METABOLIC MECHANISMS UNDERLYING FOLATE-RESPONSIVE DEVELOPMENTAL ANOMALIES

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Neural tube closure defects (NTDs) are common developmental anomalies that result from failure of the embryonic neural tube to bend and fuse during development, resulting in herniation and deterioration of nervous system tissue. Low folate status is an important environmental determinant of NTD risk, and maternal folate supplementation can prevent the occurrence of NTDs by 50-70%, but the mechanisms remain unknown. Folates function to carry and activate methyl groups for a set of anabolic reactions collectively known as one-carbon metabolism (OCM). OCM is required for the de novo biosynthesis of purines and thymylate, and for the remethylation of homocysteine to methionine. Methionine can be adenosylated to form S-adenosylmethionine, which is the universal methyl donor for cellular methylation reactions, including chromatin methylation. Impairments in OCM result in elevated homocysteine, reduced proliferation, genomic instability, and chromatin hypomethylation.

Evidence from human genetic studies indicates that NTDs are complex traits that arise from deleterious gene-nutrient interactions, although human gene candidates that account for population-wide risk have not yet been identified. In these studies, we explore NTDs in response to disruption of two folate-dependent genes, cytoplasmic serine hydroxymethyltransferase (*Shmt1*) and the gene encoding the trifunctional

enzyme MTHFD1 (*Mthfd1*). SHMT1 regulates the partitioning of one-carbons by prioritizing thymidylate biosynthesis at the expense of cellular methylation. MTHFD1 catalyzes the synthesis of 10-formylTHF, which is required for de novo purine biosynthesis. Disruption of *Shmt1* resulted in NTDs in mice that mimicked folate-responsive NTDs in humans. This is first gene within folate metabolism that when disrupted causes NTDs in mice. *Shmt1* disruption in the *splotch* mutant, a folate-responsive NTD model with impaired thymidylate biosynthesis, exacerbated and increased the frequency of NTDs, further implicating impaired thymidylate biosynthesis in NTD pathogenesis. Similarly, disruption of MTHFD1 resulted in developmental anomalies associated with the maternal, not fetal, genotype, as observed in humans. Supplementation of *Mthfd1*-deficient dams with hypoxanthine also revealed NTDs in *Mthfd1*-deficient embryos. These data implicate impaired nucleotide biosynthesis in NTD pathogenesis and highlight two new mouse models to study mechanisms underlying folate-responsive developmental anomalies.

BIOGRAPHICAL SKETCH

Anna Elisse Beaudin was born on September 4, 1978, to Jimmy and Stephanie Pflaster in Santa Monica, California. After graduating from Santa Monica High School in 1996, Anna moved to Ithaca, NY to attend Cornell University. At Cornell University, Anna majored in Biopsychology and participated in research in the laboratory of Dr. Barbara Strupp investigating the enduring cognitive effects of early lead (Pb) exposure. She completed an undergraduate honors thesis on the same topic and graduated Magna cum Laude in Psychology and with Distinction in all other subjects in May of 2000. In 2001, following a brief stint as a research assistant in the laboratory of Dr. Tim DeVoogd in the department of Psychology at Cornell, she entered the graduate program in Psychology at Brown University under the supervision of Dr. Rebecca Burwell, studying the functional neuroanatomy of the parahippocampal region. After attaining a Masters in Science, Anna decided she wanted to engage in research that was more applied to human health and disease, and so chose to pursue her graduate degree in a different field, returning to Cornell University where she joined the laboratory of Dr. Patrick Stover in Nutritional Sciences. On August 10, 2003, Anna married the love of her life, Stéphane Beaudin, in Santa Monica, California. Their son Henri Julien Beaudin was born on September 21, 2008. Anna's beloved father, Jimmy Pflaster, passed away on January 1, 2009 after a courageous battle with melanoma.

For my Papa, who always pushed me to shoot for the stars and taught me everything I know about biochemistry.

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LIST OF ABBREVIATIONS

AdoMet: S-adenosylmethionine

AdoHcy: S-adenosylhomocysteine

ATP: adenosine triphosphate

BHMT: betaine-homocysteine methyltransferase

CBS: cystathionine beta-synthase

DHFR: dihydrofolate reductase

DNA: deoxyribonucleic acid

FPGS: folylpolyglutamate synthetase

FR: folate receptor

GTP: guanosine triphosphate

α-MEM: minimal essential media, alpha modification

MTHFR: methylenetetrahydrofolate reductase

MTHFS: methenyltetrahydrofolate synthetase

NADPH: nicotinamide adenine dinucleotide phosphate

PBS: phosphate-buffered saline

RFC: reduced folate carrier

RNA: ribonucleic acid

SHMT: serine hydroxymethyltransferase

SNP: single nucleotide polymorphism

THF: tetrahydrofolate

TYMS, TS: thymidylate synthase

CHAPTER 1

FOLATE MEDIATED ONE CARBON METABOLISM AND NEURAL TUBE DEFECTS – BALANCING GENOME SYNTHESIS AND GENE EXPRESSION

Abstract

Neural tube defects (NTDs) refer to a cluster of neurodevelopmental conditions associated with failure of neural tube closure during embryonic development. Worldwide prevalence of NTDs ranges from approximately 0.5 to 60 per 10,000 births, with regional and population-specific variation in prevalence. Numerous environmental and genetic influences contribute to NTD etiology; accumulating evidence from population-based studies has demonstrated that folate status is a significant determinant of NTD risk. Folate-mediated one-carbon metabolism is essential for *de novo* nucleotide biosynthesis, methionine biosynthesis, and cellular methylation reactions. Periconceptional maternal supplementation with folic acid can prevent occurrence of NTDs in the general population by up to 70%; currently several countries fortify their food supply with folic acid for the prevention of NTDs. Despite the unambiguous impact of folate status on NTD risk, the mechanism by which folic acid protects against NTDs remains unknown. Identification of the mechanism by which folate status affects neural tube closure will assist in developing more efficacious and better targeted preventative measures. In this review, we summarize current research on the relationship between folate status and NTDs, with an emphasis on linking genetic variation, folate nutriture, and specific metabolic and/or genomic pathways that intersect to determine NTD outcomes.

Introduction

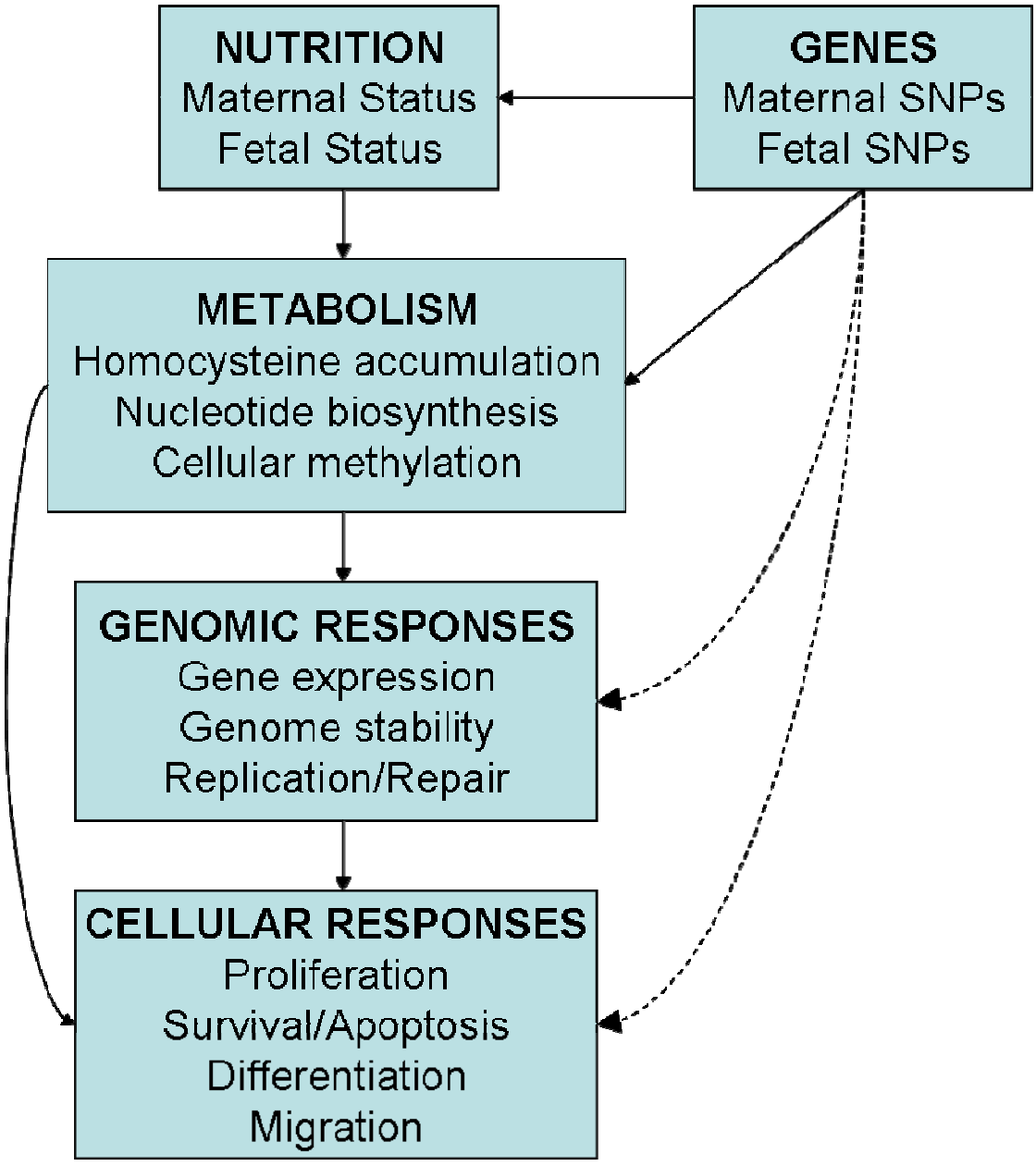
Failure of the embryonic neural tube to close completely during development results in neural tube closure defects (NTDs). NTDs are among the most common congenital birth defects, with worldwide prevalence ranging from 0.5 to 60 per 10,000 births (1). The most common and most severe NTDs include spina bifida, caused by failure of posterior neural tube closure resulting in exposure and/or herniation of the spinal chord, and anencephaly, characterized by the absence of the cranial vault and brain as the result of failure of anterior neural tube closure. Maternal supplementation with folic acid is the most effective measure known to prevent NTDs; it has been estimated that up to 70% of NTDs can be prevented by folate supplementation (2). Despite the unambiguous impact of folate status on NTD risk, however, the mechanism by which folic acid exerts this protective effect remains elusive.

This review summarizes current research on the relationships among genetic variation, folate nutriture, folate-mediated one-carbon metabolism and NTDs, with a specific focus on the metabolic and/or genomic pathways that intersect to enable proper neural tube closure (Figure 1.1).

Folate and NTDs: Historical and Current Perspectives

The association of folate status and/or impaired folate metabolism with NTDs was originally indicated by the observation that women with recurring NTD-affected pregnancies exhibited impairments in folate metabolism and/or status. In the 1960's, Hibbard and colleagues determined that mothers of infants born with CNS malformations excreted more formiminoglutamic acid (FIGLU) in urine, a biomarker of impaired folate metabolism, than matched control mothers (3). It was further demonstrated that mothers of affected infants had significantly lower erythrocyte

Figure 1.1. Linking alterations in folate status with NTDs. Alterations in folate status affect neural tube closure, but the mechanism(s) remains unknown. Disruption of folate metabolism can result in homocysteine accumulation, impaired nucleotide biosynthesis, and impaired cellular methylation. These metabolic impairments evoke genomic responses such as alterations in gene expression, genomic instability, reduced mitotic rates, and impaired DNA repair. Impairments in metabolism and/or genomic responses may influence cellular responses critical to proper neural tube closure, including cell proliferation, survival, differentiation, and migration. Single nucleotide polymorphisms (SNPs) in folate-related genes can influence both maternal and infant folate status, or alternatively SNPs can also directly disrupt metabolism. In addition, SNPs in folate-related genes may also modify genomic and/or cellular responses directly via another, unidentified mechanism.


NUTRITION
Maternal Status
Fetal Status
GENES
Maternal SNPs
Fetal SNPs
METABOLISM
Homocysteine accumulation
Nucleotide biosynthesis
Cellular methylation
GENOMIC RESPONSES
Gene expression
Genome stability
Replication/Repair
CELLULAR RESPONSES
Proliferation
Survival/Apoptosis
Differentiation
Migration

folate levels than control mothers (4,5). These findings motivated subsequent studies to determine the preventative effect of folate nutrition on pregnancy outcomes. A flurry of small observational studies in the 1980's provided preliminary support for a protective effect of periconceptional folate supplementation on NTD recurrence (6-11); however, in the majority of these studies women were consuming multivitamin supplements containing folic acid, and therefore an exclusive role for folic acid in the prevention of NTDs remained unproven.

Two landmark studies in the early 1990's provided unequivocal evidence that maternal supplementation with folic acid during pregnancy prevented both NTD recurrence (12) and occurrence (2). In 1991, the British Medical Research Council (MRC) conducted the largest multi-center randomized trial to date to determine the effectiveness of folic acid supplementation alone in preventing recurrence of NTDs as compared to multivitamin supplements. Women with a previous NTD-affected pregnancy were assigned to one of four groups: 1) 4 mgs/day folic acid alone, 2) 4 mgs/day folic acid plus multivitamin, 3) multivitamin only, or 4) no vitamin treatment. Supplementation with folic acid resulted in a 72% reduction in NTDs; vitamin supplements lacking folic acid did not significantly reduce NTD recurrence. The protective effect of supplementation with folic acid was so convincing that the study was prematurely terminated due to ethical considerations, i.e. withholding folic acid from women in the other treatment groups. Within the same year, Cziesel and Dudas (2) conducted a randomized control trial to determine whether periconceptional vitamin supplementation could prevent the first occurrence of NTDs. Women without a history of previous NTD-affected pregnancy were randomly assigned to multivitamin supplement including 800 µg of folic acid, or a trace-element control supplement without folic acid at least three months prior to conception. No NTDs were observed among 2104 pregnancies in the vitamin supplemented group, as

compared to 6 NTD-affected pregnancies in the trace element supplemented group, consistent with a 70% protective effect of supplementation with the multivitamin. Despite the use of a multivitamin supplement, it was concluded that the 800 μg of folic acid was responsible for the preventative effect observed in this study, based on the findings from the MRC trial. Additionally, it has been demonstrated that folate status can predict NTD risk (13), and that folic acid supplementation can prevent NTDs even in the absence of maternal folate deficiency (14), suggesting a genetic underpinning to NTD risk.

The results of these trials inspired a series of public health efforts to promote the use of folic acid-containing supplements among women of child-bearing age. In 1992, the U.S. Public Health Services and Institute of Medicine (IOM) issued the recommendation that all women of childbearing age consume a vitamin supplement containing 400 μg of folic acid daily, and that women with a previous NTD-affected birth who were planning another pregnancy should consume 4 mg/day folic acid to prevent NTDs. These recommendations were not effective; only 29% of women were found to be compliant with the recommendation (15). These data were not surprising considering that most pregnancies in the United States are unplanned. Furthermore, any public health intervention must be pre-emptive because supplementation after pregnancy is not effective as neural tube closure occurs around the fourth week of gestation, before most women are aware of their pregnancy. Therefore, in the late 1990's, public efforts to endorse supplement use were deemed inadequate, and the decision was made by the Food and Drug Administration to mandate fortification of enriched grain products in the US with folic acid to enable individuals to achieve an intake of 100 μg of folic acid /day (16). This fortification initiative has prevented 30% of NTDs in the general United States population (17), providing additional support for the preventative effect of folic acid supplementation on NTD occurrence.

The folate fortification policy in the United States was unique from previous fortification initiatives in that it did not seek to remedy a nutrient deficiency in the general population. Rather, it sought to target a distinct group, (i.e. women of childbearing years with genetic susceptibility to bear a child with a NTD) to achieve a health outcome (i.e. prevention of a birth defect). Despite the accumulation of decades of research and changes in public health policy, we are still mostly unaware of the mechanism(s) by which alterations in folate status and/or metabolism affect neural tube closure. It is most often assumed that supplementation with folic acid is acting either directly to correct a nutritional deficiency or indirectly to overcome metabolic impairment whose etiology is genetic and/or environmental. However, evidence that folate supplementation affords protection against NTDs even in the absence of overt maternal folate deficiency or impaired folate status suggests the possibility that folate supplementation may be exerting a protective effect via other unknown mechanisms, perhaps independent of metabolism.

Although there are no established adverse consequences associated with elevated intakes of folate in humans, the current upper limit (UL) for synthetic folic acid intake is 1 mg/day (18). This UL was established as a result of concerns that elevated folate intakes may mask the hematological diagnosis of vitamin B12 deficiency, which occurs in as many as 15% of the population 65 years and older. There was also concern that elevated folate intakes may accelerate neuropathology associated with vitamin B12 deficiency (19). In addition, although folate fortification has been a highly effective public health measure in reducing NTDs, the lack of mechanism-based understanding of this prevention leads to general concerns regarding unintended consequences resulting from the supplementation policy. Such concerns include cancer risk (20), and more recently, the adverse effects of unmetabolized folic acid accumulating in human blood (21). Further, recent advances in our

understanding of epigenetic programming and the influence of folate metabolism on epigenetic outcomes (22-24) have raised concerns regarding the possible consequences of over-supplying folate during embryonic development. Without a clear understanding of an "upper limit" for folate nutriture or knowledge regarding the lowest efficacious dose for prevention, there exists the possibility that some population subgroups may not benefit and potentially accrue risk from the fortification initiative. Identifying the mechanism(s) by which folate status affects NTD outcomes may help establish more efficacious and better targeted preventative measures that minimize unintended consequences for the entire population.

Folate-mediated One-Carbon Metabolism

Function of Folate. Tetrahydrofolates (THF), the biologically active form of folate in the body, serve as cofactors that chemically activate and carry one-carbon units. Activated one-carbon units carried on either N5 or N10 of THF can be enzymatically interconverted to different oxidation states ranging from formyl to methyl (25-27). Furthermore, folates differ by the length of their popyglutamate peptide. Serum folates contain a single glutamate residue, whereas intracellular folates contain a polyglutamate chain consisting of 5-8 glutamate peptides linked by an unusual γ-linked peptide bond (28,29). Within the cell, folate polyglutamates function as coenzymes for the reversible transfer of one-carbon units in a set of reactions collectively known as one-carbon metabolism (25,30).

Folate Absorption and Accumulation. Most dietary folates are polyglutamated and therefore must be deconjugated to monoglutamates prior to absorption and transport (31). Deconjugation is catalyzed in the intestine by the enzyme folylpoly-γ-glutamate carboxypeptidase II, encoded by the gene glutamate carboxypeptidase II (GCPII). Folate monoglutamates are then absorbed from the intestine into the

bloodstream via the reduced folate carrier (RFC) and/or a novel intestinal folate transporter (32); RFC is a facilitative anion exchanger expressed primarily in the small intestine, liver, lung, and placenta (33). Most folates in the blood are present as 5-methylTHF monoglutamate; cellular uptake of 5-methylTHF can be accomplished by the folate receptor alpha, a membrane-anchored receptor with a glycosylphosphatidylinositol moiety that mediates folate transport via an endocytic process (34,35). Although folate receptor alpha also has a high affinity for monoglutamic 5-methylTHF, it has even higher high affinity for folic acid (36,37). Once inside the cell, the addition of a polyglutamate tail to the folate derivative is catalyzed by folylpoly-gamma-glutamate synthetase; the polyglutamate moiety serves to sequester folates within the cell, as well as to increase the affinity of the THF cofactor for enzymes catalyzing one-carbon transfer reactions (25). Folic acid, which refers to the oxidized and synthetic form of folate found in fortified foods and dietary supplements, is more bioavailable than natural food folates and contains a single glutamate residue. Once transported into cells, it is enzymatically reduced to THF and is chemically identical to natural food folate.

Folate Mediated One-Carbon Metabolism. Folate mediated one-carbon metabolism (OCM) is compartmentalized in the cell, occurring primarily within the cytoplasm and the mitochondria (Figure 1.2). In the mitochondria, the primary role of one-carbon metabolism is to produce formate and glycine from serine (27,30). Formate traverses into the cytoplasm where it serves as a major source of one-carbon units for cytoplasmic one-carbon metabolism. In the cytoplasm, formate condenses with THF to form the cofactor 10-formylTHF in an ATP-dependent reaction catalyzed by the trifunctional enzyme methylenetetrahydrofolate dehydrogenase/ methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase, commonly known as C^1-THF synthase, which is transcribed from the MTHFD1 gene.

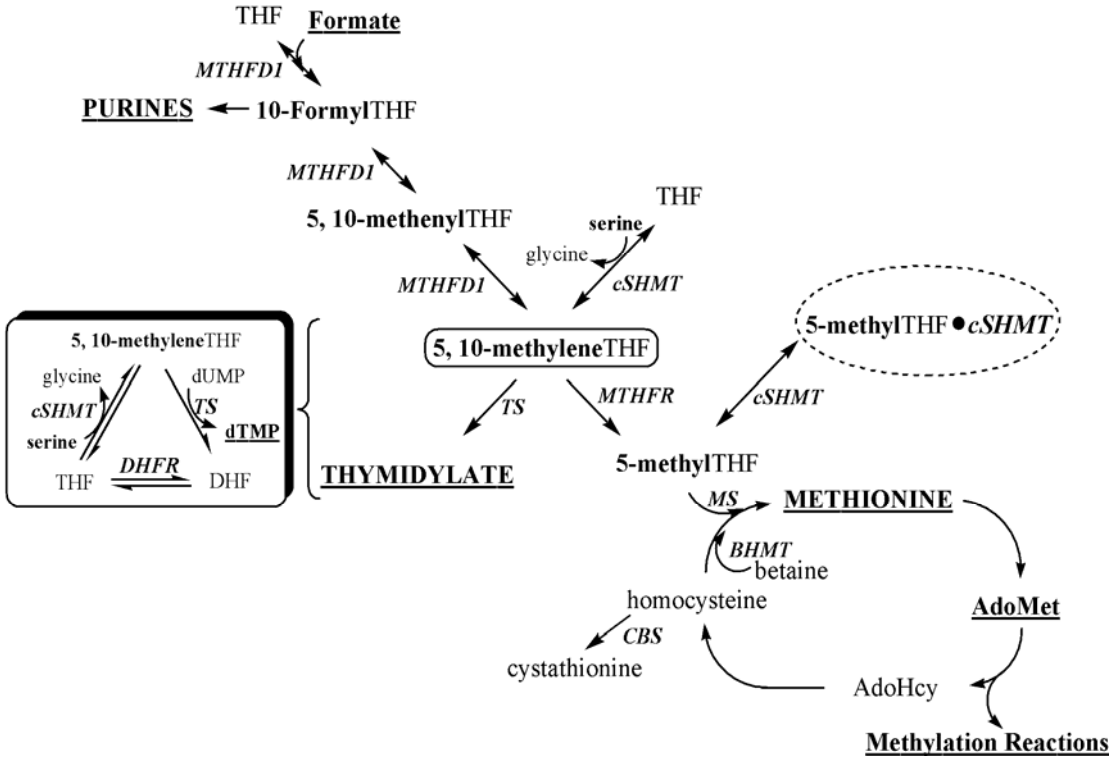


Figure 1.2. Folate-mediated one-carbon metabolism. Tetrahydrofolate (THF)-mediated one-carbon metabolism is required for the synthesis of purines, thymidylate and the remethylation of homocysteine to methionine. The hydroxymethyl group of serine is the major source of one-carbon units which are generated in the mitochondria in the form of formate, or in the cytoplasm through the activity of cytoplasmic serine hydroxymethyltransferase. Mitochondrial-derived formate can enter the cytoplasm and function as a one-carbon unit for folate metabolism. cSHMT also serves a role in inhibiting homocysteine remethylation by sequestering 5-methyl-THF in the cytoplasm. The one-carbon is labeled in "bold". The "inset" shows the thymidylate synthesis pathway which involves three enzymes, cSHMT, TS and DHFR. AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; BHMT, betaine-homocystiene methyltransferase; CBS, cystathionine- β synthase; cSHMT, cytoplasmic serine hydroxymethyltransferase; DHFR, dihydrofolate reductase; dUMP, deoxyuridine monophosphate; MTHFD1, methylenetetrahydrofolate dehydrogenase/ methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase; MTHFR, methylenetetrahydrofolate reductase; MS, methionine synthase; TS, thymidylate synthase;

10-formylTHF serves as a one-carbon donor for the *de novo* biosynthesis of purines by supplying the #2 and #8 carbons to the purine ring. Alternatively, the one-carbon moiety of 10-formylTHF can be reduced in a NADPH-dependent reduction by C^1-THF synthase to form the cofactor 5,10-methyleneTHF, which is utilized by the enzyme thymidylate synthase (TS) as a cofactor in the methylation of dUMP to form thymidylate (dTMP). 5,10-methyleneTHF can also be produced from the condensation of serine and THF in a reaction catalyzed by the enzyme cytoplasmic serine hydroxymethyltransferase (cSHMT) that preferentially directs one-carbon units towards thymidylate biosynthesis (38). The one-carbon moiety of 5,10-methyleneTHF can be further reduced to 5-methylTHF, in a reaction catalyzed by the enzyme methyleneTHF reductase (MTHFR) that irreversibly commits one-carbon units to the methionine/homocysteine remethylation cycle. 5-methylTHF donates a one-carbon to the remethylation of homocysteine to form methionine, which is catalyzed by the B-12 (cob(I)alamin)-dependent enzyme methionine synthase (MS). During the reaction catalyzed by methionine synthase, cob(I)alamin can be oxidized to cob(II)alamin, which results in inactivation of the MS-cobalamin complex. The activity of another gene, methionine synthase reductase, encoded by the gene MTRR, catalyzes the reductive methylation of cob(II)alamin, thereby restoring MS activity. Methionine can be adenylated to form S-adenosylmethionine (AdoMet) which serves as the universal methyl donor for numerous cellular methylation reactions (25,27,28,39) including the methylation of lipids, histones, DNA, RNA, and neurotransmitters, among other molecules. The product of AdoMet-dependent methylation reactions is S-adenosylhomocysteine (AdoHcy) which is consequently hydrolyzed to homocysteine. Thus, cytoplasmic one-carbon metabolism is essential for 1) the synthesis of purines and thymidylate, 2) the remethylation of homocysteine,

and 3) and the synthesis of AdoMet, which is required for cellular methylation (25,27,28,39).

