimated to have pre-neoplastic lesions (polyps and/or adenomas) by age 50[2]. If folic acid promotes the progression of pre-neoplastic lesions, the question is raised as to whether supplementation is beneficial to the entire population or merely to a subset. This thesis provides evidence that folate supplementation may be beneficial to those without hereditary predisposition or pre-disposing lesions. This would imply that, in terms of cancer prevention, fortification may only be beneficial to the young. It might therefore be suggested that folic acid supplementation should be targeted to those groups who would derive the most benefit, for example youth under the age of 20 and expectant mothers. This suggestion will have to be weighed, however, against the benefits of supplementation to the general population. Plasma homocysteine levels have decreased since fortification[140]; supplementation may therefore decrease the population risk for other major health risks such as cardiovascular disease.

5.8 Claims to originality.

1. BALB/c mice develop an overall 25% incidence of intestinal tumors after 1 year on a folate-deficient diet with *Mthfr*+/- mice developing more tumors than *Mthfr*+/+ mice. C57Bl/6 mice, both *Mthfr*+/+ and +/-, placed on the same control and folate-deficient diets as BALB/c mice do not develop intestinal tumors
2. Plk1 and Cdc25c are decreased while p-CDC2 is increased in intestines from FD compared to CD BALB/c mice. Plk1 and Cdc25c are increased while p-CDC2 is decreased in tumors compared to normal intestines from FD BALB/c mice. Plk1 and Cdc25c are not decreased and p-CDC2 is not increased in intestines from FD compared to CD C57Bl/6 mice.
3. The percent of p-H2AX nuclear foci and dUTP/dTTP ratio increased in intestines from FD compared to CD BALB/c mice, but not in intestines from FD compared to CD C57Bl/6 mice. Global DNA methylation results suggest a trend towards decreased methylation in intestines from FD compared to CD BALB/c mice ($p=0.06$), but not in intestines from FD compared to CD C57Bl/6 mice
4. Betaine supplementation to the folate-deficient diet does not reduce tumor formation in BALB/c mice. The betaine supplementation decreased plasma homocysteine, but did not increase DNA methylation, decrease the dUTP/dTTP ratio or percent p-H2AX nuclear foci, or alter the expression of *Plk*1 and *Cdc25*c in intestines from FD compared to CD BALB/c mice.
5. BALB/c mice have increased mRNA expression of *Dnmt1*, *Mat2a*, *Mthfd1*, *Mthfd2*, *Mthfr*, *Mtrr*, *Rfc1*, *Shmt1* and *Shmt2* and decreased expression of *Mtr* in the intestine compared to C57Bl/6 mice, regardless of diet or genotype.
6. The enzyme activities of MTHFR and TS are increased in intestines from BALB/c compared to C57Bl/6 mice.
7. The protein levels of MTHFD1 (in *Mthfr*+/+ mice only), MTHFD2 and MTRR (regardless of diet or genotype) are increased in intestines from BALB/c compared to C57Bl/6 mice.
8. *Foxo3* DNA may be amplified in intestines from FD compared to CD BALB/c mice. *Foxo3* mRNA may be increased in some intestines from FD compared to

CD BALB/c mice, but is not increased in intestines from FD compared to CD C57Bl/6 mice.

9. *Tdg* mRNA is increased in 4/12 intestines from FD compared to CD BALB/c mice but there is no change in the TDG protein level. *Tdg* mRNA and protein is increased in intestines from FD compared to CD C57Bl/6 mice.

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APPENDIX I

Supplementary data

Supplementary Table 4.1 Primers and PCR conditions

Gene	Forward and reverse primers 5'→3'	°C	Amplicon size (bp)	$MgCl_2$ (µl/ reaction)
Mthfr	TCTTTGAGGCCAGCACCTTCTTCA	60	119	1.5
	TACAAGCTGCCGAAGGGAAGTGTA			
Ts	TAAACAGCCATTTCCATTTTAATC	56	127	3
	AACCAAGACCTTTCCCAAAGC			
Shmt1	AGAAGATCCAGAGTGCTGTGGCAA	60	103	0
	ACAGGCTGTTCCTGTCAGAAGTCA			
Shmt2	TGGCTTGTCTCTGGGTCTTT	58	226	0
	TCCGTAGAGTCGTCGATTTTA			
Mthfd1	GGATGCAATCAAGCCCAACCTCAT	60	95	0
	AGGAATTCCCGTGTGCAATGTTGG			
Mthfd2	AATCCCGCCAGTCACTCCTATGTT	60	107	0
	AGCTCTTCCTCTGAAACTGAGGCT			
Mtr	ACGTACCCCTAAGTCAAGCCAGAA	62	400	0
	TGGACACTCTGTGCTGGCCAGAAT			
Mtrr	ATCAATCCACGGCTCTACCA	58	124	3
	AGAATACACATACTTCTGCAAT			
Rfc1	ATCTGGGTGTGCTACGTGACCTTT	60	101	0
	AGCTCTTTAGACAGGGAAGACGCA			
Pcft	CACCCGCCACCGGGAGAA	60	109	0
	AAGCCTCCCACGTTCATGTA			
Tdg	CGCGTTTAAAGTGAAAAGGAAA	62	150	0
	TCCTTTGTAAGCAGCCATTAAT			
Foxo3	CGGCTCTTGGTGTACTTGTT	62	136	1.5
	GCTTCATGCGCGTTCAGAAT			
Mat2a	GCTAAAGTGGCTTGTGAAACTGT	58	151	0
	TTCTTCCAAAGGGTTTGACTACA			
Cbs	CTTCTCCGGCATCACGATAA	62	106	3
	TTCGGATGATCGAAGATGCT			

Apex1	CAGTGCCCGCTAAAAGTCTC	62	100	0
	TCATGTTCTTCCTCGCCTAT			
Mlh1	CAAGCATCTCCGCTTTCTTC	56	108	0
	TGCTGGCCTTAGACAGTCCT			
Msh2	ACGAAGATTGGTGCCTTTTG	64	112	0
	CCTCAGTAGTGAGCGCTGTG			

APPENDIX II

Compliance forms