Homocysteine is a non-essential amino acid that is toxic when it accumulates within the cell. There are several cellular mechanisms to prevent its accumulation (Figure 1.2). In addition to remethylation via the folate-dependent pathway, homocysteine can also be remethylated to methionine via the transfer of a methyl group from betaine, a reaction catalyzed by the enzyme betaine-homocysteine methyltransferase (BHMT). The reaction catalyzed by BHMT occurs mainly in the liver and kidney (40). In some cell types and tissues, homocysteine can also be degraded by the transulfuration pathway, through which homocysteine irreversibly condenses with serine to form cystathionine. Transulfuration is catalyzed by the vitamin B6-dependent enzyme cystathionine β-synthase (CBS). The transulfuration pathway degrades homocysteine and thereby effectively removes a source of cellular methionine for protein synthesis and cellular methylation.

Stringent regulation of cellular homocysteine levels is not only important in preventing cytotoxicity, but is also critical for maintenance of cellular methylation potential and protein synthesis. The relative AdoMet:AdoHcy ratio has been suggested to be an indicator of cellular methylation potential. Accumulation of homocysteine drives the reaction catalyzed by S-adenosylhomocysteine hydrolase (SAHH), which normally favors the hydrolysis of S-adenosylhomocysteine (AdoHcy) to homocysteine, in the direction of AdoHcy synthesis (41-43). AdoHcy is a potent inhibitor of certain AdoMet-dependent methylation reactions (43). In addition, accumulation of AdoMet inhibits the enzyme MTHFR (44,45), preventing the accumulation of 5-methylTHF when cellular levels of methionine are sufficient to meet methylation demands. AdoMet also inactivates BHMT and activates CBS-mediated transulfuration (46). Thus, flux of the methionine cycle is tightly regulated

in order to preserve methionine and AdoMet synthesis and to prevent unnecessary accumulation of intermediate metabolites. Regulation of the flux of folate-derived one-carbon units into the methionine cycle through MTHFR also has implications for other folate-dependent reactions. Because the reduction of 5,10-methyleneTHF to 5-methylTHF catalyzed by MTHFR is irreversible, disruptions in the methionine cycle result in accumulation of 5-methylTHF at the expense of all other folate derivatives, a phenomenon referred to as the folate "methyl-trap". Direct causes of methyl trap include B12 deficiency, which is the cofactor for methionine synthesis, or genetic disruption in enzymes that mediate methionine synthesis. The methyl trap impairs nucleotide biosynthesis due to folate substrate depletion. Conversely, disruption of the MTHFR-mediated reduction of 5,10-methyleneTHF results in more one-carbon units available for thymidylate biosynthesis at the expense of the methionine cycle.

The efficiency of folate-mediated one-carbon metabolism also depends on the availability of THF cofactors. It has been estimated that most cellular folates are protein-bound and further, cellular folate binding capacity exceeds the intracellular concentration of folates (47). Thus, the amount of free or unbound folate cofactors in the cell is negligible, and as such, under conditions of limited availability of free folate cofactors, folate-dependent pathways compete for available cofactors (47,48). Competition for folate cofactors is greatest for the cofactor 5,10-methyleneTHF, which is utilized by three biosynthetic reactions: 1) the methylation of dUMP to form thymidylate, catalyzed by TS, 2) the synthesis of serine, catalyzed by serine hydroxymethyltransferase, and 3) the synthesis of 5-methylTHF catalyzed by MTHFR, which irreversibly commits one-carbon units to the methylation cycle. The cofactor 5,10- methyleneTHF thus represents a significant junction in the pathway, at which point one-carbons can either be utilized for thymidylate biosynthesis or committed to the methylation cycle for the remethylation of homocysteine and

AdoMet biosynthesis. This indicates that genome synthesis competes for a limiting pool of folate-activated one-carbons with chromatin methylation reactions that affect genome stability and gene expression. Experimental evidence and theoretical modeling suggest that when folate cofactors are limiting, the synthesis of 5-methylTHF for cellular methylation is favored at the expense of thymidylate biosynthesis (48,49). The exception to this preference occurs in cells which express cSHMT. Recent evidence indicates that when cSHMT is expressed, flux of one-carbon units is shifted towards thymidylate synthesis at the expense of AdoMet synthesis and cellular methylation reactions. Regulation of these pathways by cSHMT is accomplished via two mechanisms: 1) the synthesis of a separate pool of 5,10 methyleneTHF that is preferentially shuttled to thymidylate synthesis and 2) tight binding of the cofactor 5-methylTHF by cSHMT, which prohibits entry of one-carbon units to the methylation cycle thereby inhibiting methionine and AdoMet biosyntheses.

Impairments in Folate-mediated One-Carbon Metabolism – Association with Neural Tube Closure

Impairments in folate-mediated one-carbon metabolism can result from diminished folate status, single nucleotide polymorphisms (SNPs) in genes that affect folate metabolism, and/or secondary micronutrient deficiencies of nutrients that alter folate status, such as iron and other B vitamins (19,50,51). Common biomarkers of impaired folate status include elevated plasma homocysteine and increased uracil content in DNA (52). Cellular accumulation of homocysteine has been shown to elicit oxidative stress responses (53-55) and may be cytotoxic (56). In addition, homocysteine negatively regulates cellular methylation. Hypomethylation of DNA resulting from impaired folate metabolism can alter the expression of the 30% of

genes that are regulated by CpG methylation (57), whereas inability to methylate proteins, including histones, can affect chromatin structure and many cellular functions, including but not limited to neurotransmitter and myelin synthesis (58-61). Increased uracil content in DNA results from a lack of available thymidylate for DNA replication and repair. A shortage of one-carbon units for *de novo* purine or thymidylate synthesis will slow replication thereby decreasing mitotic rates as well as affecting DNA repair, reducing the proliferative capacity of the cell and promoting genomic instability (62,63).

Completion of neural tube closure requires the precise coordination of cell proliferation, survival, differentiation and migration events; any one of these events could feasibly be disrupted by impairments in folate metabolism (Figure 1.1). Although there are a number of possible mechanisms by which impairments in folate metabolism might affect neural tube closure, there is currently no direct evidence linking a specific metabolic impairment in folate metabolism with a specific cellular event that results in the failure of the neural tube to close.

Potential Mechanisms of Perturbation of Neural Tube Closure

Homocysteine Accumulation. Elevated maternal homocysteine levels have been linked to NTD-affected pregnancies (64,65) suggesting that cellular homocysteine accumulation may contribute to NTD pathogenesis. *In vitro* experiments have documented cytoxicity of homocysteine at supraphysiological doses (66,67). Homocysteine toxicity may be mediated by overactivation of NMDA receptors, resulting in glutamate excitotoxicity (56,68,69). A role for oxidative stress in the etiology of NTDs has been suggested by gene expression profiling experiments in a mouse model of diabetes that is susceptible to NTDs (70,71). Oxidative stress resulting from elevated homocysteine could potentially disrupt neural tube closure.

However, mice with deficient CBS activity exhibit hyperhomocysteinemia but do not develop NTDs (72).

Cellular Methylation. Impairments in AdoMet biosynthesis affect genomic methylation, which can alter expression of 30% of the genome regulated by CpG methylation (73). Alterations in chromatin methylation may affect embryonic development via effects on cellular differentiation (74-76) and/or cellular migration processes (77,78). DNA methylation has been implicated in cell differentiation based on several findings demonstrating the disruptive effects of the demethylating agent 5-deazacytosine on normal differentiation in a variety of cell lines and tissues (74,75,79). Loss of DNA methylation is presumed to affect cell differentiation via alterations in CpG methylation-regulated gene expression, consistent with findings that cell differentiation requires mediators of transcriptional repression of CpG-methylated genes, including methyl binding proteins and histone deacetylases (80-82) . More specifically, it has recently been posited that *de novo* methylation and associated gene silencing is required for differentiation during development (83). This is supported by data from studies in which targeted loss of the *de novo* methyltransferase enzymes Dnmt3a and Dnmt3b altered differentiation capacity in ES cells (84). Further, Dnmt3b -/- embryos display NTDs, confirming the requirement for timely cell differentiation in neural tube closure. Targeted deletion of genes that mediate methylation-mediated suppression of gene expression also result in failure of neural tube closure (85). Epigenetic regulation of cell migration could also be critical for proper neural tube closure, as appropriate migration of neural crest cells adjacent to the neuroepithelium may be required for neural tube closure (86,87). Together, these data imply that regulation of cellular differentiation and migration by genome methylation is crucial for proper neural tube closure.

Nucleotide Biosynthesis. Impairments in nucleotide biosynthesis for DNA replication and repair can decrease mitotic rates during critical morphogenetic windows and/or increase mutation rates, both of which can result in failure of neural tube closure (88). Rapid growth of the neuroepithelium during neural tube closure requires an increased dependency on *de novo* nucleotide biosynthesis to sustain rate of cell division. Genomic instability resulting from uracil misincorporation may also affect neural tube closure, as other instances of genomic instability, including centromeric and telomeric instability have been reported to cause NTDs (89-91). Although it is widely believed that folate functions to provide adequate nucleotide pools during development, the extent to which this particular deficiency might contribute to NTDs is unclear. Further, the specific effect of uracil misincorporation on genomic instability and neural tube closure has not been investigated.

Genetics of Folate Metabolism and NTDs

Family history of NTD-affected pregnancy is one of the strongest risk factors for these birth defects. Risk of NTD is elevated 3-8% in siblings of affected individuals, and there is even some evidence that risk is elevated in second and third degree relatives (92). In addition, NTDs are more common among different ethnic groups (92). Collectively, these data indicate a genetic component to NTD etiology. The realization that NTDs could be prevented by maternal folate supplementation even in the absence of pre-existing maternal folate deficiency further suggested that inherent errors in folate metabolism could contribute to folate deficiency despite proper dietary intake (14). This led to the discovery of single nucleotide polymorphisms (SNPs) in folate-related genes that impair folate status and/or metabolism without frank folate deficiency. Since then, many studies have examined the effects of SNPs in a number of folate-related genes on NTD risk, providing

evidence both for a genetic contribution to the etiology of neural tube defects and further support for a direct role of folate-mediated one-carbon metabolism in the development of NTDs.

Beyond identifying genetic risk factors for NTDs, understanding the influence of specific SNPs on folate mediated one-carbon metabolism and NTD risk may be used to provide some insight into the mechanism(s) underlying folate-NTD associations. The following genetic data and associated biochemical processes are described below as follows: 1) Genes within folate transport, 2) Genes contributing to nucleotide biosynthesis, and 3) Genes within the methionine/homocysteine cycle. A more thorough review of SNP data for each gene within the folate and methionine/homocysteine cycles can be found in a recent review by van der Linden et al (93).

Genes within Folate Transport. Genes involved in folate transport and accumulation that have been investigated in relation to NTDs include the reduced folate carrier (RFC), and folate receptors (FR) alpha and beta. Folate receptors are expressed throughout the developing neural tube and early in embryonic development (94); RFC is strongly expressed in the placenta, as well in the developing neural tube (33). No common genetic variants have been identified in the coding region of the folate receptor genes, and SNPs within non-coding regions of these genes do not show association with NTDs (95-98). A common SNP in RFC1 has been identified, A80G, that may modulate plasma folate levels (99). Investigations of NTD risk associated with this SNP have yielded mixed results, with some failing to detect an association (100,101) and others detecting an increased risk associated with case and maternal GG genotype (102). Interestingly, several of these studies have identified an increased risk of NTD-affected pregnancy among mothers carrying two G alleles that is either

only present under (103-105) or exaggerated by (105) conditions of low folate status. The responsiveness of this mutation to dietary folate provides support for a role of RFC in mediating folate-responsive NTDs. This is further suggested by investigation of the RFC gene knock-out (KO) mouse model, in which early embryonic lethality is rescued by folic acid supplementation (106). However, homozygous RFC KO mice die *in utero* prior to neural tube closure; that folic acid supplementation rescues this lethality suggests that RFC-mediated folate transport is critical for other aspects of embryonic development beyond neural tube closure. In contrast, investigation of folate receptor α KO mouse has demonstrated unequivocally that embryonic folate transport is essential for neural tube closure. Targeted deletion of the folate receptor gene, Folbp1, results in NTDs that can be rescued by maternal supplementation with pharmacological doses of folic acid (107). These are the first experimental data to indicate a direct role of embryonic folate utilization in neural tube closure. It is clear from both the human and animal data that both RFC and the high affinity folate receptor alpha are critical for embryonic development, most likely due to a high embryonic demand for folate cofactors. However, the present data do not provide additional information regarding specific metabolic mechanisms, nor is it clear how more subtle modulation of folate status might affect neural tube closure.

Genes Within the Methionine/Homocysteine Metabolic Cycle. Genes within the methionine/homocysteine remethylation cycle have been attractive candidates for genetic analysis in relation to NTDs, based on the associations between NTD risk and both elevated maternal homocysteine (64,65) and low B12 status (108). The C677T SNP in the MTHFR gene was the first genetic defect in folate metabolism to be investigated in relation to NTDs, and remains the most studied. The alanine-to-valine substitution produces a thermolabile enzyme variant associated with a 50-60%

reduction of enzymatic activity in homozygous individuals (109-111). Loss of MTHFR activity causes a reduction in 5-methylTHF that is available for homocysteine remethylation. Numerous studies have investigated the association between the MTHFR C677T SNP and NTD risk (112-120) Metanalyses of these studies have confirmed an elevated risk for both maternal (50-70% increase) and fetal (80-90% increase) TT genotypes (121-123), as well as mildly elevated risk associated with the heterozygous 677CT genotype in mothers (10% increase) and offspring (30% increase) (123) . Still, several studies have failed to identify a significant association of the MTHFR C677T SNP with NTD risk (101,115,124-127). Notwithstanding these discrepancies, the MTHFR C677T SNP continues to be investigated in relation to NTDs. Elevated homocysteine has been observed in individuals homozygous for the SNP (119,128,129), although more recently it has been suggested that hyperhomocysteinemia occurs primarily under conditions of mild to moderate folate deficiency in this genetic sub-group (130,131). Further, elevated homocysteine in individuals homozygous for the MTHFR C677T polymorphism is responsive to supplementation with folic acid (132). Because elevated homocysteine is a risk factor for NTD-affected pregnancy, it has been proposed that folate supplementation might prevent NTDs by stabilizing enzymatic activity thereby normalizing homocysteine levels. Indeed, several studies have suggested that the MTHFR C677T SNP interacts with folate status in elevating NTDs risk (114,117). Still, the frequency of the T allele in most populations cannot account fully for either 1) the 70% reduction in NTDs observed with folate supplementation, or 2) impaired folate status observed in mothers of affected offspring (133).

More recently, a second SNP within the MTHFR coding region has been investigated in relation to NTDs. The MTHFR 1298A>C transition also results in reduced enzymatic activity, although there is no demonstrated effect on homocysteine

levels. In parallel with the negligible effects on biomarkers of folate status, a limited number of studies have been unable to identify a unique contribution of the MTHFR A1298C polymorphism to NTD risk (100,101). Some preliminary investigation of the two SNPs in MTFHR in tandem suggests that possession of both polymorphisms confers elevated risk greater than that of either polymorphism alone (101,121,134). More recently, however, it has been determined that these two SNPs are almost in complete linkage disequilibrium (115,135,136); homozygosity for both SNPs is undetected at the same loci. Thus, conclusions regarding the effect of the MTHFR 1298A>C transition on NTD risk are limited.

Rozen and colleagues have created a mouse model in which the MTHFR gene has been deleted (137). Although homozygous MTHFR knock-out (KO) mice die soon after birth, live births of all three genotypes occur at expected frequencies. Further, investigation of embryonic development of these mice has failed to identify an NTD associated with loss of MTHFR, even under folate-deficient conditions (138) and despite a 10-fold elevation in homocysteine levels (137). Thus, this model cannot be used to further understand the role of MTHFR in regulating folate metabolism during neural tube closure. Further, the discrepancy in NTD phenotype between the MTHFR KO mouse and the C677T SNP suggests that the biochemical effect of the C677T SNP may modify folate metabolism and consequently NTD risk in a manner that has yet to be determined.

The tenuous association between elevated NTD risk and polymorphisms in the MTHFR gene is illustrative of current limitations in our understanding of the mechanism(s) by which impairments in folate metabolism alter neural tube closure. Although elevated homocysteine has been implicated in mediating the effect of this polymorphism on NTD risk, recent evidence also indicates that MTHFR C677T affects folate-dependent anabolic reactions beyond homocysteine accumulation. In

particular, homozygous individuals also show reduced methylation potential and some evidence for increased *de novo* thymidylate biosynthesis (139-142). Thus, reduced MTHFR activity may affect neural tube closure by altering the partitioning of one-carbon units between thymidylate and methionine synthesis in a way that is not fully understood. Regulation of the flux of one-carbon units has important implications for folate-related pathologies. For example, the MTHFR C677T is associated with an increased risk of NTDs but a decreased risk of colon cancer (143). The opposing influence of this polymorphism on these two pathologies highlights the importance of balancing the one-carbon flux between the thymidylate and methionine biosynthetic pathways and indicates that it may be the alteration in balance of one-carbon metabolism, and not one particular anabolic pathway, that increases risk for pathology.

Other candidate genes within the methionine/homocysteine remethylation pathway include methionine synthase (MS, gene name MTR) and methionine synthase reductase (MTRR), which cooperate to remethylate homocysteine to methionine via the folate-dependent pathway, and BHMT and CBS, which modulate homocysteine levels independent of the folate cycle (Figure 1.2). Examination of the evidence for an association between SNPs in MTR/MTRR and NTD risk has yielded inconsistent findings. A limited number of studies have investigated the MTR A2756G SNP in relation to NTD risk. The majority of these studies have found no association with NTD risk, either in case-control studies (102,144,145) or with the more robust transmission disequilibrium test (TDT) (146). Two studies (147,148) identified a significant risk associated with this polymorphism in NTD cases; however, in both study populations there is a very high frequency of the MTHFR TT allele (149) which may interact with the MTR A2765G polymorphism in increasing NTD risk (100,150). Interestingly, both of these studies also identified significantly elevated NTD risk

associated with homozygosity for a 66A>G polymorphism in the related gene, MTRR, in both the mothers and cases. The positive association between the MTRR A66G polymorphism and NTD risk is supported by findings from a handful of smaller, case-control studies (150,151). Recently, however, O'Leary and colleagues (152) conducted an exhaustive investigation of several MTRR polymorphisms, including A66G, in relation to NTD risk and found no association between NTDs and any genetic variation in MTRR. The large sample population and robust statistical methods, including case/control comparison, log-linear analysis, and TDT, provide a strong argument against an increased NTD risk associated with polymorphisms in MTRR. The lack of a strong association with NTD risk concurs with biochemical data indicating that MTRR SNPs have a limited or even lowering effect on homocysteine levels (153,154). In fact, Christenson et al (117) demonstrated a mild protective effect of the GG genotype, which may be consistent with a homocysteine-lowering effect. In light of the findings that neither MTR nor MTRR SNPs significantly affect homocysteine levels, it is possible that these SNPs do not have functional consequences. Thus, based on the human literature, a role of MTR/MTRR variants in neural tube closure defects has not been established.

To date, evidence from mouse models of MTR and MTRR deficiency has not provided additional insight into the influence of these genes on neural tube closure. A methionine synthase KO mouse model was created by a homologous recombination event in which a PGK cassette was inserted into the coding region of the MTR gene, resulting in a null allele (155). Nullizygous MTR KO embryos did not survive to birth, and examination of genotype distributions at different stages during embryonic development revealed that nullizygous embryos were being resorbed prior to neural tube closure in the mouse, preventing examination of the effect of MS deletion on neural tube closure. Maternal treatment with pharmacological doses of folic acid

failed to rescue early embryonic lethality. Further, no developmental anomalies have been observed in heterozygous MTR KO mice, suggesting that partial loss of methionine synthase is not a risk factor for NTDs in mice. To avoid early embryonic lethality associated with total loss of MS activity, Elmore and colleagues recently generated a mouse model with reduced MTRR activity, created by the insertion of a gene trap vector within the MTRR gene (156). Since MTRR activity is required to maintain MS activity, hypomorphic expression of MTRR gene results in reduced MS activity. Homozygous $MTRR^{gt/gt}$ mice survive to birth and display a very mild postnatal phenotype, despite a robust metabolic phenotype that includes highly elevated plasma homocysteine, reduced plasma methionine, and tissue-specific alterations in AdoMet/AdoHcy ratio. Although no NTD has been observed in these mice, examination of embryonic development under conditions of folate deficiency may provide better insight into the role of folate-dependent methionine synthesis in NTD etiology.

Evidence relating genetic alterations in BHMT and CBS do not provide support for a role of homocysteine accumulation in NTD pathology. One common variant in the BHMT gene, A742G, has been identified; however, two independent studies failed to detect a significant association with NTD risk (157,158). The BHMT A742G SNP has not shown a strong relationship with homocysteine levels (159,160) indicating that it may not have functional relevance. One other recent study investigated a novel intronic SNP in BHMT that was associated with an increased risk of NTDs in mothers taking periconceptional folate supplements (98). However, the biochemical effect of this particular SNP has not been elucidated, precluding any insight into a metabolic mechanism. No mouse model of BHMT deletion has been described to date, and therefore little information exists regarding its role in embryonic development. BHMT is not expressed in the developing embryo until after

neurulation (161), suggesting that the BHMT-dependent homocysteine remethylation may not play a role in embryonic homocysteine metabolism during neural tube closure.

The severe clinical manifestation of CBS deficiency (162) in humans indicates that CBS is essential for normal embryonic development; however, such severe deficiencies have not been investigated in relation to NTDs. CBS is expressed early in mammalian development and throughout the developing neural tube (163,164), suggesting that it may be a factor in neural tube closure. Two common polymorphisms have been assessed in relation to NTDs; an insertional allele in exon 8, 844ins68, and a transition mutation T833C, both of which tend to segregate together in cis (165). No studies to date have identified an association between either mutation and NTDs (126,134,166,167), consistent with the lack of effect of these polymorphisms on homocysteine levels (131,168). More recently, another polymorphism in a non-coding region of CBS, a 31 bp variable-number tandem repeat (VNTR) has been identified which has been shown to modulate homocysteine levels (169,170); however, one study to date has failed to detect an association with NTDs (171). Further investigation of this polymorphism is warranted. A mouse knock-out model for CBS was generated (72); however, homozygous CBS KO mice survived to birth and exhibited early postnatal lethality despite extremely elevated homocysteine levels. The absence of NTDs in the CBS KO mouse suggests that the homocysteine transulfuration pathway mediated by the CBS may not be directly involved in the pathogenesis of NTDs.

Collectively, the lack of evidence from both human SNP association studies and knock-out animal models of genes that metabolize homocysteine do not support an independent role for homocysteine remethylation and/or homocysteine accumulation in neural tube closure defects. Alternatively, the lack of an association

between SNPs in genes that regulate homocysteine methylation and NTDs may reflect the lack of penetrance with respect to alterations in homocysteine levels. Further, the minimal effects of these SNPs on homocysteine levels suggest the possibility that genetic mutations that produce a greater impairment in homocysteine metabolism may be incompatible with embryonic survival. This notion is supported by studies of methionine synthase, in which homozygous deletion of this gene in the mouse is embryonic lethal. Survival of CBS homozygous KO mice, however, suggests that homocysteine transulfuration may be expendable during embryonic development.

Genes Contributing To Nucleotide Biosynthesis. Impairments in *de novo* nucleotide biosynthesis represent one of the earliest proposed mechanisms to account for the associations between folate metabolism and neural tube closure defects. The embryonic neural tube is the sight of rapid and widespread cell division, and thus there is an increased demand for *de novo* nucleotide synthesis. Of the genes involved in one-carbon metabolism, MTHFD1, which encodes the trifunctional enzyme C^1-THF synthase, provides the most direct contribution to nucleotide biosynthesis by catalyzing the synthesis of 10-formylTHF and 5,10-methyleneTHF, the cofactors for *de novo* purine and thymidylate biosynthesis, respectively. Brody et al (172) identified a SNP in the coding region of MTHFD1, R653Q, that is strongly associated with maternal risk for NTDs. The association between homozygosity for the Q allele and maternal, but not infant, risk was recently confirmed in a separate cohort (173). Analysis of the same SNP in an Italian population identified homozygosity for the polymorphism in cases as a risk for NTDs (174). The lack of observed association between fetal genotype and NTDs in the Irish population may be due to transmission of the Q allele from homozygous mothers to fetuses resulting in a synergistic phenotype that causes early lethality (173). Indeed, there were fewer QQ cases

observed in the population than expected. It is still unclear how the R563Q polymorphism is altering metabolic outcomes, since neither plasma folate, red blood cell folate nor homocysteine levels are altered in QQ mothers. The metabolic effect of the polymorphism on cases has not been investigated. However, the strength of the association between the polymorphism and NTDs in the absence of effects on folate and homocysteine levels suggests that this polymorphism is affecting neural tube closure via alterations in a folate-dependent anabolic pathway other than the methylation cycle. *De novo* nucleotide biosynthesis is the most likely candidate pathway, but this remains to be established.

Genes that have been investigated in relation to thymidylate biosynthesis specifically include cSHMT and TS. Limited examination of a common variant allele in cSHMT, C1420T, has not yielded an association with NTD risk in two studies, although limited power may have precluded the detection of an association (100,175). Another study by Relton and colleagues (101) identified a protective effect associated with the T allele in case mothers, which may be consistent with mildly elevated homocysteine levels observed in CC mothers (175); however, this finding remains to be confirmed. In contrast to many other SNPs that have been examined in relation to NTDs, the biochemical effect of the C1420T polymorphism on cSHMT function has been partially elucidated. Recently, Woeller et al (176) demonstrated that cSHMT can be imported to the nucleus via SUMOylation, where it may function to directly shuttle 5,10 methyleneTHF into the nuclear thymidylate biosynthesis pathway. The authors further demonstrated that the C1420T polymorphism inhibits SUMOylation of cSHMT *in vitro*. Prevention of cSHMT nuclear import may have implications for *de novo* thymidylate biosynthesis and/or flux of one-carbon units, since cSHMT has been previously shown to regulate the flux between thymidylate biosynthesis and cellular methylation (38). Thus, further investigation of the role of this enzyme in NTD

pathogenesis is warranted. Recently, a mouse model in which exon 8 of the cSHMT gene was replaced with a β-geo cassette has been generated that results in total loss of cSHMT protein in homozygous mutants (MacFarlane et al; unpublished). Homozygous mutants are viable; thus, the examination of the role of cSHMT in embryonic development and neural tube closure can be examined.

Only two studies have examined polymorphisms in TS in relation to NTDs. Volcik et al (177) examined two polymorphisms outside of the coding region of TS -- a 28-bp tandem repeat in the promoter enhancer region (termed TSER) and a 6-bp deletion in the 3' UTR – in relation to spina bifida in a large, ethnically heterogenous population. Homozygosity for the double-repeat allele (TSER 2/2) was not significantly associated with risk of spina bifida when examined in the entire study population; however, when the population was segregated by ethnicity, the risk for spina bifida in non-hispanic whites was increased 4-fold in the presence of the TSER 2/2 genotype. Further, risk for NTD was even greater in this ethnic group when the 6-bp deletion in the 3' UTR was absent. In contrast, Wilding et al (178) found no association between the TSER polymorphism and NTD risk in a white population in the UK. The likelihood of detecting associations across different ethnic populations in these studies was likely influenced by differences in the frequency of the various repeat alleles in the control populations (178). TS expression is lower in the presence of the 2X repeat as compared to the 3X repeat allele (179,180), but its effects on folate and homocysteine levels are uncertain. Trihn and colleagues (181) observed reduced plasma folate and mildly elevated homocysteine under low folate conditions associated with TSER 3/3 genotype in a Singapore Chinese population, whereas Brown et al (182) observed no effect of the TSER polymorphism on either serum folate or homocysteine levels in young Irish adults. Thus, clearer delineation of the effect of the TSER polymorphism on folate and homocysteine levels among different

populations may help clarify population differences in NTD risk.

The role of dihydrofolate reductase (DHFR) in neural tube closure has recently been investigated, following the discovery of a novel 19-bp deletion polymorphism in intron1 of the DHFR gene (183). Johnson et al (2004) first investigated the relationship between this polymorphism and risk of spina bifida in a mostly Caucasian population in the United States. Maternal homozygosity for the 19-bp deletion allele was associated with an increased risk for spina bifida in the offspring, although there was no risk associated with spina bifida cases. Two more recent studies examining the 19-bp deletion allele in relation to NTD risk failed to replicate this finding. Van der Linden and colleagues found no association between the deletion allele and risk for spina bifida, either in mothers or cases (184). The authors also investigated DHFR expression levels in lymphoblast cell lines derived from spina bifida cases, as well as plasma folate and homocysteine in all subjects. No differences in DHFR expression were found in response to the 19-bp deletion and further no differences in plasma folate or homocysteine were observed, even when examined in combination with the MTHFR C677T genotype. In sharp contrast, Parle-McDermott et al conducted very similar analyses in an Irish population, and found a protective effect of the 19-bp deletion allele in case mothers, consistent with a 1.6-fold increase in DHFR expression associated with the polymorphism in generic lymphoblast cell lines (185). The reason for the discrepancy between these two studies remains to be determined; indeed, further investigation of the biochemical effect of the DHFR polymorphism as well as the association with NTD risk is warranted. Incidentally, the study by Parle-McDermott et al investigated the entire DHFR gene for additional polymorphisms, and failed to detect any within the coding region. Together, these findings do not provide support for a specific role of DHFR in mediating neural tube closure.

Limited examination of the role of genes contributing to nucleotide

biosynthesis suggests that nucleotide biosynthesis, either in the mother or the fetus, may be a determinant of proper neural tube closure. This is particularly evident in the case of MTHFD1, in which the R653Q polymorphism is strongly associated with maternal risk for NTD-affected pregnancy. Investigation of the specific effect of this SNP on MTHFD1 function and folate-mediated one-carbon metabolism will shed more light on the metabolic mechanism(s) underlying elevated maternal risk. Further examination of polymorphisms in cSHMT, TS, and DHFR in larger and more diverse populations will provide more information regarding the role of these genes in neural tube closure. Thus far, limited power to detect associations between SNPs in these genes and NTD risk, as well as inadequate understanding regarding the biochemical effects of these SNPs on enzymatic function, restrict our ability to draw further conclusions.

Summary. Presently, SNPs in several genes associated with various aspects of folate-mediated one-carbon metabolism have been explored in relation to NTDs. Although the precise contribution of these genetic variants to NTD risk in the population remains undetermined, it is clear that genetic variation in genes regulating folate-mediated one-carbon metabolism underlies failure of neural tube closure. Further investigation of the interactions of these SNPs with folate status, as well as the identification of specific metabolic and/or genomic impairments caused by SNPs in different folate-related genes, will provide more information as to the underlying mechanisms of folate-responsive NTDs. In addition, further examination of the unique contributions of maternal and fetal genotype, using both more robust TDT analysis and animal models, will aid in determining how folate supplementation affords protection against NTDs. Lastly, continuing investigation of gene-gene interactions in relation to NTD risk will help to identify populations at the greatest

risks for NTDs and provide important information regarding the interactions among different folate-dependent anabolic pathways in NTD pathogenesis.

Linking Metabolism and NTDs: Can We Deduce a Mechanism from Animal Models?

To date, evidence from analysis of human SNPs and mutant mouse models within the folate and methionine/homocysteine pathways has not provided sufficient information to establish the metabolic and/or genomic mechanism(s) underlying human folate-responsive neural tube closure defects. Additional research is required in the area of gene-nutrient and gene-gene interactions within these models to establish causal risk factors and the associated mechanisms. In general, conclusions drawn from human genetic studies of folate and NTDs are limited by the numerous confounding environmental and genetic influences within a given population. In addition, the metabolic and genomic outcomes that can be investigated in humans are restricted. Animal models are more suitable for investigating links between specific biochemical/metabolic impairments, cellular responses, and pathological outcomes. Further, the use of animal models allows for more complete assessment of pathology, especially related to fetal outcomes. Unfortunately, since the majority of mouse models in which folate-related genes have been disrupted do not exhibit NTDs, their utility in exploring metabolic mechanism(s) underlying NTDs is limited. Here we review data in which folate and/or homocysteine status has been experimentally manipulated in wild-type mice or in mouse models with genetic mutations that cause NTDs in order to better understand the role of folate metabolism in neural tube closure.

Models of Folate Deficiency and NTDs. Investigators have long sought a mouse model of maternal dietary folate deficiency to investigate mechanism underlying neurodevelopmental anomalies. Surprisingly, folate deficiency alone does not produce NTDs in mice (186,187). Heid et al (186) assessed implantations, resorptions, and NTDs in litters at gestational day 12 from dams fed folate-deficient diets ranging from 45-2255 nm/kg folic acid. Doses below 181 nmol/kg were incompatible with implantation; doses between 221 and 453 nmol/kg resulted in 100% resorption rate. Doses within a range that produced about 75% resorptions did not produce NTDs in viable embryos. In 2002, Burgoon et al (187) fed dams a modified diet without folic acid that also contained succinyl sulfathiazole (SS), an antibiotic that reduces gut flora capable of generating folates, in an effort to completely eliminate sources of folate. Although some implantations survived past gestational day 11-12, there was still no evidence for NTDs in this model. Thus, is appears that folate deficiency in mice is insufficient for generating NTDs, likely due to the ability of mice to resorb fetuses that are not viable.

Models of Hyperhomocysteinemia and NTDs. In light of the failure of folate deficiency to induce NTDs in a mouse model, direct supplementation with homocysteine, either via maternal diet or embryo culture, has been investigated as a direct cause of NTDs in mice. There is a body of evidence from studies in chick embryos indicating that homocysteine may be a teratogen at high levels (for review see (188). Besides a number of developmental defects outside of the nervous system, homocysteine has also been shown to induce NTDS in cultured chick embryos, an effect that is also folate-responsive (189,190). Potential cellular mechanisms underlying NTDs in this model have been investigated; homocysteine has been shown to inhibit neural crest outgrowth *in vivo* (191) and neural crest differentiation *in vitro*

(192). It is important to note that the majority of these studies have administered very high doses of homocysteine, far above those that have been associated with NTD risk in human pregnancy. In contrast to findings in chick embryos, several recent studies have provided evidence against a direct role of homocysteine in neural tube closure defects in mice, either in cultured embryos (193,194) or via maternal administration with homocysteine (195). The most recent study by Bennett and colleagues assessed NTDs in response to varying doses of homocysteine in several different mouse strains susceptible to teratogen-induced NTDs, providing a persuasive argument against a direct role of homocysteine in NTD pathogenesis. Furthermore, in the study by Burgoon et al (187) in which pregnant mice were fed a folate-deficient diet with SS, elevation of plasma homocysteine within a range comparable to levels observed in humans also failed to induce NTDs. Collectively, these data do not support a direct role of homocysteine in NTD pathogenesis, at least in a mammalian model, and the absence of phenotype prevents further study of the mechanism(s) by which alterations in homocysteine/folate metabolism affect neural tube closure.

Alteration in Methylation Cycle and NTDs. Although evidence is weak for a direct influence of homocysteine on neural tube closure defects, it is possible that homocysteine accumulation may alter neural tube closure via perturbation of the methionine cycle. Recent examination of this hypothesis by Dunlevy and colleagues has provided preliminary support for a direct role of methionine and methionine-dependent cellular methylation in neural tube closure. In two separate studies, the authors demonstrated that culture of neurulation-stage mouse embryos with excess methionine or inhibitors of methionine adenosyl transferase (MAT) resulted in an exencephalic phenotype at high frequency (196,197). Supplementation of culture media with 5mM methionine or the Adomet inhibitors ethionine or cycloleucine

reduced the AdoMet/AdoHcy ratio, suggesting that the NTD phenotype might be linked to reduction in cellular methylation, in particular DNA hypomethylation. Possible cellular mechanisms underlying failure of neural tube closure in this model was also examined via morphological investigation of affected embryos. Reduced cranial mesenchymal density was observed in embryos cultured with methionine or ethionine, although the direct relevance to the NTD phenotype was not immediately apparent. Although culture with cycloleucine did affect neuroepithelial density, the absence of the same effect in methionine- and ethionine-treated embryos raises doubt as to the ultimate cause of this morphological defect. Furthermore, no differences in cell proliferation or survival were detected in any affected embryos. The lack of an obvious defect in neuroepithelial integrity suggests that the NTD phenotype observed in this study may be due to another as yet unidentified mechanism. In addition, the supraphysiological doses of methionine utilized poses questions regarding the relevance of these effects to human-responsive NTDs. Nonetheless, these data are one of the few to link a specific metabolic impairment (e.g. altered cellular methylation potential) with failure of neural tube closure, and justify further investigation into this potential mechanism.

Alterations in Folate Metabolism in Mouse Models of NTDs

Numerous genetic mutations outside the folate/methionine pathways have been identified that cause NTDs in mice. The majority of these mutations fall within well-defined developmental pathways. Impairments in folate metabolism and/or responsiveness to dietary manipulation of folate or methionine have been examined in a handful of these mutants (for review see (198). The present review will focus on those mouse models that have demonstrated responsiveness to folate or methionine, since these bear the most relevance to human-responsive NTDs. Additionally, these

models make strong candidates for studying metabolic impairments underlying folate-responsive human NTDs because like human NTDs they show dose-dependent responsiveness to folic acid in the absence of frank maternal folate deficiency. By addressing mechanisms associated with folate supplementation and/or deficiency in mouse models with genetic mutations outside the folate pathways we hope to gain insight into 1) metabolic/cellular outcomes associated with folate supplementation on a folate-replete background, and 2) interaction of folate metabolism with key developmental pathways.

Pax3 (Splotch). Rescue of NTD phenotype by folate supplementation has been demonstrated in the *splotch* mutant both in cultured embryos (199) and with maternal folate supplementation (199,200). The *splotch* mutant carries a deletion allele in the gene encoding the transcription factor Pax3; homozygous *splotch* mutants display a spina bifida phenotype that is 100% penetrant with occasional exencephaly, whereas heterozygote *splotch* mutants are identified by white belly splotch indicative of neural crest migration defects in the absence of neural tube closure defects. Fleming and Copp (1998) investigated alterations in folate metabolism in *splotch* mutants; using the deoxyuridine (dU) suppression assay, they determined that *splotch* mutant embryos were impaired in their ability to generate thymidine *de novo*. Further, supplementation of the culture media with either thymidine or folic acid ameliorated impairments in thymidylate biosynthesis while simultaneously preventing NTDs in homozygous *splotch* embryos. NTD rescue was also demonstrated with embryonic supplementation of the same compounds *in utero*. Interestingly, supplementation of culture media with methionine produced NTDs in almost 50% of *splotch* heterozygotes *and* exacerbated the impairment in *de novo* thymidine biosynthesis. Rescue of NTD phenotype by folic acid and exacerbation with methionine were

replicated *in utero* in the study by Wlodarczyk and colleagues; however, thymidine supplementation was not protective against NTDs in this study. Exploration of folate metabolism and folate responsiveness in the *splotch* mutant to date yields several insights. First, rescue of cultured embryos with folic acid or thymidylate provides additional support for a direct role of embryonic folate utilization in neural tube closure. Second, the data from Fleming and Copp provide the first line of evidence directly linking a specific metabolic impairment (e.g. impaired thymidylate biosynthesis) within the folate pathway with NTDs. That this impairment was also responsive to supplementation with folic acid strengthens its significance in relation to folate-responsive human NTDs. Recently, impaired *de novo* thymidylate synthesis was also confirmed in human embryos with NTDs (201) further strengthening the evidence for a causal relationship between impaired thymidylate biosynthesis and NTDs. Third, the opposing influences of methionine and thymidine supplementation on NTD outcomes in this model again suggests that maintaining the proper balance between methionine synthesis and nucleotide biosynthesis is critical for proper neural tube closure. Lastly, Wlodarczyk and colleagues note that the supplementation method that exerted the greatest protective effect on NTD risk (i.e. 200mg/kg dietary supplementation) also resulted in 6-fold increase in the number of resorptions. These findings emphasize potential negative outcomes associated with elevated folate intake in the absence of genetic and/or other risk factors.

Cited2. Mutation of Cited2, a member of family of nuclear transcriptional activators, causes exencephaly in 80% of homozygous embryos. Barbera et al (202) demonstrated that maternal periconceptional supplementation with moderate doses of folic acid could prevent 75% of NTDs in homozygotes. Impaired *de novo* thymidylate biosynthesis was investigated in cultured embryos using the dU suppression test;

however, despite responsiveness to folic acid supplementation, Cited2 mutants did not display impaired thymidylate biosynthesis. No other metabolic outcomes were evaluated in this study. Characterization of the cellular defect underlying exencephaly in the Cited2 mutant implicated massive cell death in the rostral neuroepithelium. Interestingly, cell death was not rescued by folic acid supplementation, indicating that folic acid was preventing NTDs by some other unidentified mechanism. In the absence of an impairment in folate-dependent thymidylate synthesis, these data suggest the possibility that folate may be exert a protective effect independent of one-carbon metabolism. At a cellular level, the authors suggest that folic acid may be acting to stimulate cell proliferation in an effort to compensate for massive cell death.

Crooked Tail (Cd). The crooked tail (Cd) mutant was identified as a spontaneous mutation that produced a kinked tail in heterozygous progeny and exencephaly in homozygotes. Folate-responsiveness was investigated in the Cd mutant (203) using a series of folic acid doses within a range comparable to those recommended for prevention of NTDs in humans. Exencephaly in Cd homozygotes showed a dose-dependent response to maternal supplementation with folic acid, with a 59% protective effect akin to that observed with human NTDs. Recently, Carter and colleagues mapped the location of the Cd mutation to the gene LRP6, encoding a co-receptor within the Wnt signaling pathway (204). The Cd mutation has been characterized as a single nucleotide missense mutation that alters regulation of Wnt signaling. This is the first evidence indicating that an essential developmental signaling pathway interacts with folate status. It remains to be determined 1) whether the Cd mutation is directly associated with impairments in folate-mediated on carbon metabolism, and 2) whether folate supplementation rescues NTD phenotype by rescuing metabolic impairments. Recent investigation of folate and homocysteine

levels in adult Cd mice did not reveal obvious metabolic impairments, although the similarity in metabolic profile of Cd homozygous mice as compared to wild-type and heterozygous littermates on folate-deficient diets suggests a defect in intracellular folate utilization (205). Further investigation of the interaction between the Cd mutation and folate status should provide novel insights into a potential role of folate metabolism in developmental signaling and/or the effects of altered developmental signaling on folate-mediated one-carbon metabolism.

Cart1 and Axd. Two other mutants have demonstrated responsiveness to supplementation within the folate/methionine cycles, although there has been little examination of the basis for responsiveness in these models. Mice mutant for the Cart1 gene, which encodes cartiledge homeoprotein 1, develop exencephaly associated with loss of forebrain mesenchyme. Intraperitoneal injection of pregnant females with folic acid around the time of neural tube closure prevented about 80% of NTDs in a small sample of homozygous embryos, although neither the cellular or metabolic bases for this robust effect were explored (206). Further, the dependence of the phenotype on mesenchymal integrity, not neuroepithelial integrity, and the absence of Cart1 expression in the neuroepithelium makes this model less appealing for studying the role of folate-mediated one-carbon metabolism in neural tube closure.

Another NTD mutant, the Axd mutant, demonstrates responsiveness to supraphysiological doses of methionine, but not to supraphysiological doses of folic acid and vitamin B12 (207). Variable NTD phenotypes ranging from spina bifida to curly tail in embryos from heterozygous Axd mating were prevented by almost 50% in dams supplementated with i.p. injections of 180mg/kg methionine during the period of neurulation. Due to the extremely high doses used in these studies, relevance to human supplementation is limited. Still, the responsiveness of the NTD phenotype in Axd mutants to methionine supplementation, but not to folate supplementation,

provides additional support for the requirement of balancing flux of one-carbon units between the thymidylate and methionine biosynthetic pathways.

Conclusions

In summary, despite decades of research exploring the relationship between folate nutriture, genetic variation, and NTDs, we are still mostly unaware of the metabolic and/or genomic mechanism(s) by which alterations in folate metabolism affect neural tube closure. Data from genetic studies and studies of animal models thus far indicate that disruption in both nucleotide biosynthesis and cellular methylation may underlie NTD pathogenesis, although concrete evidence regarding the specific metabolic and/or genomic defects is lacking. In addition, we know relatively little concerning how metabolic/genomic outcomes influence cellular processes during neural tube closure. Because folate-dependent anabolic reactions compete for folate-derived one-carbon units, it may be that under conditions of low-folate status, a sensitive regulation of flux is required to achieve normal neurulation. Further exploration of human SNPs in a context of gene-nutrient and gene-gene interactions may provide greater insight into how genetic alterations in folate-related genes affect flux of one-carbon metabolism and thereby contribute to NTD risk. In addition, continued investigation of folate metabolism and folate responsiveness in mouse models will yield more information regarding metabolic derangement underlying failure of neural tube closure. Although several of the mouse models in which folate-related genes have been mutated exhibit early pre-neurulation lethality, exploration of heterozygous KO mice under conditions of low folate status may provide a better model of human genetic variation and deficiency. Lastly, manipulation of genes known to regulate flux of folate-mediated one-carbon

metabolism, i.e. cSHMT, may provide more opportunity for determining the metabolic and/or genomic underpinning of folate-responsive NTDs.

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CHAPTER 2

INSIGHTS INTO METABOLIC MECHANISMS UNDERLYING FOLATE-RESPONSIVE NEURAL TUBE DEFECTS: A MINI-REVIEW

Abstract

Neural tube closure defects (NTDs), including anencephaly and spina bifida, arise from the failure of neurulation during early embryonic development. NTDs are common birth defects with a heterogenous and multifactorial etiology with interacting genetic and environmental risk factors. Although the mechanisms resulting in failure of neural tube closure are unknown, up to 70% of NTDs can be prevented by maternal folic acid supplementation. However, the metabolic mechanisms underlying the association between folic acid and NTD pathogenesis have not been identified. This review summarizes our current understanding of the mechanism(s) by which impairments in folate metabolism might ultimately lead to failure of neural tube closure, with an emphasis on untangling the relative contributions of nutritional deficiency and genetic risk factors to NTD pathogenesis.

Introduction

Failure of the neural tube to fuse in its entirety during early embryogenesis results in a cluster of common developmental anomalies known as neural tube closure defects (NTDs). NTDs usually present with herniation and exposure of nervous tissue in the cranial region (termed anencephaly) or the spinal region (termed spina bifida). These malformations are severe, irreversible and debilitating; anencephaly is incompatible with postnatal survival whereas spina bifida results in a life-long

disability and often necessitates multiple surgical interventions. Although the morphogenic processes underlying both normal neurulation and its failure continue to be an area of active investigation, the ultimate causes and the associated biological mechanisms of neural tube closure defects in mammals remain unknown. Over the past two decades, numerous genetic and environmental risk factors have been identified; however, the strongest association to date is that between the B-vitamin folate and NTD risk. Early clinical observations in the 1960's led to an understanding that reduced maternal folate status was associated with elevated NTD risk (1-3). Subsequent studies identified elevated maternal homocysteine, which is a biomarker of impaired folate status and/or metabolism, as a risk factor for NTDs (4,5). Later, randomized control trials and population-wide fortification initiatives (6-8) verified the efficacy of folic acid supplementation in reducing both NTD occurrence (9) and recurrence (10) by up to 70%. Despite several decades of epidemiological research indicating that folate is intimately linked to NTD risk, the metabolic mechanisms underlying the pathogenesis of folate-responsive NTDs have yet to be identified.

Although maternal folate status is linked to NTD risk, certain individuals within populations are at greater risk than others independent of folate status, indicating that folate deficiency alone is not sufficient to cause an NTD-affected pregnancy. A genetic component to NTDs has been long recognized; family history is one of the strongest risk factors for NTDs (11). In addition, NTDs are more common among certain ethnic groups and in individuals with a previous NTD-affected pregnancy (12). Because folate status contributes to NTD risk, investigation of genetic risk factors in humans has focused primarily on genetic variation within genes that encode proteins that bind, transport, process and/or metabolize folate, with the assumption that genetically-induced alterations in folate status and/or metabolism are likely to contribute to NTD pathogenesis. Although polymorphisms have been

identified in folate-related genes that contribute to risk for an NTD-affected pregnancy, the total genetic variation identified to date that contributes to NTD risk does not account for the overall genetic contribution to NTD incidence observed in human populations. Commonly, epidemiological studies focus on synergistic gene-diet interactions, and the identification of polymorphisms within folate-related genes that interact with low folate status to confer risk for developmental anomalies including NTDs.

The profile of NTD pathogenesis is emerging as an interaction between predisposing genetic factors with primary or secondary nutrient deficiencies (Figure 2.1). Although it was originally hypothesized that maternal folate supplementation lowered risk for NTDs by correcting a primary folate deficiency, it has become apparent that this explanation may not account for many cases of NTD prevention. Furthermore, folic acid supplementation may prevent NTDs even in the absence of overt maternal folate deficiency, because most women with an NTD-affected pregnancy are not folate deficient (13-15). Increased folate intake, in the form of folic acid supplements or fortified food, may compensate for genetically-linked impairments in folate utilization and/or secondary nutrient deficiencies, not correct folate deficiency per say. Thus, investigations into gene-nutrient interactions that result in NTD pathogenesis now distinguish among: 1) single gene variants that affect folate status alone, 2) single gene variants that affect folate utilization and/or metabolism, and 3) single gene variants that affect both folate status and metabolism/utilization. Impairments in folate status can be the result of dietary folate deficiency, but can also result from genetic variation that impacts cellular folate accumulation including its absorption, cellular transport,

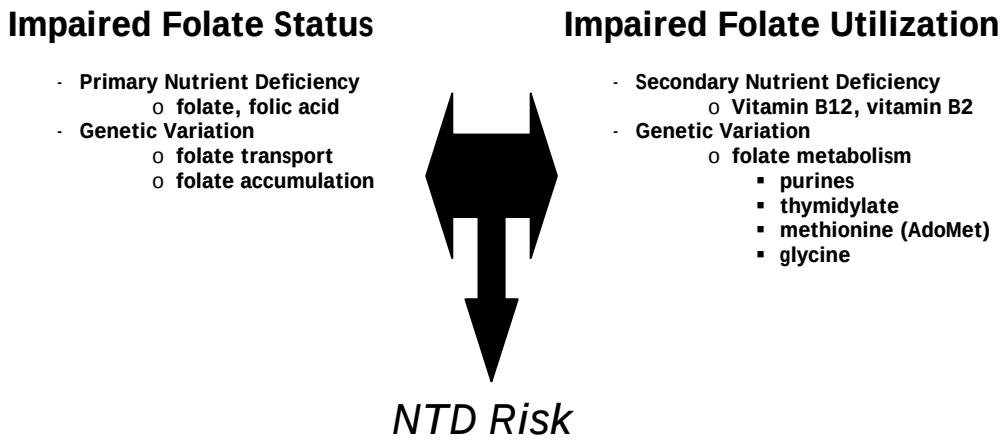

Figure 2.1. **Gene nutrient interactions in neural tube closure defects.** Low folate status interacts with impairments in one-carbon metabolism (folate utilization) to create risk for NTDs. Both folate status and folate utilization are compromised by interactions among genetic and environmental (nutritional) factors.

processing, retention and degradation (16). Likewise, impairments in folate utilization/metabolism can result from genetic variation that affects the activity and/or stability of folate-dependent metabolic enzymes, but can also result from secondary deficiencies of nutrients intimately linked to folate metabolism, such as vitamin B12 and choline. Thus, both genetic risk factors and nutrient deficiencies contribute to impairments in folate status and/or impairments in folate utilization (Figure 2.1). Untangling the relative contributions of the genetic and nutritional components of NTD risk will be required to identify the specific biological pathways that lead to NTD pathogenesis, which will enable the design of better targeted and efficacious interventions for NTD prevention. In this review, our current understanding of the mechanisms underlying NTD pathogenesis in the context of folate-mediated one-carbon metabolism is summarized.

Folate-mediated One Carbon Metabolism

In the cell, folates function as a family of metabolic cofactors that carry and chemically activate single carbons, referred to as one-carbon units, for a variety of anabolic and catabolic reactions collectively known as folate-mediated one-carbon metabolism (OCM) (Figure 2.2). Folate-activated one-carbons are carried by tetrahydrofolate (THF), the metabolically active form of folates. THF carries one-carbons at three different oxidation states ranging from formaldehyde to methanol, and the one-carbon forms of folate can be interconverted enzymatically (17-19). Cellular folate cofactors also contain a poly-γ-glutamate peptide that varies in length in cells from 3 to 9 glutamate residues. In the gut, the poly-γ-glutamate peptide is hydrolyzed leaving monoglutamated folate derivatives that are transported across the intestinal mucosa. Folate monoglutamates, predominately in the form of 5-methylTHF, are present in serum and transported into cells. Both retention of folate in the cell, and

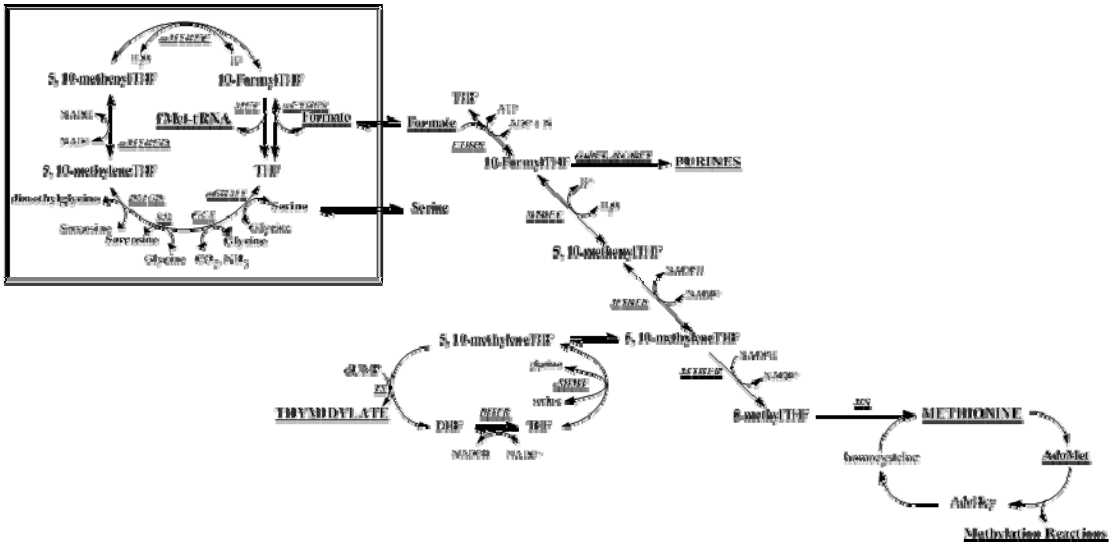

Figure 2.2: **Compartmentation of folate-mediated one-carbon metabolism in the cytoplasm and mitochondria.** One-carbon metabolism in the cytoplasm is required for the *de novo* synthesis of purines and thymidylate, and for the remethylation of homocysteine to methionine. One-carbon metabolism in mitochondria is required to generate formate for one-carbon metabolism in the cytoplasm, to generate the amino acid glycine, and to synthesize formylmethionyl-tRNA for protein synthesis in mitochondria. FTHFS, 10-Formyltetrahydrofolate Synthetase; MTHFC, Methenyltetrahydrofolate Cyclohydrolase; MTHFD, Methylenetetrahydrofolate Dehydrogenase; MTHFR, Methylenetetrahydrofolate Reductase; GARFT, Phosphoribosylglycinamide Formyltransferase; AICARFT, Phosphoribosylaminoimidazolecarboxamide formyltransferase; FTCT, Glycine Formiminotransferase/ Formimidoyltetrahydrofolate Cyclodeaminase & Glutamate Formiminotransferase/ Formimidoyltetrahydrofolate Cyclodeaminase; cSHMT, Cytoplasmic Serine Hydroxymethyltransferase; TS, Thymidylate Synthase; DHFR, Dihydrofolate Reductase; MS, Methionine Synthase, mSHMT, Mitochondrial Serine Hydroxymethyltransferase; GSC, Glycine Cleavage System; SD, Sarcosine Dehydrogenase; DMGD, Dimethylglycine Dehydrogense; mMTHFD, Mitochondrial Methylenetetrahydrofolate Dehydrogenase; mMTHFC, Mitochondrial Methenyltetrahydrofolate Cyclohydrolase; M-tRNA-FT, Methionyl-tRNA Formyltransferase; mFTHFS, Mitochondrial Formyltetrahydrofolate Synthetase.

their conversion to functional cofactors, requires the reestablishment of the poly- γ - glutamate peptide (20,21).

Folate-mediated one-carbon metabolism is a metabolic network of interdependent pathways that is compartmentalized in the mitochondria, the cytoplasm, and the nucleus. Folate metabolism in mitochondria is required for the production of formate, glycine and ^{fmet}tRNA from the catabolism of choline, serine and glycine (18,22). Once formed, formate traverses from the mitochondria to the cytoplasm where it is serves as a primary source of one-carbon units for cytoplasmic OCM. Folate-mediated one-carbon metabolism in the cytoplasm is essential for: 1) *de novo* purine biosynthesis, 2) *de novo* thymidylate biosynthesis, and 3) the remethylation of homocysteine to form methionine. Methionine is required for the biosynthesis of S-adenosylmethionine (AdoMet), which is a cofactor that serves as the universal one-carbon donor for cellular methylation reactions including methylation of chromatin, proteins, and lipids, and other small molecules (19,20). During S-phase, the enzymes that constitute the *de novo* thymidylate biosynthesis cycle, serine hydroxymethyltransferase, thymidylate synthase and dihydrofolate reductase, are modified by the small ubiquitin-related modifier (SUMO) and transported to the nucleus for nuclear thymidylate biosynthesis (23,24). Impairments in folate-mediated OCM can result from diminished folate status, single nucleotide polymorphisms in genes that encode folate-metabolizing enzymes, or secondary micronutrient deficiencies that alter folate status, including other B vitamins (25-27). Biomarkers of impaired one-carbon metabolism include diminished capacity to synthesize thymidylate *de novo* leading to increased uracil content into DNA (28), elevated serum homocysteine (29), and DNA hypomethylation (30,31) .

Folate Transport

Sources of dietary folates include food folates, which contain a polyglutamate peptide, and folic acid, a synthetic dietary supplement and fortificant. Folic acid is a monoglutamic and oxidized form of folate that, unlike natural reduced folates, is chemically stable. Most folic acid is readily absorbed and converted to THF within the enterocytes where it becomes chemically indistinguishable from natural food folates (32). Polyglutamated food folate derivatives must first be converted to monoglutamate derivatives by the enzyme folylpoly-γ-glutamate carboxypeptidase II (FGCP; gene name, GCPII) in the gut prior to absorption through the intestine (33), which is accomplished through a recently identified intestinal folate receptor (PCFT) (34). Serum folates are present in the form of monoglutamated 5-methyl THF, which is taken up at the cell surface either by the reduced folate carrier (RFC), a facilitative anion-exchange carrier, or by an endocytotic process mediated by one of two folate receptors, FRα and FRβ (35). The folate receptor is a membrane-anchored receptor with a GPI-moiety that has a high affinity for 5-methyl THF (35). Once inside the cell, polyglutamation of folate cofactors is catalyzed by the enzyme folylpoly-γ-glutamate synthetase (FPGS), resulting in the sequestration of folates within the cell. The polyglutamate peptide also increases the affinity of THF cofactors for folate-dependent enzymes (17,19). Within the cell, 5-methylTHF is the most abundant folate derivative.

Studies to date have not provided conclusive evidence that common polymorphisms within genes that encode proteins that mediate folate transport and absorption affect folate status. There have not been any variants identified within the coding regions of the folate receptor genes, and investigations of polymorphisms in non-coding regions of FRα and FRβ have not yielded an association with NTD risk (36-39).. Coding variants within genes that impair folate transport and accumulation

may not be compatible with life and result in embryonic lethality. Nonetheless, genetic variation within the folate receptor genes does not confer risk for NTDs in humans. A common single nucleotide polymorphism (SNP) in the RFC1 gene has shown a moderate association with NTD risk under conditions of folate deficiency (40-42), although with low penetrance. With regard to genes encoding proteins that mediate folate processing, no polymorphisms have been identified within the gene encoding FPGS that confer risk for NTDs in human populations. Similarly, a single variant identified in GCPII does not affect NTD risk in humans (43). Thus, there is little evidence that genetic variation disrupts folate transport, absorption, processing and retention to a degree that independently contributes to NTD risk in human populations. However, given that NTDs are complex traits, genetic alterations of these processes might exacerbate nutritional deficiencies or metabolic impairments, and thereby sensitize population subgroups with genetically-induced impairments in one-carbon metabolism.

A definitive and causal role of embryonic folate deficiency in NTD pathogenesis has been demonstrated by the observation that genetic deletion of the gene encoding FRα, Folr1, results in NTDs (44). However, investigation of one-carbon metabolism in this mouse model has not provided mechanistic information on the identity of the one-carbon pathway component that underlies NTD pathogenesis. There are no differences observed in homocysteine levels in FolR1$^{+/-}$ dams maintained on a normal diet (44), nor are there any differences in DNA methylation in FolR1$^{+/-}$ embryos or FolR1$^{-/-}$ embryos rescued to GD15.5 with folinic acid (45). Furthermore, that NTDs in nullizygous FolR1 KO embryos can be rescued by maternal supplementation with folic acid (44) provides further support for the concept that genetic disruption of folate transport affects folate status in the absence of alterations in folate utilization, because reductions in transport capacity can be overcome by

maternal vitamin supplementation. A similar finding has been observed with deletion of the gene encoding RFC in mice. Deletion of RFC results in early embryonic lethality; however, embryonic survival can be rescued up until GD12 by maternal folic acid supplementation (46). Collectively, these data suggest that adequate folate status mediated by cellular folate uptake is required for embryonic development and neural tube closure. However, in the absence of an association between human genes involved in folate uptakes and NTD risk, these models do not provide further insight into specific causes that underlie human folate-responsive NTDs.

Folate Metabolism in the Mitochondria

The primary roles of folate metabolism in mitochondria is to generate formate and glycine from the enzymatic cleavage of serine; in certain tissues glycine can also be catabolized to generate formate through mitochondrial folate metabolism (47). This pathway is initiated by the mitochondrial isoform of serine hydoxymethyltransferase (mSHMT; gene name *Shmt2*), which catalyzes the conversion of serine and THF to form glycine and methylene-THF. Methylene-THF can also be synthesized from glycine by the glycine cleavage system (GCS) (48), as well as from of the catabolism of sarcosine and dimethylglycine (49). Methylene-THF is oxidized to produce methenyl-THF in a reaction catalyzed by methylenetetrahydrofolate dehydrogenase (MTHFD), which is subsequently hydrolyzed to 10-Formyl THF by the enzyme methenyltetrahydrofolate cyclohydrolase (MTHFC). The formyl group of 10-formyl-THF is hydrolyzed to generate free formate and THF to complete the cycle, in a reaction catalyzed by formyltetrahydrofolate synthetase (FTHFS) (50). Formate then traverses into the cytoplasm and serves as a major source of one-carbon units for cytoplasmic OCM. Some of the genes encoding the enzymes that catalyze the generation of formate from 5,10-methylene THF in the mitochondria have yet to be identified (47).

To date, there have been few reports and little investigation into the role of mitochondrial folate metabolism in NTD pathogenesis, this includes investigations of human genetic susceptibility and genetically manipulated mouse models. One limitation is the paucity of knowledge regarding the identity of the genes and enzymes that regulate OCM in the mitochondria, and the degree to which the capacity of mitochondrial OCM, including formate production, affects cytoplasmic OCM. Furthermore, mitochondrial OCM plays different metabolic roles in different tissues and cell types. Whereas it has been shown in certain cell types that mitochondrial OCM is an essential source of glycine (47), it remains to be established definitively that formate derived from mitochondrial OCM is essential for cytoplasmic OCM. Recently, a mouse KO model of the gene encoding the cytoplasmic SHMT isoform (cSHMT; *Shmt1*) was generated and was shown to be both viable and fertile (51). The viability of this mouse model indicates that one-carbon units generated in the cytoplasm by cSHMT, through the expression of *Shmt1*, are not essential for growth and survival. This mouse models emphasizes the importance of mitochondrial OCM in the production of one-carbon units for folate-dependent anabolic reactions in the cytoplasm. Further investigation into the role of mitochondrial OCM in regulating folate-dependent anabolic pathways in the cytoplasm is warranted. In addition, exploration of human SNPs in mitochondrial folate-dependent enzymes and the creation of mouse models with disruptions in genes encoding the mitochondrial folate pathway will shed light on the potential contribution of mitochondrial folate metabolism to NTD pathogenesis.

Folate Metabolism in the Cytoplasm

Cytoplasmic OCM is essential for the *de novo* biosynthesis of nucleotides as well as AdoMet-dependent cellular methylation reactions (Figure 2.2). The purine

biosynthesis pathway utilizes the cofactor 10-formylTHF as the one-carbon donor for the #2 and #8 carbons in the purine ring. *De novo* thymidylate biosynthesis utilizes methylene-THF as a cofactor for the methylation of dUMP to form dTMP, in a reaction catalyzed by the enzyme thymidylate synthase (TS). The one-carbons carried by 10-formyl-THF and methylene-THF can be generated in the cytoplasm from formate via the action of the ATP- and NADPH-dependent trifunctional enzyme which contains FTHFS/MTHFC/MTHFD activities encoded by the *MTHFD1* gene. Methylene-THF can also be synthesized from the PLP-dependent enzymatic conversion of serine and THF, catalyzed by the enzyme cytoplasmic SHMT (cSHMT, gene name *Shmt1*), a reaction that also generates glycine (Figure 2.2). Methylene-THF can be irreversibly reduced by the enzyme methylene-THF reductase (MTHFR) to yield 5- methylTHF, which serves as the cofactor for the B-12 dependent remethylation of homocysteine to form methionine, catalyzed by methionine synthase (MS; gene name *MTR*). Methionine can be adenosylated to form S-adenosylmethionine (AdoMet), which is the one carbon donor for cellular methylation reactions. The transfer of the one-carbon from AdoMet yields the intermediate S-adenosylhomocysteine (AdoHcy), which is hydrolyzed to homocysteine and adenosine by the enzyme S-adenosylhomocysteine hydrolase (SAHH).

Although much is known about OCM and its anabolic pathways in the cytoplasm, deciphering the causal metabolic pathway associated with NTD risk has been challenging. Within the cell, the concentration of folate-binding proteins and enzymes far exceeds the concentration of folate cofactors, and thus all cellular folate is protein bound (16). This indicates that folate-dependent anabolic reactions in the cytoplasm compete for a limiting pool of folate-derived one-carbon units and folate cofactors (16,52). This competition is greatest for the two anabolic reactions that utilize 5,10-methyleneTHF, thymidylate biosynthesis and homocysteine remethylation

(leading to AdoMet biosynthesis). These two pathways are sensitive to folate deficiency, and therefore it is challenging to determine independently the impact of either thymidylate synthesis or homocysteine remethylation (and cellular methylation reactions) on NTD risk. It has been suggested by mathematical modeling (53) and experimental data (52) that under normal cellular conditions, the biosynthesis of 5-methylTHF is favored over the biosynthesis of thymidylate.

Purine Biosynthesis

Rapid cell proliferation is essential for neural tube closure, thereby requiring increased rates and dependence on *de novo* nucleotide biosynthesis. However, although the thymidylate biosynthesis and homocysteine remethylation pathways are highly sensitive to folate status, the purine biosynthetic pathway appears to be less sensitive to conditions of folate deficiency (54). The "higher priority" conferred to purine biosynthesis within the OCM network may be due to the dependency of most organisms on maintenance of *de novo* purine biosynthesis for development and survival. The purine biosynthetic machinery utilizes exclusively 10-formyl-THF as the folate cofactor for the biosynthesis of the purine ring. In addition to 10-formylTHF, there are two other folate derivatives found in cells at the level of oxidation of formate: 5-formyl-THF and 5,10-methenyl-THF. These folate derivatives participate in a futile cycle that involves the SHMT-catalyzed synthesis of 5-formyl-THF from methenyl-THF, and the ATP-dependent conversion of 5-formyl-THF back to methenyl-THF by the enzyme methenyl-THF synthetase (MTHFS) (55,56). Although neither of these folate derivatives serve as substrates for folate-dependent anabolic reactions, recent evidence indicates that regulation of MTHFS and 5-formyl levels may impact cellular folate accumulation and regulate purine biosynthesis. Anguera et al (57) demonstrated that increased MTHFS expression depleted cellular

folate concentration, and Field et al (54) showed that MTHFS expression enhanced purine biosynthesis through the sequestration and potential shunting of 10-formyl-THF into the purine biosynthetic pathway. Furthermore, the binding of 10-formylTHF to MTHFS inhibits 5-formyl-THF metabolism, enabling its accumulation. In this regard, 5-formylTHF may function as a storage form of folate in the cell that can be quickly mobilized for purine biosynthesis (54). Thus, ongoing investigations of *de novo* purine biosynthesis and its regulation are elucidating mechanisms by which purine biosynthesis is protected within the OCM network even during periods of folate deficiency. Disruptions in synthesis, utilization or distribution of the formyl folate cofactors by *MTHFD1* and *MTHFS* could therefore potentially alter purine biosynthesis and/or folate status.

Information regarding the potential contribution of folate-dependent enzymes that regulate or provide substrates for purine biosynthesis to NTD risk is very limited because very few variants within these enzymes have been investigated in relation to NTDs in humans. There have been no SNPs identified within the gene encoding MTHFS that have been investigated in relation to NTDs, although the data described above warrant such investigation. The most studied variant associated with purine biosynthesis is the R653Q transition in *MTHFD1*. A strong association has been found between *MTHFD1* R653Q and an increased maternal risk for NTDs (58-60), although negative results have also been reported (61,62). Despite this strong association, the metabolic basis for increased risk associated with the SNP has not been elucidated. The R653Q SNP does not appear to affect total folate levels (58), arguing against an effect mediated by changes in folate status. Furthermore, the SNP has not been found to be associated with elevated plasma homocysteine (58,63), suggesting that metabolic disruption is likely located at the level of purine and/or thymidylate biosynthesis. Further investigation of the biochemical effect of the SNP

on enzyme function and cellular folate utilization may provide insight into the mechanism by which this SNP confers NTD risk. In addition, the use of animal models to elucidate the effects of *MTHFD1* deficiency on folate status and utilization will be useful in ascertaining the role of this potential genetic component in NTD pathogenesis.

Thymidylate Biosynthesis

Thymidylate synthase (TS) catalyzes the transfer of a one-carbon moiety from 5,10-methylene-THF to uridylate (dUMP) to form thymidylate (dTMP) *de novo*. In this reaction, folate serves both as a source of an activated one-carbon, but also as a source of reducing equivalents. The substrate 5,10-methylene-THF is synthesized both from the MTHFD-catalyzed reduction of methenyl-THF, and by the PLP-dependent conversion of serine and THF to form glycine and methyleneTHF, catalyzed by cSHMT (Figure 2.2). The transfer of the one-carbon from methylene-THF to dUMP also involves the oxidation of THF to dihydrofolate, which is recycled back into folate pools by the enzyme dihydrofolate reductase (DHFR). Decreased capacity to synthesize thymidylate *de novo* due to folate deficiency is associated with increased rates of uracil misincorporation into DNA (28). Uracil misincorporation can result in increased genomic instability (64,65). Other instances of genomic instability, including telomeric and centromeric instability, have been reported to cause NTDs in mice (66-68). In addition, impairments in *de novo* thymidylate biosynthesis affect DNA replication, reducing proliferative capacity essential for neurulation.

Recent evidence suggests that the partitioning of one-carbon units towards *de novo* thymidylate biosynthesis represents a point of sensitive regulation within cytoplasmic OCM. The thymidylate biosynthesis machinery, including cSHMT, TS and DHFR, was shown to translocate to the nucleus at S-phase following modification

of the enzymes with the small ubiquitin-like modifier (SUMO) (23,24). Compartmentation of *de novo* thymidylate biosynthesis in the nucleus enables the preferential shunting of cSHMT-derived one carbon units into the thymidylate biosynthesis pathway. Other studies have demonstrated that cSHMT activity is rate limiting for *de novo* thymidylate biosynthesis (69). In addition to stimulating thymidylate biosynthesis, cSHMT also regulates 5-methyl-THF utilization in the cytoplasm (69). 5-MethylTHF is bound tightly by cSHMT, preventing its use for the remethylation of homocysteine to methionine, which impairs AdoMet biosynthesis and ultimately cellular methylation reactions (Figure 2.2). Therefore, cSHMT mediates the competition between thymidylate biosynthesis and homocysteine remethylation for folate-activated one-carbons. In cells that do not express cSHMT, data indicate that the partitioning of one-carbon units in the form of methylene-THF is favored in the direction of the generation of 5-methylTHF at the expense of thymidylate biosynthesis (52,53). However, increased cSHMT expression favors one-carbon flux in the direction of thymidylate biosynthesis at the expense of cellular methylation. Recently, our lab generated a mouse model with a null cSHMT allele that confirms the metabolic role of cSHMT (51). Loss of cSHMT expression does not affect folate status, but does result in an enhanced methylation potential (as indicated by the hepatic AdoMet/AdoHcy ratio) and increased uracil levels in nuclear DNA. Together, these data indicate that cSHMT regulates folate utilization by balancing the partitioning of one-carbon units between thymidylate biosynthesis and cellular methylation, in the absence of effects on folate status.

Investigation of the thymidylate biosynthesis pathway as a genetic risk factor for NTDs in humans has been inconclusive. The only human SNPs in the TS gene that have been investigated in relation to human NTD risk are present in non-coding regions, and the only two studies have yielded conflicting findings (70,71). The effect

of a common SNP in the promoter/enhancer region of the TS gene has yet to be clearly delineated, and the effect of altered TS expression on OCM and folate status have yet to be investigated in a mouse model. As with genes involved in purine biosynthesis, it is likely that mutations within the TS gene that markedly affect function are associated with gestational lethality. Recently, a non-coding deletion allele in the gene encoding DHFR was investigated in relation to NTDs; however, results from three studies have been inconclusive in determining a role for DHFR in mediating NTD risk (72-74). A handful of studies have investigated the effect of the common L474F polymorphism within the gene encoding cSHMT in relation to NTD risk. However, the scope and size of the studies thus far has been inadequate to fully determine a potential association with NTD risk (75-77). Interestingly, the biochemical effect of the L474F cSHMT variant has recently been determined. The L474F variant is impaired in its ability to undergo SUMO modification and SUMO-dependent nuclear localization (23). These data suggest that the SNP may interfere with the ability of cSHMT to preferentially partition one-carbon units to thymidylate biosynthesis, ultimately modifying the utilization of folate cofactors in the cytoplasm. Further investigation of the SNP in relation to NTDs in humans should shed light on the importance of cSHMT-mediated regulation of folate utilization in human NTD risk.

Although the incidence of NTDs in response to genetic deletion of folate-related genes mediating *de novo* thymidylate biosynthesis in mice has not yet been explored, there is some evidence from other folate-responsive mouse models to suggest that impairments in *de novo* thymidylate biosynthesis might underlie NTD pathogenesis. Fleming and Copp (78) investigated metabolic alterations and folate responsiveness in the *splotch* mouse model of NTDs. Homozygous *splotch* embryos, which display a completely penetrant NTD phenotype, exhibited impairments in *de*

novo thymidylate biosynthesis as evidenced by a reduction in dU suppression values. Supplementation of culture media with either folic acid or thymidine prevented NTDs in homozygous *splotch* embryos, directly implicating impairments in thymidylate biosynthesis in NTD pathogenesis in this folate-responsive mouse model. Surprisingly, supplementation of cultured *splotch* embryos with methionine exacerbated impairments in dU suppression and caused NTDs in heterozygous *splotch* embryos, which do not otherwise develop NTDs. Rescue of NTDs in homozygous *splotch* embryos by folic acid supplementation and exacerbation by methionine supplementation was also observed in another study *in vivo* (79). More recently, impaired de novo thymidylate biosynthesis was also observed in human embryos with NTDs (80). Collectively, these data provide preliminary evidence that *de novo* thymidylate biosynthesis is crucial for proper neural tube closure. In addition, maintaining a balance of utilization between various folate-dependent anabolic pathways in the cytoplasm (e.g. methionine biosynthesis and thymidylate biosynthesis) may influence neural tube closure via direct effects on the thymidylate biosynthesis pathway.

Homocysteine remethylation/methylation cycle

Much of the focus on identifying a metabolic basis for folate-responsive NTDs has centered on the role of homocysteine and the methylation cycle, consistent with the results from human epidemiological studies that have linked moderately elevated maternal homocysteine with NTD risk. Supplementation with folic acid alleviates hyperhomocysteinemia, leading to the hypothesis that impairments in the homocysteine remethylation cycle were causal in NTD pathogenesis. In addition to being a biomarker for impaired folate status and reduced methylation potential (the AdoMet/AdoHcy ratio), homocysteine at elevated levels is cytotoxic (81,82), and also

negatively affects cellular methylation through the accumulation of AdoHcy, which is an inhibitor of cellular methylation reactions (83-85). Altered gene expression resulting from chromatin hypomethylation has been proposed as a mechanism underlying many folate-related pathologies, including NTDs (86). Likewise, the direct modification of proteins by homocysteine has also been implicated in these developmental anomalies (87). The regulation of homocysteine remethylation and AdoMet biosynthesis is complex, and includes feedback mechanisms that prevent both the accumulation of homocysteine and maintenance of both AdoMet and AdoHcy concentrations for cellular methylation reactions (84). For example, AdoMet is an allosteric, feedback inhibitor of MTHFR (88,89), and its accumulation inhibits MTHFR activity, thereby preventing the synthesis of 5-methylTHF from the methylene-THF cofactor when AdoMet levels are adequate for cellular methylation reactions.

In addition to the folate-dependent remethylation of homocysteine to methionine, two other folate-independent pathways exist that function to prevent the accumulation of homocysteine in certain tissues and cell types: homocysteine can be remethylated to methionine by the transfer of a one-carbon moiety from betaine, catalyzed by the enzyme betaine homocysteinemethyltransferase (BHMT), and homocysteine can be eliminated via the transulfuration pathway, in which cystathionine β-synthase (CBS) catalyzes the condensation of serine and homocysteine to form cystathionine. Transulfuration simultaneously degrades homocysteine while removing a source for methionine biosynthesis in the cell. It is important to note that BHMT and CBS are not expressed in all tissues (84,90,91), whereas folate-dependent methionine synthase expression is more ubiquitous (91). The activities of the BHMT-mediated methylation pathway and the transsulfuration pathway are also regulated via feedback mechanisms associated with the cellular

methylation cycle; accumulation of AdoMet inhibits BHMT-mediated methionine biosynthesis while stimulating CBS-mediated transulfuration (92).

Because of the strong association between elevated homocysteine and NTD risk, genes involved in homocysteine metabolism and the methylation cycle have been at the center of the search for candidate genes involved in NTD pathogenesis. The most prominent of these is MTHFR, for which two human SNPs have been identified that are associated with NTD risk, MTFHR C677T and MTHFR A1298C. The MTHFR C677T SNP has been well-characterized and codes for a thermolabile enzyme with a 50-60% reduction in enzymatic activity in individuals homozygous for the T allele (93-95). To date, the C677T SNP has been widely considered the most attractive genetic candidate underlying folate-responsive NTDs based on three findings: 1) it has consistently demonstrated a strong association with NTD occurrence in the human epidemiological literature, with both maternal and fetal alleles contributing to risk (96-98), 2) it is associated with elevated homocysteine, a predictor of NTD risk (99-101), and 3) it interacts with folate status in determining both impairments in metabolism and NTD risk (102-107). Furthermore, the strength of the association between the MTHFR C677T SNP and NTD risk has reciprocally bolstered the hypothesis that metabolic disruptions in homocysteine homeostasis underlie NTD pathogenesis.

Despite MTHFR having the strongest association in the literature with NTD risk, the penetrance of the SNP cannot account for NTD incidence in the population; the frequency of the T allele in the general population does not account for the level of reduction of NTD risk observed in response to folic acid supplementation (108). Furthermore, there is no direct evidence that the association between the MTHFR C677T SNP and NTD risk is related to metabolic perturbations of the homocysteine remethylation cycle. Deletion of the MTHFR gene in the mouse germ line is

associated with both elevated homocysteine as well as alterations in cellular methylation potential and global DNA hypomethylation (109) , yet does not result in NTDs *in vivo,* even in response to maternal folate deficiency (110). Therefore, there is no causal evidence that reduced MTHFR activity induces NTD risk by impairing the homocysteine remethylation cycle. However, in addition to its inhibitory effects on homocysteine remethylation, the MTHFR SNP also impairs folate status. Data from several studies have revealed that the T allele is associated with reductions in serum and red blood cell folate levels (111-114). The observed reduction of total folates in individuals with reduced MTHFR activity may result from the accumulation of the less stable formylated derivatives of folate at the expense of 5-methylTHF, resulting in increased folate turnover (115,116). Therefore, the C677T SNP may influence NTD risk by impairing folate status, as opposed to impairing folate metabolism via the methylation cycle. This suggestion has been corroborated by the finding that MTHFR KO mice also display reduced plasma folate levels, in addition to an alteration in percentage of methylated folate derivatives (117) . Because folate deficiency alone does not induce NTDs in mice (118,119), data from studies of the MTHFR KO mouse model support the notion that alterations in MTHFR activity in humans may influence neurulation indirectly via alterations in folate status and/or the distribution of folate cofactors that ultimately impinge upon other folate-dependent anabolic pathways beyond the methylation cycle.

Other studies support the conclusion that the accumulation of homocysteine is not an underlying cause of NTDs. Examination of SNPs in other genes regulating cellular homocysteine accumulation, including MS, CBS, and BHMT, has not provided convincing evidence that these genes are associated with NTD risk in humans (120-126). Similarly, mouse models with deletions of genes that regulate homocysteine accumulation fail to support the hypothesis that homocysteine

accumulation underlies NTD pathogenesis. Genetic deletion of CBS in mice produces highly elevated homocysteine levels but these mice do not exhibit NTDs (127). Another mouse model with reduced expression of the gene MTRR, which regulates methionine synthase activity, also produces a robust metabolic phenotype, including elevated plasma homocysteine, reduced plasma methionine, and tissue-specific alterations in methylation potential, but do not exhibit a developmental phenotype (128). Lastly, hyperhomocysteinemia induced in culture or *in vivo* by nutritional manipulation does not affect neural tube closure in mice (129-131). Together, these data do not provide support for a direct role of homocysteine in NTD pathogenesis, and instead indicate that elevated homocysteine is merely a biomarker for impairments in folate status which disrupt the function of the entire folate-dependent OCM network.

Notwithstanding the lack of evidence for a role of homocysteine in NTD pathogenesis, vitamin B12 status has been found to be moderately associated with NTD risk (132-134). Many of these studies have been limited by the failure or inability to statistically account for maternal folate status, which is often closely linked to maternal B12 status. Recently, the association between vitamin B12 status and NTD risk was examined in a folic acid fortified population. Vitamin B12 levels in the lowest quartile were associated with a tripling of NTD risk (133). Severe vitamin B12 deficiency impairs the activity of MS, and leads to both impairment of the homocysteine remethylation cycle as well as impaired nucleotide biosynthesis. The impairment in nucleotide biosynthesis is indirect; lack of MS activity can result in the accumulation of cellular folate as 5-methylTHF at the expense of other folate cofactors, a condition referred to as a methyl trap. The methyl trap occurs because the reduction of methylene-THF to 5-methyl-THF catalyzed by MTHFR is irreversible, and MS is the only folate-dependent enzyme to use 5-methylTHF as a cofactor (Figure

2.2). Vitamin B12 deficiency also diminishes AdoMet levels, resulting in the activation of MTHFR activity thereby enhancing the methyl trap. Therefore, vitamin B12 deficiency may influence NTD risk by altering the distribution of folate cofactors thereby affecting the entire OCM network. Future investigation of the association between vitamin B12 deficiency, cellular folate utilization, and NTD risk is required to determine the precise metabolic perturbation underlying NTD pathogenesis.

Conclusions

Although maternal folic acid supplementation/fortification is effective for the prevention of NTDs, the mechanisms underlying folate-responsive NTDs remain unknown. Continued investigation of gene-gene interactions and gene-nutrient interactions in humans will provide greater insight into the influence of genetic perturbations on both folate status and folate utilization in NTD pathogenesis. This examination of potential genetic risk factors is currently limited by gaps in knowledge of the enzymes and associated genes involved in OCM, especially in mitochondria, and the metabolic sequelae associated with specific gene-nutrient interactions. Furthermore, there is still much knowledge to be gained concerning the regulation of cellular folate status, metabolism/utilization and the impact of alterations in the flux of folates through the metabolic network on all metabolic, genomic, and cellular outcomes. As an example, it is still unclear if the C677T polymorphism in MTHFR increases human NTD risk by influencing homocysteine levels, distribution of folate cofactors in the cell, and/or depressing cellular folate status. In addition, the generation and detailed metabolic characterization of genetically altered mice that model impairments in specific folate-dependent anabolic pathways and their regulation is necessary to provide greater insight into the effects of altered folate metabolism on genomic, cellular and developmental outcomes and ultimately result in

the establishment of the underlying mechanisms of developmental anomalies. Determining the precise metabolic impairments that result in NTDs will enable the design of improved nutritional interventions that target both susceptible subgroups of the population and the metabolic pathway that causes neural tube closure defects.

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CHAPTER 3

SHMT1 AND THE METABOLIC ORIGIN OF FOLATE-RESPONSIVE NEURAL TUBE DEFECTS

Abstract

The specific metabolic disruption that causes human folate-responsive neural tube closure defects (NTDs) is unknown. In this study, disruption of the gene encoding cytoplasmic serine hydroxymethyltransferase (*Shmt1*) in mice is shown to induce NTDs. SHMT1 regulates folate metabolism by providing folate-activated one-carbons for *de novo* thymidylate biosynthesis in the nucleus. *Shmt1* is expressed in the embryonic neuroepithelium and neural crest during neural tube closure in mice and *Shmt1*$^{-/-}$ embryos exhibited exencephaly. Maternal and fetal genotype and maternal diet contributed to NTD incidence, mimicking gene-nutrient interactions contributing to folate-responsive human NTDs. *Shmt1* disruption on the *Pax3*$^{sp/+}$ background exacerbated NTD frequency and severity. *Pax3* deficiency impaired *de novo* thymidylate and purine biosynthesis, and altered levels of Shmt1 and thymidylate synthase protein. *Shmt1* is the only gene encoding a folate-metabolizing enzyme that affects neural tube closure in mice. These results provide evidence that repression of *Shmt1* expression causes NTDs by impairing thymidylate biosynthesis.

Introduction

During embryogenesis, the neuroepithelium bends, fuses and cavitates to form the embryonic neural tube through the process of neurulation, which ultimately gives rise to the central nervous system. Failure of neurulation results in a spectrum of common developmental anomalies collectively referred to as neural tube closure

defects (NTDs). The most common and severe NTDs include spina bifida, a condition caused by defective caudal neurulation resulting in herniation and exposure of spinal tissue, and anencephaly, a failure of rostral neurulation with consequent deterioration of brain tissue due to *in utero* exposure. Worldwide prevalence of human NTDs ranges from <1 to 9 per 1000 births (1).

NTDs are complex traits that result from deleterious gene-nutrient interactions. One of the strongest environmental determinants of NTD risk is maternal folate status, but the majority of NTD-affected pregnancies are not associated with overt maternal folate deficiency (2-4). Rather, nutritional folate deficiency interacts with genetic variants that compromise folate metabolism to confer increased NTD risk (5-8). Maternal folic acid supplementation reduces both NTD occurrence and recurrence (9,10). Currently, over 20 countries have taken measures to fortify their food supply with folic acid to reduce the incidence of NTDs, which has resulted in a 20-80% reduction in NTD prevalence (11-13).

The specific metabolic pathway(s) within folate-mediated one-carbon metabolism that affect neural tube closure are unknown. Folates function as a family of enzyme cofactors that carry and activate one-carbons to support a network of anabolic reactions collectively known as one carbon metabolism (OCM, Figure 3.1). Folate-mediated OCM is essential for the *de novo* biosynthesis of purines and thymidylate (dTMP), and for the remethylation of homocysteine to form methionine. Methionine can be adenosylated to form *S*-adenosylmethionine (AdoMet), the universal methyl donor for numerous cellular methylation reactions, including chromatin methylation (Figure 3.1). Impairments in OCM can be caused by low folate status resulting either from dietary folate deficiency or genetic variations that disrupt cellular folate accumulation or uptake. OCM can also be impaired by genetic variations or secondary nutrient deficiencies that affect the activity and/or stability of

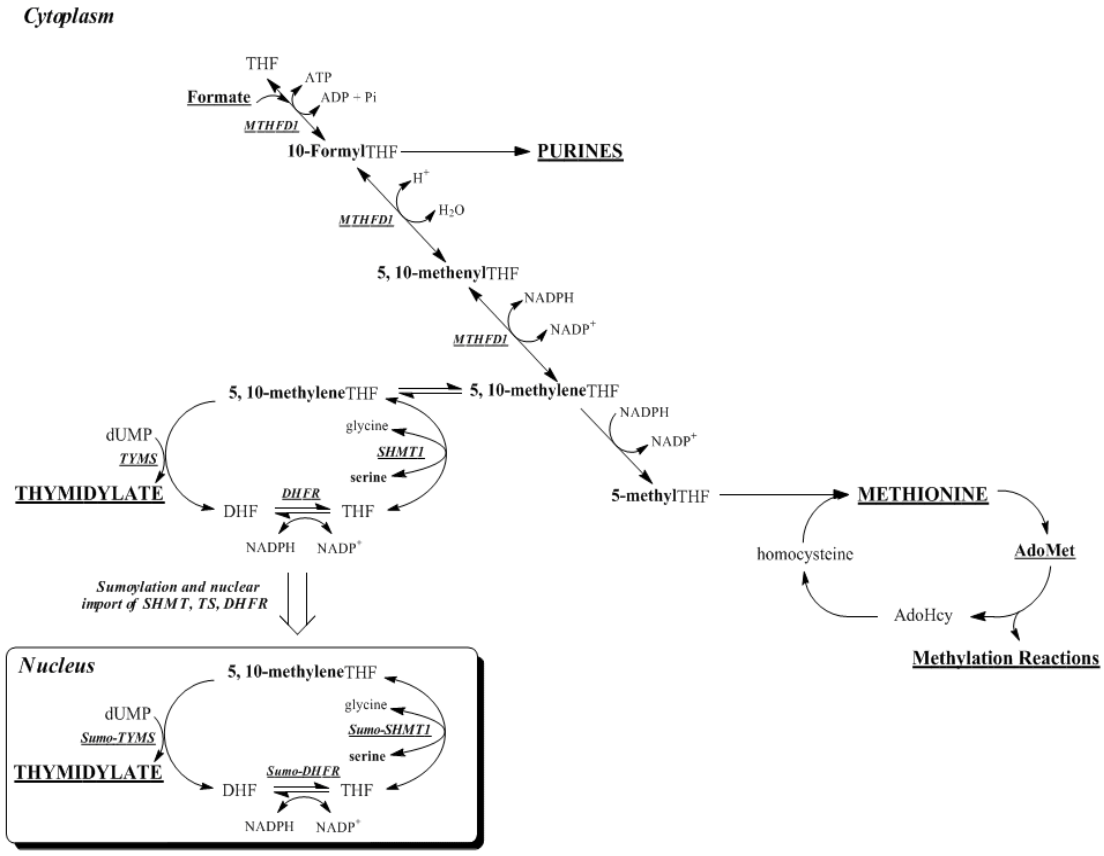


Figure 3.1. Compartmentation of folate-mediated one-carbon metabolism in the cytoplasm, and nucleus. One-carbon metabolism in the cytoplasm is required for the de novo synthesis of purines and thymidylate, and for the remethylation of homocysteine to methionine. One-carbon metabolism in the nucleus synthesizes dTMP from dUMP and serine. MTHFD1,

folate-dependent metabolic enzymes thereby disrupting cellular folate utilization. Because the anabolic pathways within the one-carbon network are tightly coupled and compete for a limiting pool of folate cofactors (14,15), impairments in OCM often disrupt simultaneously *de novo* nucleotide biosynthesis and cellular methylation potential, resulting in reduced proliferative capacity, increased DNA uracil content, elevated plasma homocysteine, reduced cellular methylation capacity (as indicated by the ratio of AdoMet/AdoHcy) and chromatin hypomethylation (16).

Most studies to date have focused on the role of homocysteine remethylation and cellular methylation in the etiology of NTD pathogenesis. Association studies have identified elevated homocysteine (17,18), vitamin B12 deficiency (19-21) and gene variants that encode enzymes in the homocysteine remethylation pathway (22-24) as risk factors for an NTD-affected pregnancy. Most notably, the 677C→T polymorphism in the gene encoding the enzyme methylenetetrahydrofolate reductase (MTHFR) is associated with elevated homocysteine (25,26), impaired folate accumulation (27,28), and NTD risk, with both maternal and fetal genotype contributing to risk (22,29). However, disruption of genes that encode enzymes which are required for homocysteine remethylation, including MTHFR and methionine synthase (Figure 3.1), have not yielded NTDs in mice (30-32).

Over 150 genes have been identified that influence neural tube closure in mice, including genes associated with apoptosis, cell-cell communication, gene transcription, and major developmental signaling pathways (33). Only a subset of these NTD mouse models are responsive to maternal folate (34-37). Of these, only the *splotch* mutant (*Pax3*^{sp}), which carries a point mutation in the gene encoding the nuclear transcription factor Pax3, has demonstrated impairments in OCM. Homozygous *splotch* embryos exhibit fully penetrant spina bifida and impaired *de novo* thymidylate biosynthesis, although the mechanisms are unknown. NTDs in the

splotch mutant can be rescued with supplemental dietary folic acid or thymidine, but are exacerbated by methionine supplementation (34,35). Amelioration of impaired thymidylate biosynthesis and NTD occurence with either folic acid or thymidine supplementation suggests that folic acid may prevent NTDs in the *splotch* mutant by rescuing *de novo* thymidylate synthesis. However, Pax3 has not been linked to human NTD pathogenesis (38-40).

The thymidylate biosynthesis pathway is comprised of three enzymes which function in the cytoplasm and the nucleus: serine hydroxymethyltransferase, thymidylate synthase and dihydrofolate reductase (41) (Figure 3.1). The enzyme serine hydroxymethyltransferase (SHMT1) provides folate-activated one-carbon moieties for thymidylate synthesis by catalyzing the transfer of the hydroxymethyl group of serine to tetrahydrofolate (THF) to form glycine and the cofactor 5,10-methyleneTHF (42). During S phase, these cytoplasmic enzymes undergo SUMO-dependent translocation into the nucleus to provide dTTP for DNA replication (41,43) (Figure 3.1) . In the cytoplasm, methyleneTHF required for thymidylate biosynthesis can be derived either from SHMT1- or MTHFD1-catalyzed reactions. *Shmt1* is not essential in mice, but its expression enhances the efficiency of *de novo* thymidylate biosynthesis (44,45). To determine directly if impaired *de novo* thymidylate biosynthesis increases risk for NTDs, the contribution of *Shmt1* to NTDs was investigated in mice.

Results

Shmt1 *is expressed in the developing neural tube and paraxial structures*

Shmt1 transcription was examined by monitoring β-galactosidase activity generated from the endogenous *Shmt1* promoter in $Shmt1^{flox/flox}$ embryos across the period from gastrulation through neuropore closure and early organogenesis (day

E12). Early expression is most robust in splanchnopleure, especially in the yolk sac and allantois (Figure 3.2A,B), within the neural plate and cardiac loop myocardium. Craniofacial neural crest mesenchymal populations are also labeled (Figure 3.2B). Within the neuroepithelium, expression is initially diffuse and uniform along the length of the body and also along the medio-lateral (future ventro-dorsal, basal-alar) axis of the forming brain and spinal cord. Non-neural surface ectoderm is not labeled.

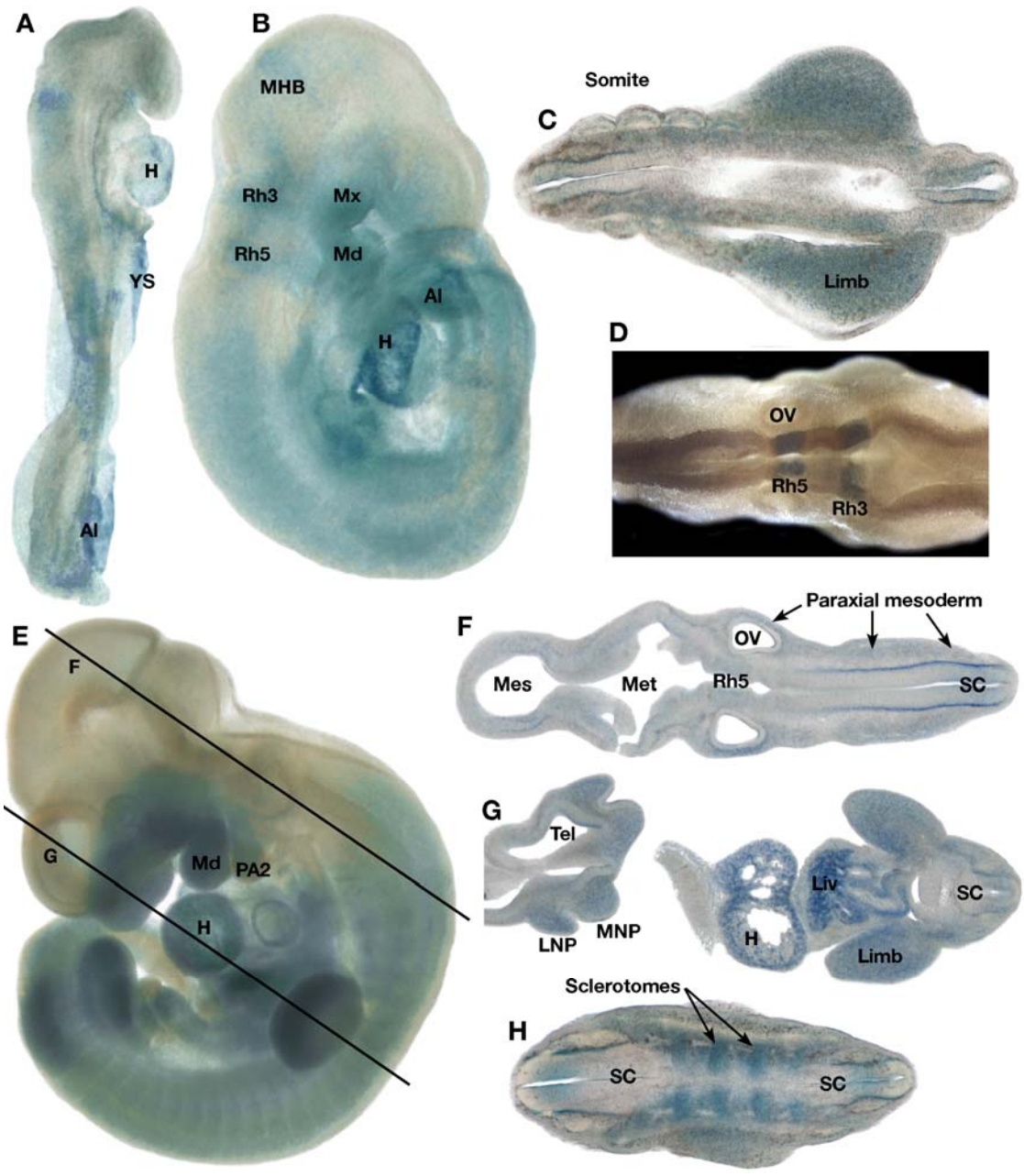
Widespread expression in the neuroepithelium decreases rapidly in a rostro-caudal sequence following neural tube closure, with expression remaining primarily in the more caudal, continually forming parts of the neural tube, including but not limited to the region of the caudal neuropore. The exceptions to this loss of *Shmt1* expression occur at the midbrain-hindbrain boundary and within rhombomeres 3 and 5 (Figure 3.2B, D), where elevated levels persist to E10.25 (rhombomere 3) and E11 (rhombomere 5).

Beginning at E10.5, neural tube *Shmt1* expression is reinitiated selectively along the central nervous system and becomes robust by E12-13. Within the brain, this is strongest in the alar and basal regions of the telencephalon (Figure 3.3), but seen also in the future thalamic regions of the diencephalon, cortical regions of the mesencephalon, and subventricular columns along the ventral hindbrain. The rhombic lip also shows elevated expression.

Also beginning at day E10.5, expression of *Shmt1* becomes elevated along the full length of the spinal cord. This is most pronounced in a longitudinal band located dorsal to the sulcus limitans and excluding the dorsal neural plate (Figure 3.2G, H). Expression is greatest at the region of the cervical flexure (Figure 3.3A). This wave of expression is transient, and is largely gone by E13.

Olfactory, and otic epithelia, both derived from surface ectoderm, lack *Shmt1* expression during their initial stages of invagination and differentiation, though the

Figure 3.2. *Shmt1* expression in embryos from E8-E13.
(A) lateral view of an E8 (5 somite) embryo, showing diffuse labeling throughout the forming neural tube and also in the yolk sac (YS), allantoic stalk (Al), and heart (H).
(B) By E9.5, expression in the brain is restricted to the midbrain-hindbrain boundary (MHB) region, and rhombomeres 3 and 5 (Rh3, Rh5), but is still present in the spinal cord. Expression is present in craniofacial neural crest populations, best seen in the mandibular (Md) and maxillary (Mx) prominences.
(C) is a coronal section through the caudal flexure of an E10, 20-somite embryo, showing diffuse labeling in the spinal cord (SC), somites, and limb mesoderm.
(D) is a coronal section through the hindbrain of a E9.25 embryo, showing *Shmt1* expression restricted to rhombomeres 3 and 5.
(E-G) An E10.5 embryo, with (F) and (G) sections at the levels indicated. Diffuse labeling in the telencephalic lobes (Tel), midbrain (Mes) and rostral hindbrain (metencephalon, Met) are evident, as is *Shmt1* expression in the lateral and medial nasal processes (LNP, MNP) derived from craniofacial neural crest.
(H) The restriction of spinal cord expression to a longitudinal band located dorsal to the sulcus limitans is shown in a caudal coronal section from an E11.5 embryo.


A
H
YS
Al
B
MHB
Rh3
Mx
Rh5
Md
Al
H
Somite
C
Limb
D
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Rh5
Rh3
E
F
G
Md
PA2
H
Paraxial mesoderm
F
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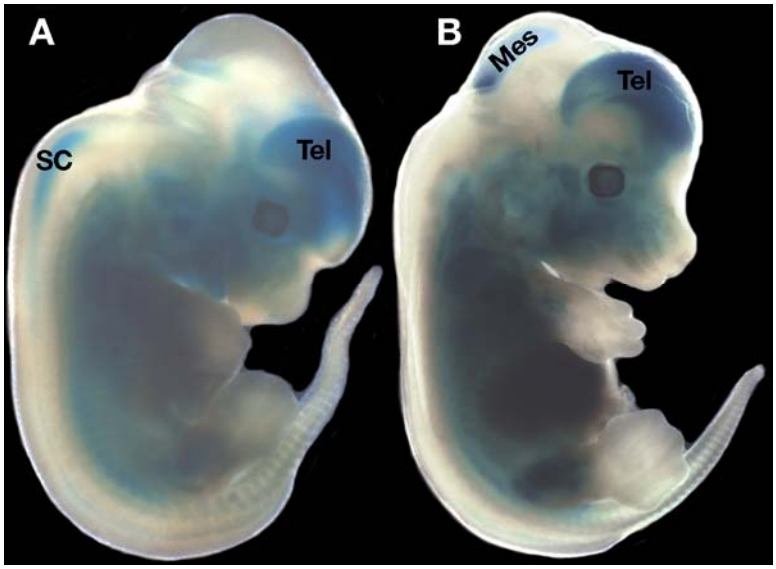


Figure 3.3. Whole embryo views of *Shmt1* expression in (A) E12 and (B) E13 embryos.

chondrogenic mesenchymal tissues surrounding these are well labeled. In contrast, the brain-derived optic vesicle epithelium is initially diffusely labeled. As the optic cup forms, expression becomes restricted to the anterior (limbic) and optic stalk regions.

Paraxial mesoderm, which underlies most of the neural plate and later surrounds the brain (excluding prosencephalon) and spinal cord, lacks prominent *Shmt1* expression until after neural tube closure. With the separation of distinct somitic compartments, both the pre-chondrogenic sclerotome and pre-myogenic myotome regions express *Shmt1*. Early limb bud mesoderm is uniformly positive, but this quickly falls in the core, skeletogenic regions of developing limbs (Figure 3.2C).

Expression of *Shmt1* is evident in craniofacial neural crest populations as they emerge from neural fold epithelium, and remains high during the formation of pharyngeal (branchial) arches and frontonasal prominences through days E9-10.5 (Figure 3.2B). Expression levels then fall off except in skeletogenic and odontogenic mesenchymal populations.

Shmt1 *deficiency sensitizes embryos to exencephaly*

The effect of *Shmt1* disruption on neural tube closure was determined in litters derived from crosses of $Shmt1^{+/-}$ males to $Shmt1^{+/+}$, $Shmt1^{+/-}$, or $Shmt1^{-/-}$ female mice maintained on either congenic 129SvEv or C57Bl/6 backgrounds. Dams were fed either a modified AIN93G diet lacking folate and choline (FCD) or control AIN93G diet from weaning through gestation. Litters were harvested at E11.5, when neural tube closure was completed, and individual embryos were examined for the presence of NTDs (Tables 3.1-3.4). Incidence of NTDs on the 129SvEv background is described below; however, NTDs were also observed albeit at a lower frequency on the C57Bl/6 background (Tables 3.3, 3.4). A low frequency of exencephaly was

Table 3.1 Frequency of NTDs observed in E11.5 embryos isolated from crosses of *Shmt1*-deficient mice on a 129/SvEv background as a function of maternal *Shmt1* genotype and diet. Main effects of maternal *Shmt1* genotype and maternal diet were determined by analysis of variance. Both maternal *Shmt1* genotype ($p < 0.05$) and maternal diet ($p < 0.01$) significantly influenced NTD occurrence.

Diet	Maternal Genotype	No. litters	No. embryos	No. NTDs (%)
Control	*Shmt1*$^{+/+}$	8	44	0
	Shmt1$^{+/-}$	27	149	1 (<1%)
	Shmt1$^{-/-}$	8	39	0
FCD	*Shmt1*$^{+/+}$	19	101	1 (1%)
	Shmt1$^{+/-}$	36	180	7 (3.9%)
	Shmt1$^{-/-}$	16	85	11 (13%)

Table 3.2. Frequency of NTDs observed in E11.5 embryos isolated from crosses of *Shmt1*-deficient dams on a 129/SvEv background maintained on the FCD diet, as a function of maternal and embryonic *Shmt1* genotype.

		Embryonic *Shmt1* Genotype		
Maternal *Shmt1* genotype		*Shmt1*$^{+/+}$	*Shmt1*$^{+/-}$	*Shmt1*$^{-/-}$
Shmt1$^{+/+}$	No. NTDs (%)	0	1 (1.8%)	-
	No. Embryos	46	55	-
Shmt1$^{+/-}$	No. NTDs (%)	0	3 (3.4%)	4 (8.2%)
	No. Embryos	43	88	49
Shmt1$^{-/-}$	No. NTDs (%)	-	2 (4.8%)	9 (21%)
	No. Embryos	-	42	43

Table 3.3. Frequency of NTDs observed in E11.5 embryos isolated from crosses of *Shmt1*-deficient dams on a C57Bl/6 background as a function of maternal *Shmt1* genotype and diet.

Diet	Maternal Genotype	No. litters	No. embryos	No. NTDs (%)
Control	*Shmt1*$^{+/+}$	4	27	0
	Shmt1$^{+/-}$	28	209	0
	Shmt1$^{-/-}$	10	78	0
FCD	*Shmt1*$^{+/+}$	32	239	0
	Shmt1$^{+/-}$	38	195	2 (1%)
	Shmt1$^{-/-}$	25	184	6 (3.4%)

Table 3.4. Frequency of NTDs observed in E11.5 embryos isolated from crosses of *Shmt1*-deficient mice on a C57Bl/6 background maintain on the FCD diet, as a function of maternal and embryonic *Shmt1* genotype.

		Embryonic *Shmt1* Genotype		
Maternal *Shmt1* genotype		*Shmt1*$^{+/+}$	*Shmt1*$^{+/-}$	*Shmt1*$^{-/-}$
Shmt1$^{+/+}$	No. NTDs (%)	0	0	-
	No. Embryos	122	117	-
Shmt1$^{+/-}$	No. NTDs (%)	0	1 (1%)	1 (2%)
	No. Embryos	45	103	47
Shmt1$^{-/-}$	No. NTDs (%)	-	2 (1.7%)	4 (5.7%)
	No. Embryos	-	114	70

observed in embryos harvested from crosses of *Shmt1*$^{+/-}$ mice fed the FCD diet (Figure 3.4A-D, Tables 3.1, 3.2). Exencephaly was primarily observed in litters from dams maintained on the FCD diet, as supported by a significant main effect of maternal diet ($p < 0.01$), although a single *Shmt1*$^{-/-}$ embryo with exencephaly was uncovered from a dam maintained on the control diet. In all cases, exencephalic embryos were either *Shmt1*$^{+/-}$ or *Shmt1*$^{-/-}$, whereas no NTDs were observed in *Shmt1*$^{+/+}$ littermates from any litters examined. The frequency of exencephaly was higher for *Shmt1*$^{-/-}$ littermates (8.1%, 4 of 49 embryos) as compared to *Shmt1*$^{+/-}$ embryos (3.4%, 3 of 88 embryos) in litters isolated from *Shmt1*$^{+/-}$ dams fed the FCD diet, indicating that complete loss of *Shmt1* in the developing embryo was associated with a greater risk of exencephaly as compared to partial loss ($p = 0.09$, Table 3.2). Loss of *Shmt1* expression in the dam also resulted in an increased frequency of exencephaly, as evidenced by a significant main effect of maternal genotype ($p < 0.05$). Only 1 *Shmt1*$^{+/-}$ embryo with exencephaly (1%; 1 of 101 embryos) was uncovered from crosses involving *Shmt1*$^{+/+}$ dams fed the FCD diet. Approximately 10% (7/70) of embryos recovered from crosses of *Shmt1*$^{-/-}$ dams fed the FCD diet exhibited exencephaly as compared to 4% (7/180) observed in crosses of *Shmt1*$^{+/-}$ dams fed the FCD diet. The increased frequency of exencephaly observed in litters from *Shmt1*$^{-/-}$ dams was due to a greater proportion of *Shmt1*$^{-/-}$ embryos developing exencephaly (18.4%, 9 of 43).

The number of observed *Shmt1*$^{+/+}$, *Shmt1*$^{+/-}$, and *Shmt1*$^{-/-}$ embryos at E11.5 did not deviate from expected values for any of the crosses examined (data not shown). Maternal diet ($F = 21.5$, $p < 0.0001$) and embryonic *Shmt1* deficiency ($F = 4.45$, $p = 0.01$) significantly affected embryonic length as measured at E11.5 (Figure 3.5A,B). Embryos isolated from dams fed the FCD diet had significantly shorter crown-rump lengths, as did *Shmt1*$^{-/-}$ embryos isolated from dams fed either diet. Analyses of log-

Figure 3.4. **Neural tube defects in *Shmt1*-deficient embryos.**
(A and B). At E11.5, *Shmt1*$^{+/-}$ embryos from dams fed the FCD diet exhibit failure of rostral neural tube closure at the midbrain/hindbrain boundary (B). All wild-type littermates (A) are unaffected. Arrows indicate extent of lesions.
(C and D). At E14.5, affected *Shmt1*$^{+/-}$ embryos (D) exhibit prominent exencephaly.
(E and F). H&E stained transverse sections of the hindbrain region in E10.5 embryos reveal complete failure of closure in the hindbrain region in E11.5 *Shmt1*$^{+/-}$ embryos (E).

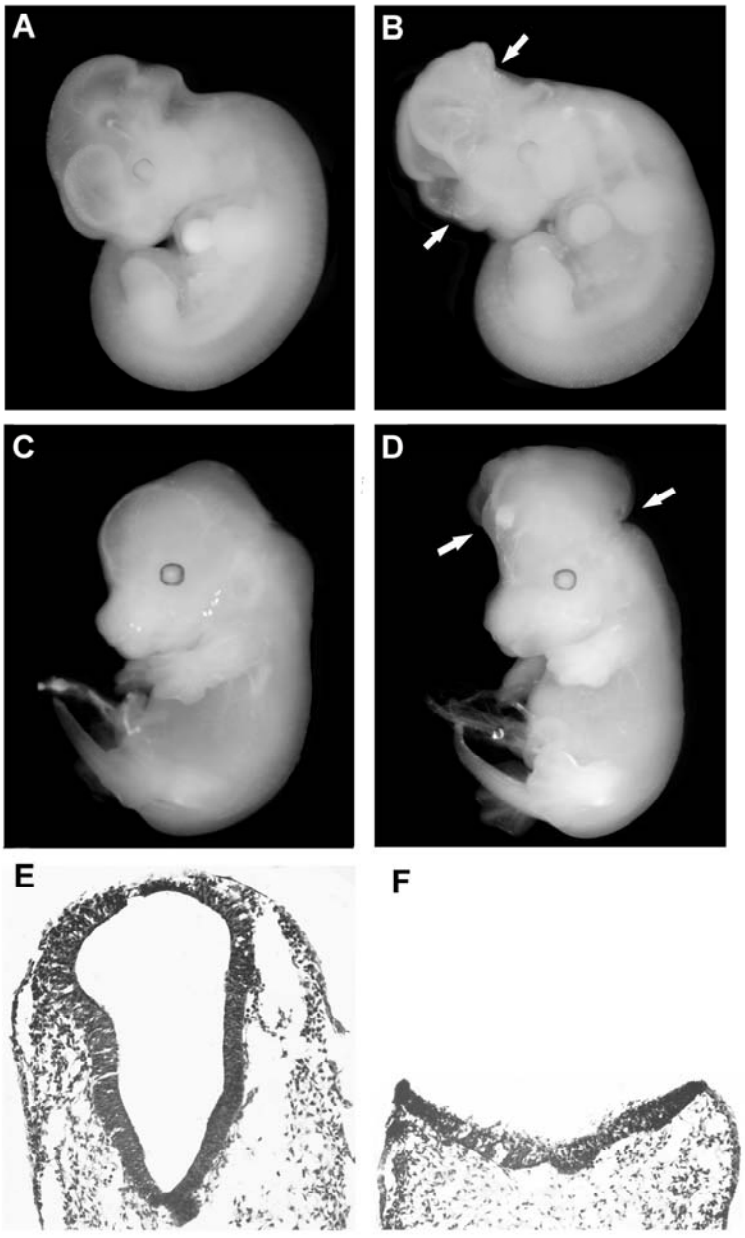
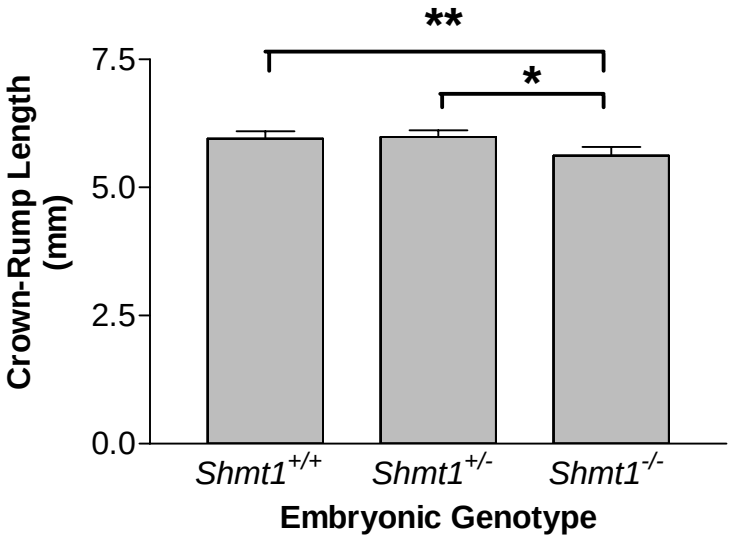
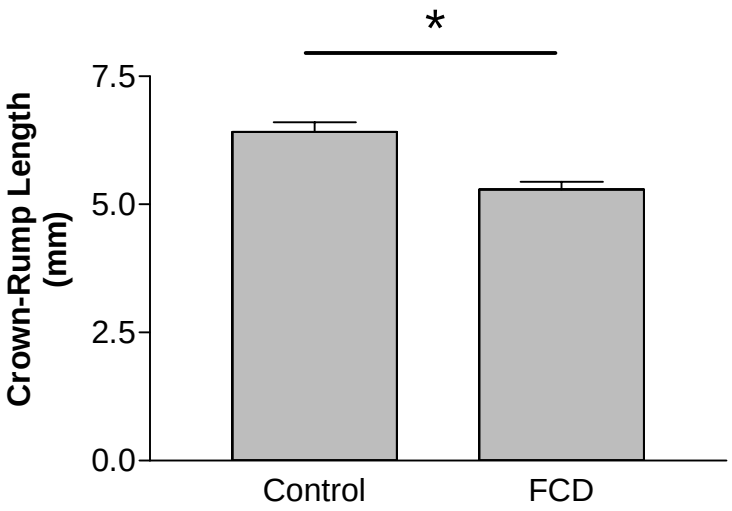

A
B
C
D
E
F

Figure 3.5. Crown-rump length of embryos derived from crosses of *Shmt1*-deficient mice.
(A) Crown-rump length as a function of embryonic *Shmt1* genotype. *, $p = 0.003$; **, $p = 0.03$.
(B)Crown-rump length as a function of maternal diet. *, $p = < 0.0001$.

A


**
*
7.5
5.0
2.5
0.0
Crown-Rump Length (mm)
Shmt1+/+
Shmt1+/-
Shmt1-/-
Embryonic Genotype

B


*
7.5
5.0
2.5
0.0
Crown-Rump Length (mm)
Control
FCD

transformed resorption rates indicated a significant effect of maternal diet ($F = 7.52$, $p < 0.01$), with significantly higher resorption rates associated with the FCD diet as compared to the control diet (24% vs 8% resorptions, respectively). In addition, there was also a trend toward an effect of maternal genotype ($F = 3.15$, $p = 0.054$) on resorption rates. *Shmt1*$^{+/-}$ females had significantly higher resorption rates than *Shmt1*$^{+/+}$ females (20% vs 12% , $p = 0.03$), but the comparison between *Shmt1*$^{-/-}$ females and *Shmt1*$^{+/+}$ females (23% vs 12%) did not reach significance. Neither maternal *Shmt1* genotype nor maternal diet significantly affected the weight of female mice at onset of gestation ($p > 0.05$, data not shown).

Maternal Shmt1 *deficiency does not influence markers of folate status*

Shmt1 genotype did not influence red blood cell (RBC) folate levels in pregnant dams ($p > 0.05$, data not shown), whereas the FCD diet resulted in a significant reduction in RBC folate levels ($F = 2637.18$, $p < 0.0001$, Figure 3.6). RBC folate levels were reduced approximately 80% in female mice maintained on the FCD diet as compared to the control diet. Similarly, maternal *Shmt1* genotype did not influence plasma homocysteine levels or the levels of several metabolites associated with homocysteine remethylation and metabolism (Table 3.5). The FCD diet resulted in plasma homocysteine levels that were elevated more than three-fold relative to levels on the control diet (49 vs 15 μM). The FCD diet also significantly affected levels of all metabolites associated with homocysteine remethylation (Table 3.5).

Shmt1 *and* Pax3 *interact in neural tube closure*

Shmt1 expression was examined in *splotch* embryos, a NTD model with impaired *de novo* thymidylate biosynthesis. There was a gene-dosage dependent increase in SHMT1 protein levels in E10.5 embryo extracts from *Pax3*$^{sp/+}$ crosses (Figure 3.7A). To determine whether *Shmt1* was misexpressed in *splotch* embryos, *Pax3*$^{sp/+}$, *Shmt1*$^{flox/flox}$ mice were intercrossed and embryos were stained for the

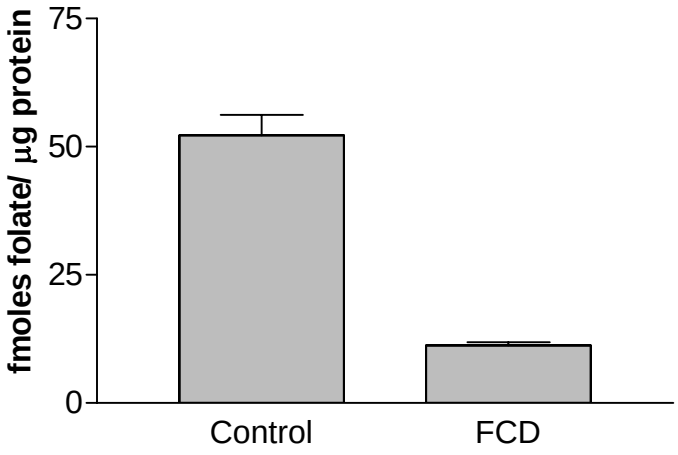


Figure 3.6. Levels of red blood cell folate in pregnant dams fed the control or FCD AIN93G diets. Data were analyzed by analysis of variance. Red blood cell folate levels were significantly reduced in females fed the FCD diet ($F = 2637.18$, $p < 0.0001$).

Table 3.5. Plasma metabolites in *Shmt1*-deficient dams. Differences between genotype and diet and genotype x diet interactions were analyzed by two-way analysis of variance procedure in SAS. Data represent mean ± SEM. a, Shmt1+/- on the FCD diet is significantly different from all other groups.

Metabolite	Diet						p value		
	Control			FCD			Diet effect	Genotype effect	Diet X genotype effect
	Shmt1 $^{+/+}$	Shmt1$^{+/-}$	Shmt1^{-} $^{/-}$	Shmt1$^{+/+}$	Shmt1$^{+/-}$	Shmt1$^{-/-}$			
Homocysteine (μM)	19.8 ± 5.3	15.2 ± 4.1	10.5 ± 1.1	44.1 ± 5.5	49.8 ± 7.8	54.6 ± 7.9	< 0.0001	NS	NS
Cysteine (μM)	200 ± 6	208 ± 4	211 ± 7	180 ± 7	184 ± 5	184 ± 4	< 0.0001	NS	NS
Cystathionine (μM)	1282 ± 225	1100 ± 176	1034 ± 66	2090 ± 223	2608 ± 283	2826 ± 355	< 0.0001	NS	NS
α - Aminobutyric Acid (μM)	11.3 ± 1.0	9.5 ± 1.0	11.3 ± 1.4	8.3 ± 0.5	8.7 ± 0.7	7.9 ± 0.4	0.003	NS	NS
Methionine (μM)	45.8 ± 2.7	47.3 ± 3.3	50.5 ± 1.7	51.4 ± 5.2	60 ± 6	58 ± 3.7	0.0148	NS	NS
Glycine (μM)	63.5 ± 7.0	53.1 ± 2.7	53.1 ± 4.1	61.1 ± 6.7	77.6 ± 10.5	74.1 ± 18.3	0.067	NS	NS
Serine (μM)	108 ± 8	109.± 7	117 ± 7	132 ± 9	153 ± 13	158 ± 12	< 0.0001	NS	NS
Dimethylglycine (μM)	5.8 ± 0.5	6.4 ± 0.5	6.7 ± 0.6	3.9 ± 0.3	3.0 ± 0.4	3.3 ± 0.3	< 0.0001	NS	NS
Methylglycine (μM)	0.42 ± 0.03	0.33 ± 0.01	0.40 ± 0.04	0.41 ± 0.03	0.61 ± 0.08	0.45 ± 0.06	0.006	NS	0.009[a]

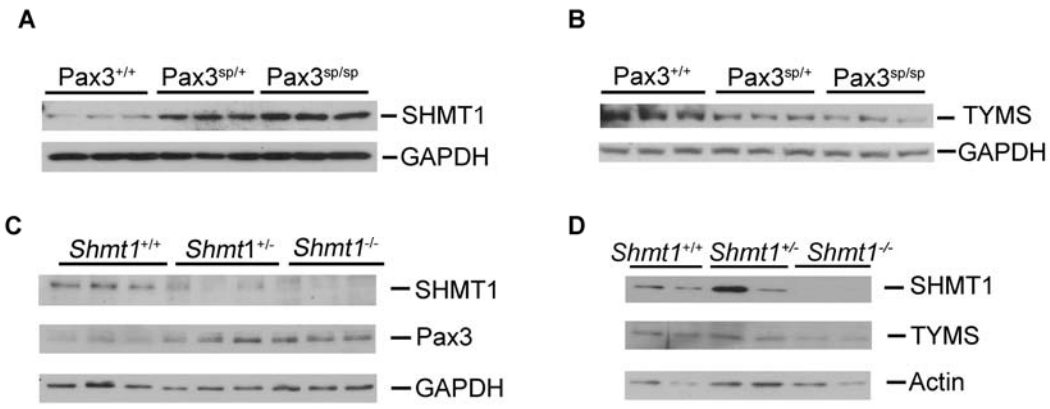


Figure 3.7. Interaction of *Shmt1* and *Pax3*.
(A and B) Protein extracts from E10.5 embryos isolated from *splotch* crosses were analyzed by western blot with a polyclonal anti-SHMT1 antibody (A) and a monoclonal anti-TYMS antibody (B). GAPDH was probed with a polyclonal antibody to verify equal loading in both experiments. Both SHMT1 and TYMS protein levels are responsive to the *splotch* mutation.
(C and D) Protein extracts from E10.5 embryos isolated from crosses of *Shmt1*$^{+/-}$ dams on the FCD diet were analyzed by western blot using a polyclonal antibody to Pax3 (C) and TYMS (D). GAPDH and actin were probed as to verify equal loading. Both Pax3 and TYMS protein levels are responsive to *Shmt1* deficiency.

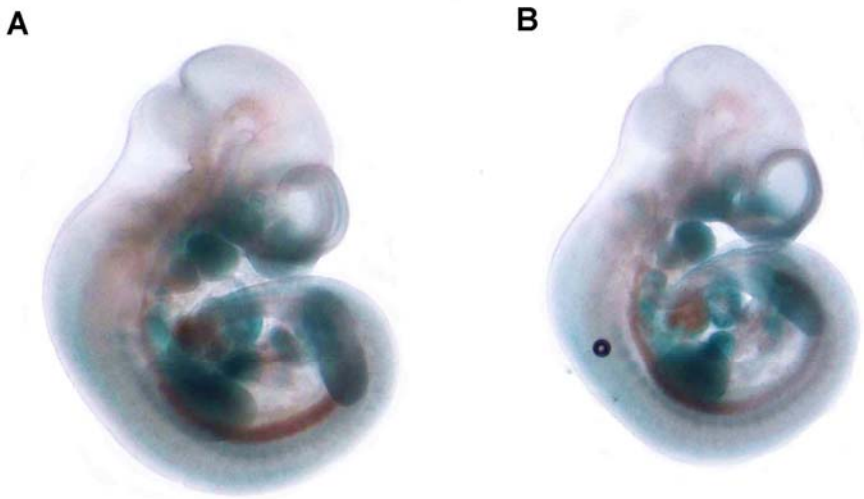


Figure 3.8. *Shmt1* is not misexpressed in *Pax3*$^{sp/sp}$ embryos.
Blue stain indicates the presence of β-galactosidase staining in *Pax3*$^{+/+}$, *Shmt1*$^{flox/flox}$ (A) and *Pax3*$^{sp/sp}$, *Shmt1*$^{flox/flox}$ embryos (B).

presence of β-galactosidase. Comparison of *Pax3*$^{+/+}$ and *Pax3*$^{sp/sp}$ littermates did not indicate alterations in of *Shmt1*expression patterns, indicating that the elevated SHMT1 protein in *splotch* embryos was not associated with altered domains of expression (Figure 3.8). Thymidylate synthase (TYMS) was also examined in *splotch* embryos by western blot in the same embryo extracts. TYMS protein levels were reduced in a gene-dosage dependent manner in response to the *splotch* mutation (Figure 3.7B). Examination of *Shmt1* and *TYMS* message levels by real-time PCR did not reveal any genotype differences (data not shown), indicating that the *splotch* mutation affects SHMT1 and TYMS protein levels post-transcriptionally.

Alterations in Pax3 and TYMS protein levels were examined in *Shmt1*-deficient embryos. A gene-dosage dependent increase in Pax3 protein levels was observed in response to *Shmt1* deficiency (Figure 3.7C). Disruption of *Shmt1*also resulted in reduced TYMS protein levels in *Shmt1*$^{-/-}$ embryos (Figure 3.7D).

Shmt1 *deficiency exacerbates the NTD phenotype in* splotch *embryos*

The effect of *Shmt1* deficiency on NTDs in *splotch* embryos was examined in compound *Pax3*, *Shmt1* mutant embryos generated from crosses of *Pax3*$^{sp/+}$, *Shmt1*$^{+/-}$ and *Pax3*$^{sp/+}$, *Shmt1*$^{-/-}$ female mice maintained on a standard rodent diet. Previous studies have shown that *Pax3*$^{sp/sp}$ embryos display a spina bifida phenotype that is 100% penetrant with occasional exencephaly, whereas *Pax3*$^{sp/+}$embryos do not display NTDs but do exhibit a white belly splotch indicative of neural crest migration defects (46). Post-neurulation stage embryos (E11.5) were harvested from compound mutant crosses and examined for presence and extent of NTDs (cranial, spinal, or combined cranial and spinal, Table 3.6). Maternal *Shmt1* deficiency and embryonic sex significantly influenced the incidence of combined cranial and spinal lesions in *Pax3*$^{sp/sp}$embryos (Figure 3.9A). Embryos isolated from crosses of *Pax3*$^{sp/+}$, *Shmt1*$^{-/-}$ dams fed the standard rodent diet had a significantly higher incidence of combined

Table 3.6. Frequency of NTDs and genotype distribution in embryos derived from crosses of C57Bl/6J-*Pax3*$^{sp/+}$ dams and *Shmt1*- and *Pax3*- deficient dams. The number of NTDs is indicated in parentheses.

	Embryonic genotype (*Pax3, Shmt1*)								
Cross	+/+	sp/+	sp/sp	+/+	sp/+	sp/sp	+/+	sp/+	sp/sp
	+/+	+/+	+/+	+/-	+/-	+/-	-/-	-/-	-/-
Pax3$^{sp/+}$ *x Pax3*$^{sp/+}$	49	84	41 (40)	--	--	--	--	--	--
Pax3$^{sp/+}$*Shmt1*$^{+/-}$ *x Pax3*$^{sp/+}$*,Shmt1* $^{+/-}$	7	33	9 (9)	32(1)	60(4)	23 (22)	12	20	12 (12)
Pax3$^{sp/+}$*,Shmt1*$^{-/-}$ *x Pax3*$^{sp/+}$*,Shmt1* $^{+/-}$	--	--	--	16(1)	26 (1)	11 (11)	21	28 (2)	17 (17)

Figure 3.9. *Shmt1* deficiency exacerbates NTDs in *splotch* mutants
(A) Incidence of spinal and combined cranial + spinal lesions in $Pax3^{sp/sp}$ embryos isolated from compound mutant crosses (*Pax3*, *Shmt1*) and C57Bl/6J-$Pax3^{sp}$ crosses maintained on a standard rodent diet.
(B and C) Spina bifida in *Shmt1*-deficient $Pax3^{+/+}$ (B) and $Pax3^{sp/+}$ (C) embryos derived from compound mutant crosses maintained on a standard rodent diet.
(D and E) Exencephaly in *Shmt1*-deficient $Pax3^{+/+}$ (D) and $Pax3^{sp/+}$ (E) embryos derived from compound mutant crosses maintained on a standard rodent diet.
(F and G). Craniorachiscisis in *Shmt1*-deficient $Pax^{sp/+}$ (F) and $Pax3^{sp/sp}$ (G) embryos derived from compound mutant crosses maintained on the FCD diet.

A

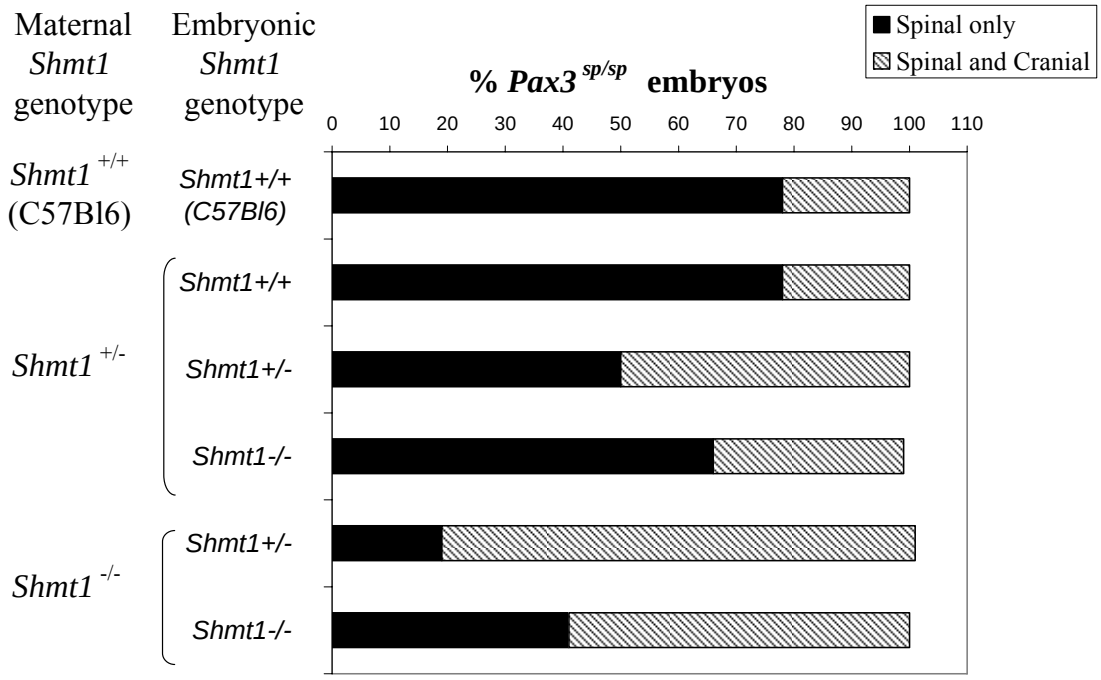
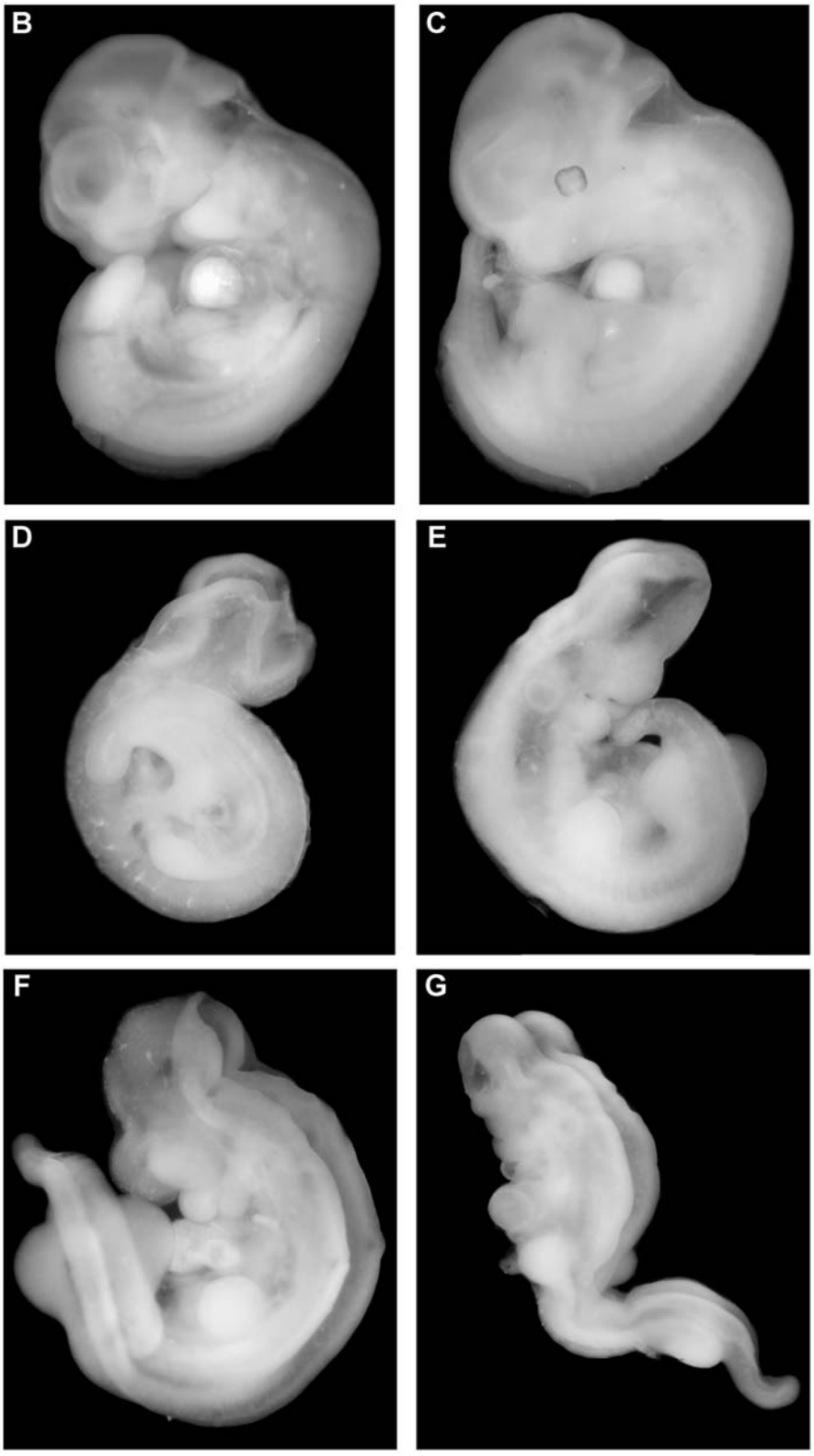

Maternal Shmt1 genotype
Embryonic Shmt1 genotype
Spinal only
Spinal and Cranial
% Pax3 sp/sp embryos
0
10
20
30
40
50
60
70
80
90
100
110
Shmt1 +/+ (C57Bl6)
Shmt1+/+ (C57Bl6)
Shmt1 +/-
Shmt1+/+
Shmt1+/-
Shmt1-/-
Shmt1 -/-
Shmt1+/-
Shmt1-/-

Figure 3.9, continued.



lesions as compared to embryos isolated from crosses of *Pax3*$^{sp/+}$, *Shmt1*$^{+/-}$ dams fed the standard rodent diet (70.5% vs 38%, $p < 0.05$). In addition, female embryos were significantly more likely to exhibit the combined phenotype (48% vs 21%, $p < 0.01$), as previously described (47,48). There was not sufficient statistical power to assess the contribution of embryonic *Shmt1* genotype to the incidence of the combined phenotype, due to a limited number of *Shmt1*$^{+/+}$,*Pax3*$^{sp/sp}$ embryos for comparison (n = 9). However, when combined incidence of cranial and spinal lesions in compound mutant embryos was compared to incidence in embryos isolated from crosses of C57Bl/6J-*Pax3*$^{sp/+}$ mice, *Pax3*$^{sp/sp}$, *Shmt1*$^{+/-}$ and *Pax3*$^{sp/sp}$, *Shmt1*$^{-/-}$ embryos had significantly higher incidence of combined spina bifida and exencephaly (46% and 66%, respectively) as compared to embryos isolated from crosses involving the *splotch* mutation alone (22%; $p = 0.04$ and $p = 0.001$, respectively), whereas incidence of combined lesions in *Pax3*$^{sp/sp}$, *Shmt1*$^{+/+}$ embryos isolated from compound mutant crosses (22%) did not differ significantly from the effects of the *splotch* mutation alone ($p > 0.95$).

In addition to increasing the severity of NTDs observed in *Pax3*$^{sp/sp}$ embryos, *Shmt1* deficiency also resulted in instances of *Pax3*$^{sp/+}$ and *Pax3*$^{+/+}$ littermates with spina bifida or exencephaly under folate-replete conditions (Table 3.6, Figure 3.9B-E). *Pax3*$^{sp/+}$ and *Pax3*$^{+/+}$ embryos with NTDs were either *Shmt1*$^{+/-}$ or *Shmt1*$^{-/-}$ in all cases. Approximately 3-5% of *Pax3*$^{sp/+}$ and *Pax3*$^{+/+}$ embryos from crosses of *Pax3*$^{sp/+}$, *Shmt1*$^{+/-}$ and *Pax3*$^{sp/+}$, *Shmt1*$^{-/-}$ dams displayed NTDs. In most cases, the lesion observed was exencephaly, but isolated spina bifida was also observed in a few cases (Figure 3.9B,C); the severity of these lesions was comparable to that observed in *Pax3*$^{sp/sp}$ embryos. Whereas lesions induced by *Shmt1* deficiency alone were confined to the cranial region, embryonic *Shmt1* deficiency resulted in spina bifida in response to either embryonic or maternal *Pax3* deficiency. Furthermore, embryonic or maternal

Pax3 deficiency was sufficient to induce spina bifida or exencephaly in *Shmt1*-deficient embryos in the absence of dietary folate deficiency. Although a low frequency of spontaneous NTDs in $Pax3^{sp/+}$ embryos has been previously reported (49,50), NTDs were not observed in any $Pax3^{sp/+}$ or $Pax3^{+/+}$ embryos that were $Shmt1^{+/+}$. In addition, there have not been previous reports of spontaneous NTDs in $Pax3^{+/+}$ littermates isolated from *splotch* matings.

The effect of dietary folate deficiency on NTD extent and frequency in compound *Pax3*, *Shmt1* mutants was examined (Table 3.7). Compound mutant dams were placed on the FCD diet from weaning through gestation, and embryos were harvested as previously described. Impaired folate status both exacerbated and increased the frequency of lesions observed in response to combined *Shmt1* and *Pax3* deficiency. Notably, there were several instances of craniorachiscisis observed, in which the entire length of the neural tube was open. Craniorachischisis was observed in *Shmt1*-deficient $Pax3^{sp/+}$ and $Pax3^{sp/sp}$ embryos (Figure 3.9F,G) . To our knowledge, craniorachischisis has not been observed previously in response to the *splotch* mutation alone. Furthermore, the frequency of NTDs observed in $Pax3^{sp/+}$ littermates rose from 3-5% to 11-14% in response to dietary folate deficiency in compound *Pax3, Shmt1* mutant crosses (Table 3.7). The majority of NTDs in $Pax^{+/+}$ and $Pax3^{sp/+}$ littermates were confined to the cranial region.

Metabolic disruption of folate-mediated OCM in splotch *MEFs*

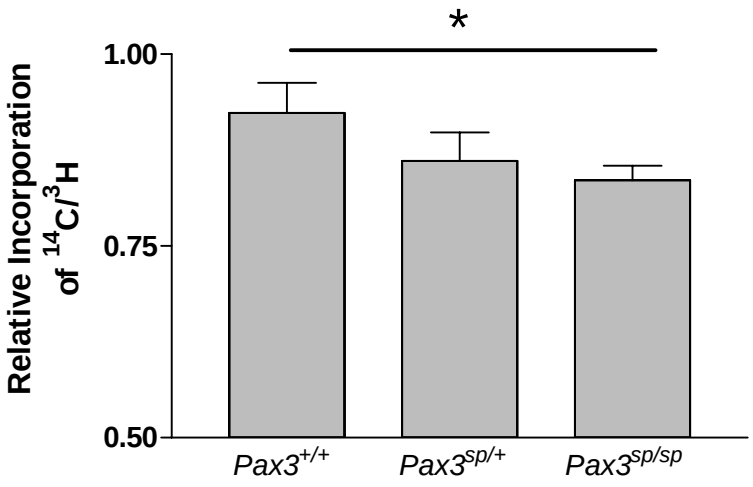
The metabolic phenotype associated with *Pax3* deficiency was determined in MEF cell lines generated from *splotch* matings. Impairments in *de novo* thymidylate biosynthesis were observed in a gene-dosage dependent manner in response to the *splotch* mutation, consistent with a previous report (35) (Figure 3.10A). Relative incorporation of ^{14}C-uridine as compared to ^{3}H-thymidine, which reflects the efficiency of *de novo* thymidylate biosynthesis, was significantly reduced in $Pax3^{sp/sp}$

Table 3.7. Frequency and types of NTDs in embryos isolated from crosses of *Shmt1*- and *Pax3*- deficient dams maintained on the FCD diet as a function of embryonic *Pax3* genotype. NTDs in $Pax3^{+/+}$ and $Pax3^{sp/+}$ embryos were only observed in *Shmt1*-deficient embryos.

Cross	Embryonic *Pax3* genotype	No. Embryos	No. NTDs (%)	Spinal	Cranial	Spinal and Cranial	Craniorachiscisis
$Pax3^{sp/+}$,$Shmt1^{+/-}$ x $Pax3^{sp/+}$,$Shmt1^{+/-}$	$Pax3^{+/+}$	10	1 (10%)	0	1	0	0
	$Pax3^{sp/+}$	28	4 (14%)	0	3	0	1
	$Pax3^{sp/sp}$	12	12 (100%)	4	1	6	1
$Pax3^{sp/+}$,$Shmt1^{-/-}$ x $Pax3^{sp/+}$,$Shmt1^{+/-}$	$Pax3^{+/+}$	5	0	0	0	0	0
	$Pax3^{sp/+}$	18	2 (11%)	1	0	0	1
	$Pax3^{sp/sp}$	7	7 (100%)	1	2	4	0

Figure 3.10. Metabolic disruption of folate-mediated OCM in *splotch* MEFs.
(A) *Pax3*$^{+/+}$, *Pax3*$^{sp/+}$, and *Pax3*$^{sp/sp}$ MEF cells were cultured to confluency in α-minimal essential medium supplemented with ^{14}C-dUMP and ^{3}H-thymidine. Efficiency of *de novo* thymidylate biosynthesis was determined by the relative enrichment of ^{14}C-dUMP (10 μM))/ ^{3}H-thymidine (500 nm) in nuclear DNA. All values represent the average of triplicate measures of three different cell lines per genotype. Results shown are mean ± SD. Genotype differences were determined using a one-tailed t-test.
(B) *Pax3*$^{+/+}$, *Pax3*$^{sp/+}$, and *Pax3*$^{sp/sp}$ MEF cells were cultured to confluency in α-minimal essential medium supplemented with ^{14}C-formate and ^{3}H-hypoxanthine. Efficiency of *de novo* purine biosynthesis was determined by the relative enrichment of ^{14}C-formate (20 μM) / ^{3}H-hypoxanthine (2 nm) in nuclear DNA. All values are the average of triplicate measures of three different cell lines per genotype. Results shown are mean ± SD. Genotype differences were determined using a one-tailed t-test.
(C) Relative ratio of AdoMet to AdoHcy in *Pax3*$^{+/+}$, *Pax3*$^{sp/+}$, and *Pax3*$^{sp/sp}$ MEF lines. N= 2-3 lines per genotype. Results shown are mean ± SD. Genotype differences were determined using a one-tailed t-test.
* indicates $p < 0.05$. ** indicates $p < 0.01$.

A


*
1.00
0.75
0.50
Relative Incorporation of $^{14}C/^{3}H$
Pax3+/+
Pax3sp/+
Pax3sp/sp

B

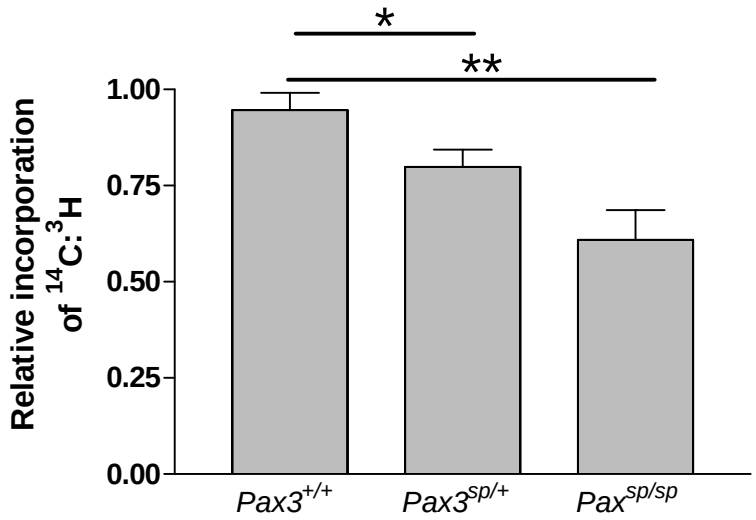
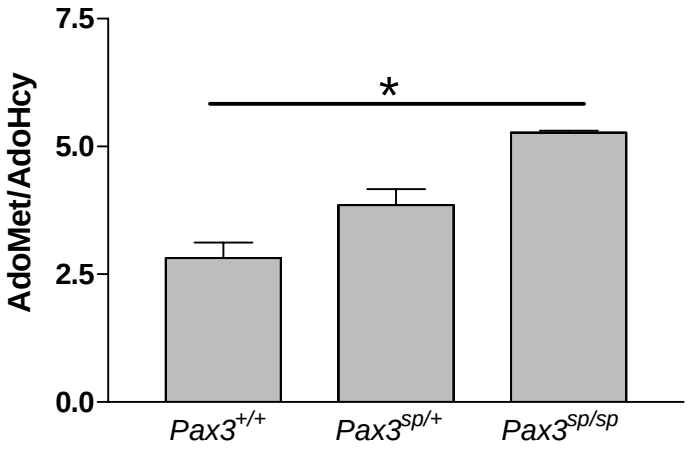

*
**
1.00
0.75
0.50
0.25
0.00
Relative incorporation of $^{14}C:^{3}H$
Pax3+/+
Pax3sp/+
Paxsp/sp

Figure 3.10, continued

C



MEF lines, as compared to *Pax3*$^{+/+}$ lines (p = 0.05). Rates of *de novo* thymidylate biosynthesis in the *Pax3*$^{sp/+}$ MEF cell lines was intermediate between the *Pax3*$^{+/+}$ and *Pax3*$^{sp/sp}$ MEF cell lines, but the comparison to the *Pax3*$^{+/+}$ lines did not reach statistical significance. Surprisingly, *Pax3*-deficient MEF lines also exhibited statistically significant impairment in *de novo* purine biosynthesis, as evidenced by a decrease in the relative ratio of incorporation of ^{14}C-formate to ^{3}H-hypoxanthine (Figure 3.10B). In this case, both *Pax3*$^{sp/+}$ and *Pax3*$^{sp/sp}$ lines exhibited reduced rates of *de novo* purine biosynthesis compared to *Pax3*$^{+/+}$ lines (p = 0.04 and 0.01, respectively), but the impairment was greatest for the *Pax3*$^{sp/sp}$ lines.

The effect of *Pax3* deficiency on methionine biosynthesis and cellular methylation was determined. The relative ratio of AdoMet to AdoHcy, which serves as an indicator of the cellular methylation potential, was increased in a gene-dosage dependent manner in response to the *splotch* mutation, although only the comparison between *Pax3*$^{sp/sp}$ lines and *Pax3*$^{+/+}$ lines was statistically significant ($p < 0.01$, Figure 3.10C).

Discussion

This study is the first to demonstrate that changes in the expression of a folate-dependent enzyme sensitizes mice to folate-responsive NTDs, and the results implicate impaired thymidylate synthesis and *Shmt1* in the etiology of folate-responsive NTDs. In the cytoplasm, SHMT1 regulates the partitioning of one-carbon units between thymidylate biosynthesis and homocysteine remethylation (45), whereas in the nucleus SHMT1 is the source of folate-activated one-carbons for thymidylate synthesis (Figure 1). Consequently, *de novo* thymidylate biosynthesis is impaired in *Shmt1* null fibroblasts (MacFarlane et al, unpublished) and loss of *Shmt1* expression increases uracil misincorporation into genomic DNA in livers of *Shmt1*$^{+/-}$ mice (44).

Shmt1 is highly penetrant relative to NTD risk as haploinsufficiency is sufficient to induce NTDs in litters of folate- and choline-deficient dams..

Although *Shmt1* is not expressed in all cells (51), it is expressed throughout the developing neural plate during folding and closure stages. This ubiquitous expression is transient, and after neural tube closure secondary centers of *Shmt1* expression appear in both brain and spinal cord regions. Some of these, e.g. the midbrain-hindbrain boundary and rhombomeres 3 and 5 are sites at which position-determining gene expressions occur (52,53) Other sites, e.g. the alar zone of the spinal cord, are more closely associated with neuronal specification. The presence of SHMT1 throughout neurula-stage neuroepithelium supports its likely contribution to nucleotide biosynthesis at sites of rapid proliferation, although later sites of expression appear more closely related to differentiation-dependent processes

Shmt1-deficient mice recapitulated the NTD risk profile observed in humans, which involves interactions among embryonic genotype, maternal diet and maternal genotype. Only embryonic *Shmt1* deficiency was essential for NTD pathogenesis in this study, but NTD penetrance was increased by maternal folate/choline deficiency and maternal *Shmt1* disruption in both *Shmt1*-deficient crosses and compound *Shmt1*, *Pax3* mutant crosses. Decreased maternal *Shmt1* expression increased the frequency of exencephaly in *Shmt1*-deficient embryos, and induced more extensive lesions in compound *Pax3, Shmt1* mutant embryos. NTDs occurred predominantly under folate/choline-deficient conditions in *Shmt1*-deficient embryos, with maternal folate deficiency resulting in instances of craniorachischisis and three-fold elevations in NTD incidence among $Pax3^{sp/+}$ and $Pax3^{+/+}$embryos deficient for *Shmt1*. The highest incidence of NTDs was observed in $Shmt1^{-/-}$ embryos harvested from $Shmt1^{-/-}$ dams fed the folate/choline deficient diet. In contrast, only one exencephalic embryo was

recovered from a *Shmt1*$^{+/+}$ dam, indicating that maternal *Shmt1* expression protects against NTDs in *Shmt1*-deficient embryos.

The mouse models investigated in this study do not support a prominent role for homocysteine or cellular methylation in the etiology of NTDs. *Shmt1*-deficient dams did not exhibit elevated plasma homocysteine, and AdoMet/AdoHcy levels were previously shown to be unchanged or mildly elevated in *Shmt1*-deficient mice fed the folate/choline deficient diet (44). Similarly, the homocysteine remethylation pathway is not implicated in NTD pathogenesis in the *splotch* NTD model. In this study, the ratio of AdoMet/AdoHcy was elevated in both *Pax3*$^{sp/+}$ and Pax$^{sp/sp}$ MEFS in a gene-dosage dependent manner. AdoMet and AdoHcy levels were also shown to be similar in wild type and *splotch* embryos in a recent study (54). Methionine supplementation *in vivo* or in culture is not protective but rather increases the frequency of more extensive defects in *Pax3*$^{sp/sp}$ embryos and also causes occasional NTDs in *Pax3*$^{sp/+}$ embryos (34,35). Lastly, exposure of *splotch* embryos to excess homocysteine in culture does not exacerbate NTD frequency or severity in *Pax3*$^{sp/sp}$ embryos or cause NTDs in *Pax3*$^{sp/+}$ or *Pax3*$^{+/+}$embryos (54), suggesting that the exacerbating effect of methionine is not mediated by elevated homocysteine levels. Together, these data do not support a role for elevated homocysteine or impaired cellular methylation as an underlying cause of NTDs in these two folate-responsive NTD models.

The impairment of *de novo* thymidylate biosynthesis in the *Pax3* model was validated and the underlying mechanism(s) extended in this study. We confirmed previous studies that *Pax3* deficiency impairs *de novo* thymidylate biosynthesis in *splotch* MEFs. Furthermore, we demonstrated that *Pax3* deficiency decreased TYMS enzyme levels in *splotch* embryos, indicating that TYMS is a downstream target of Pax3. We also demonstrated that *Shmt1* deficiency decreased TYMS enzyme levels. The common effects of *Pax3* and *Shmt1* deficiency on TYMS protein levels and *de*

novo thymidylate biosynthesis support the role of impaired thymidylate biosynthesis in the etiology of NTDs, as *Shmt1* and *Pax3* deficiency interact to increase the frequency and severity of NTDs in *Pax3*$^{sp/sp}$ embryos, and cause NTDs in Pax$^{sp/+}$ and *Pax3*$^{+/+}$ embryos in the absence of folate deficiency. Of interest, Pax3 and SHMT1 protein levels appear to be mutually regulated and compensatory. *Shmt1* deficiency increased Pax3 protein levels and SHMT1 protein levels were similarly elevated in response to *Pax3* deficiency in *splotch* embryos. Because *Shmt1* deficiency exacerbated NTDs in *Pax3*-deficient embryos, and both *Pax3* and *Shmt1* deficiency are associated with reduced TYMS levels, the elevation in SHMT1 protein levels observed in *Pax3*-deficient embryos may function to compensate partially for reduced TYMS protein levels by increasing the concentration of methyleneTHF, the required cofactor and substrate for *de novo* thymidylate biosynthesis. Similarly, the increased Pax3 levels observed in *Shmt1*-deficient embryos may function to elevate TYMS levels and thereby increase rates of thymidylate biosynthesis. Although the molecular mechanism underlying the interactions between Pax3 and SHMT1 remains to be established, the independent associations of *Shmt1* and *Pax3* with TYMS levels and *de novo* thymidylate biosynthesis indicates that the observed reciprocal regulation functions as a compensatory mechanism that normalizes impairments in *de novo* thymidylate biosynthesis, thereby affording some protection against NTDs. When this protective mechanism is disrupted in compound *Pax3*, *Shmt1* mutants, the NTD phenotype is exacerbated. Impairments in *de novo* thymidylate biosynthesis may disrupt neural tube closure by reducing proliferative capacity or increasing genomic instability via misincorporation of uracil into DNA (55). Both reduced proliferative capacity and genomic instability cause NTDs in mice (56-59)

The influence of maternal *Shmt1* genotype on NTD occurrence indicates a role for a maternally-derived soluble factor or metabolite in NTD risk. Maternally-derived

thymidine nucleotides or nucleosides have been implicated in NTD pathogenesis based on data from previous studies, as maternal thymidylate supplementation was shown to prevent NTDs in some but not all studies of *Pax3*-deficient *splotch* embryos (34,35). In this study, *Pax3* deficiency in MEFs was shown to impair both *de novo* thymidylate and purine biosynthesis, and the magnitude in the impairment in *de novo* purine biosynthesis was greater than that observed for thymidylate biosynthesis. Therefore, the conflicting experimental results present in the literature regarding maternal thymidylate supplementation on NTD prevention may be accounted for by impairments in both purine and thymidylate biosynthesis in *splotch* mutants. Unlike the *splotch* model, previous studies have shown that *Shmt1* disruption does not impair *de novo* purine biosynthesis (MacFarlane et al, unpublished). The differential effects of *Pax3* and *Shmt1* on nucleotide biosynthesis may account for the disparity in NTD phenotype between these two folate-responsive NTD models; *Shmt1* deficiency alone is associated only with exencephaly, whereas *Pax3* deficiency causes 100% penetrant spina bifida and low frequency exencephaly. *Shmt1* deficiency at both maternal and embryonic levels increased the frequency of exencephaly in *splotch* embryos, whereas the only instances of spina bifida observed in *Shmt1*-deficient embryos occurred in litters derived from $Pax3^{sp/+}$ dams. These data suggest that impaired purine biosynthesis therefore results in spina bifida, whereas impairments in thymidylate biosynthesis result in exencephaly.

Decreased *Mthfr* expression in mice does not cause NTDs (30) and *Mthfr* does not interact with *Pax3* (49), although *MTHFR* is recognized as a candidate gene underlying human NTD risk. In addition to disrupting the homocysteine remethylation pathway, the common *MTHFR* 677C→T variant alters the distribution of cellular folate derivatives (60-63), and reduces markedly serum and red blood cell folate levels (28,64,65) in humans. Similarly, mice with reduced *Mthfr* expression

exhibit reduced plasma folate levels and altered distribution of methylated folate derivatives (66). The discrepancy between the results from human genetic association studies that have identified *MTHFR* gene variants as NTD risk factors, and the lack of NTDs resulting from *Mthfr* disruption in mice, indicates that *MTHFR* affects NTD risk by modifying folate status, not homocysteine remethylation. Previous studies have shown that reduced folate status is not sufficient to induce NTDs in mice (67,68). By impairing folate status, the *MTHFR* 677C→T variant may sensitize genetically-susceptible individuals to NTDs without directly contributing to causal metabolic impairments in nucleotide biosynthesis.

We demonstrate that a 50% decrease in *Shmt1* expression is sufficient to induce NTDs in mice, and therefore factors that affect *Shmt1* expression are candidate risk factors for NTDs. Cellular SHMT1 activity and levels vary over a wide range. SHMT1 synthesizes it's own tightly-binding inhibitor, 5-formylTHF (69), and binds 5-methylTHF as an inhibitor with high affinity (45). *Shmt1* expression is also regulated by zinc (70), retinoic acid (71), heavy chain ferritin (72,73), and c-myc (74). Whereas most screens for human NTD candidate genes have focused on the identification of gene variants, this study demonstrates that relatively modest changes in SHMT1 expression confer increased NTD risk, thereby accounting for the paucity of human NTD candidate genes in the folate metabolic network identified to date.

Experimental Procedures

Mouse models

Shmt1$^{flox/+}$ and *Shmt1*$^{-/-}$ mice are viable and fertile, and their generation has been described previously (44). *Shmt1*$^{flox/+}$ and *Shmt1*$^{+/-}$ lines are maintained as a heterozygote breeding colonies on either congenic 129/SvEv or C57Bl/6 backgrounds. Heterozygous C57Bl/6J-*Pax3*^{sp} mice were obtained from Jackson Laboratories (Bar Harbor, ME). Embryonic *Shmt1* expression was examined in embryos derived from

crosses of B6;129(P2)-*Shmt1*$^{flox/flox}$ mice, which express β-galactosidase from an IRES/ β-geo cassette inserted within intron 8 of the *Shmt1* gene (44). NTDs were examined in litters harvested from crosses of either 129SvEv-*Shmt1*(N10+) or C57Bl/6-*Shmt1*(N10+) mice. *Shmt1* expression patterns in *Pax3*-deficient embryos were examined in *Pax3*$^{sp/+}$, *Shmt1*$^{flox/flox}$ mice generated from crosses between *Shmt1*$^{flox/flox}$ mice and *Pax3*$^{sp/+}$ mice. To determine the impact of *Shmt1* deficiency on NTDs in the *splotch* mutant, *Pax3*, *Shmt1* compound mutants were obtained by crossing *Shmt1*$^{+/-}$ mice on a mixed C57Bl6/ 129Sv/EV/ Balb/c background to *Pax3*$^{Sp/+}$ mice. F1 compound heterozygotes (*Pax3*$^{sp/+}$, *Shmt1*$^{+/-}$) were then used for colony breeding. *Pax3*$^{sp/+}$, *Shmt1*$^{+/-}$ or *Pax3*$^{sp/+}$, *Shmt1*$^{-/-}$ female mice were then crossed to *Pax3*$^{sp/+}$, *Shmt1*$^{+/-}$ male mice for timed mating experiments as described below.

Experimental animals and diets

All animal experiments were approved by the Cornell Institutional Animal Care and Use Committee according to the guidelines of the Animal Welfare Act and all applicable federal and state laws. Mice were maintained on a 12 hour light/dark cycle in a temperature controlled room. For studies investigating *Shmt1* deficiency and neural tube closure, female mice were randomly assigned to either an experimental AIN93G diet lacking folate and choline (FCD) or an AIN93G (Control) diet (Dyets, Bethlehem, PA) at weaning. Choline was excluded from the experimental diet in addition to folate to increase stress on the homocysteine remethylation cycle. Choline synthesis requires methyl groups in the form of S-adenosylmethionine and it provides a source of four one-carbons for homocysteine remethylation through its catabolism. Studies investigating the interaction between *Shmt1* and *Pax3* in *Pax3*, *Shmt1* compound mutants were conducted in mice fed either a standard rodent chow (Harlan) or the FCD diet. For all studies, dams were maintained on diet from weaning throughout the breeding period and for the duration of gestation, until killed. For all

studies, virgin female mice aged 70-120 days were housed overnight with males. The following morning, females were examined for the presence of a vaginal plug. Nine o'clock a.m. the day of the plug was designated as gestational day 0.5 (E0.5). Pregnant females were sacrificed between E9.5-E11.5. Gravid uteri were removed and all implants and resorption sites were recorded. Embryos were dissected free of extra embryonic membranes under a dissecting microscope, examined for presence of NTDs, and measured for crown-rump length. All yolk sacs were collected for subsequent genotyping. Embryos extracted for later use in biochemical assays or for protein extraction were taken at E9.5-10.5 and rapidly frozen in liquid nitrogen followed by storage at -80ºC. Embryos examined for morphological abnormalities were derived at E11.5, fixed 24 hours in 10% neutral buffered formalin, dehydrated in an ethanol series, and stored in 70% ethanol. For all metabolic studies, pregnant mice were sacrificed at E9.5-10.5 by cervical dislocation and blood was collected by cardiac puncture. For morphological analysis, pregnant mice were sacrificed by carbon dioxide-induced asphyxiation.

Genotype analysis

DNA was isolated from tail tissue or yolk sacs using the Qiagen DNeasy kit protocol for animal tissue (Qiagen). Genotyping for the *splotch* mutation and sex was performed using established protocols (75,76). Genotyping for *Shmt1*^{flox} and *Shmt1*$^{+/-}$ alleles was performed using a previously described protocol (44).

Histology

Embryos were processed for histological analysis following gross morphological examination. Tissues were cryoprotected in 30% sucrose in phosphate-buffered saline, embedded in O.C.T. (Tissue-Tek)) and frozen in isopentane cooled in liquid nitrogen. Thirty-micrometer thick cryosections were affixed to plus charged slides, and stained with hematoxylin and eosin.

Determination of plasma metabolites

Plasma levels of homocysteine, cystathionine, cysteine, α - aminobutyric acid, methionine, glycine, serine, N,N-dimethylglycine and N-methylglycine were determined by stable isotope dilution capillary gas chromatography mass spectrometry as previously described (77,78)

Analysis of red blood cell folate levels

Folate levels in red blood cell samples was quantified using a *Lactobacillus casei* microbiological assay as previously described (79).

Western blot

Western blot analyses were performed in triplicate using 15-30 μg of protein extracted from individual embryos as described elsewhere (44). The primary antibodies were diluted in 5% nonfat skim milk in PBS as follows: monoclonal mouse anti-mouse thymidylate synthase (TYMS) (Zymed, 1:2000), monoclonal mouse anti-mouse GAPDH (Novus, 1:40,000), polyclonal goat anti-mouse Pax3 (Santa Cruz, 1:3000), polyclonal rabbit anti-mouse actin (Abcam. 1:40,000) and polyclonal sheep anti-mouse SHMT1 (1:10000,(80). Relative levels of SHMT1, TYMS, and Pax3 protein were normalized to GAPDH or actin as a loading control.

Generation of mouse embryonic fibroblast cell lines

Mouse embryonic fibroblast (MEF) cell lines were generated from embryos isolated 10-14 days post-coitus from crosses of $Pax3^{sp/+}$ mice. Genotyping of MEF lines was completed both at the time of generation using nuclear DNA isolated from embryo heads, as well as from cell pellets, using the genotyping protocols described above. MEFs were incubated at 37° C in Defined Minimal Essential Media (Hyclone) with 10% fetal bovine serum. MEFs were grown to confluency and frozen down in fetal bovine serum with 10% dimethyl sulfoxide for later use.

Nucleotide biosynthesis assays

MEFs were plated in triplicate in 6-well plates at a density of 1 $X10^4$ cells per well. A formate suppression assay, which measures the relative efficiency of *de novo* purine nucleotide synthesis relative to synthesis from the purine nucleotide salvage pathway, was performed as previously described (81). Similarly, a deoxyuridine (dU) suppression assay was performed to determine the relative efficiency of *de novo* thymidylate biosynthesis. The dU surpression assay was a modification of that used by Fleming and Copp (35), in which relative rates of de novo thymidine biosynthesis were determined by the relative ratio of incorporation of [^{3}H] thymidine (500 nM) as compared to radiolabeled deoxyuridine monophosphate ([^{14}C] dUMP, 10 μM)) in nuclear DNA. Preparation of media for nucleotide biosynthesis assays has been described elsewhere (81). Cells were grown at 37°C, 5% CO2 in tracer media until confluent. Cells were harvested by trypsinization and DNA was isolated from cell pellets using the Qiagen DNeasy kit protocol for harvested cells. Isotope incorporation into nuclear DNA was quantified using a Beckman LS6500 scintillation counter in dual dpm mode.

Determination of S-adenosylmethionine (AdoMet) and S-Adenosylhomocysteine (AdoHcy) Concentrations

Total levels of AdoMet and AdoHcy were measured in frozen MEF cell pellets by an established HPLC procedure (45,82).

Statistical analyses

Analyses of NTD incidence and embryonic crown-rump length were conducted using repeated-measured analysis of variance (PROC MIXED or PROC GENMOD, SAS, Cary, NC) in which independent variables included maternal *Shmt1* genotype and maternal diet, and litter was considered as a repeated measure.

Embryonic *Shmt1* genotype was excluded from the model when assumptions of variance were violated due to the absence of exencephaly observed in *Shmt1*$^{+/+}$ embryos. Embryonic sex was also included in the model, as gender modifies the incidence of exencephaly (33). Analysis of total litter resorptions and implants was analyzed by analysis of variance in which litter was considered as the unit of analysis. Independent variables in the model included maternal *Shmt1* genotype and diet. Resorption rate was calculated as the ratio of resorptions/ total implants per litter and log-transformation was applied to normalize the data. Specific comparisons within main effects were assessed by comparisons of least-squared means. Chi-squared analyses were used to assess any deviation from expected genotype ratios based on Mendelian inheritance. For cell culture assays, genotype differences were assessed using a one-tailed t-test.

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CHAPTER 4

MATERNAL MTHFD1 HAPLOINSUFFICIENCY IMPAIRS FERTILITY, NEURAL TUBE CLOSURE, AND FETAL GROWTH

Abstract

The folate-dependent gene *MTHFD1* catalyzes the activation of one-carbons required for the synthesis of purines, thymidylate, and methionine. A common human polymorphism in *MTHFD1* (A1298C) increases maternal risk for adverse pregnancy outcomes including neural tube defects (NTDs), but the mechanism(s) are unknown. Here we demonstrate that maternal *Mthfd1* disruption caused by a gene-trapped (gt) allele caused fetal growth restriction, and gestational supplementation of pregnant *Mthfd1*$^{gt/+}$ females with hypoxanthine resulted in rescue of early fetal loss and occasional NTDs in *Mthfd1*$^{gt/+}$ embryos. Pregnant *Mthfd1*$^{gt/+}$ females exhibited reductions in red blood cell folate and plasma methionine levels. These results suggest that *Mthfd1* disruption affects fetal growth and neural tube closure by disrupting both folate status and cellular folate utilization.

Introduction

Maternal folate status is a penetrant environmental risk factor for several adverse outcomes in pregnancy. Maternal folic acid supplementation reduces both neural tube defect (NTD) occurrence and recurrence (1,2), and has been suggested to reduce the risk of other anomalies including orofacial clefts, limb defects, and congenital heart defects (3-7). Currently, over 20 countries worldwide have taken measures to fortify their food supply with folic acid for the prevention of NTDs, which reduced NTD prevalence by 20-80% (8-10). However, overt folate deficiency is not

present in a majority of women whose pregnancies are affected by NTDs or other development anomalies (11-13). Instead, nutritional folate deficiency interacts with genetic variants that compromise folate metabolism and confer risk for NTDs and other developmental anomalies (14-20).

Folates function in the cell to provide activated one-carbons (1Cs) for the *de novo* biosynthesis of purines and thymidylate and for the remethylation of homocysteine to methionine (Figure 4.1). Adenosylation of methionine produces S-adenosylmethionine (AdoMet), which serves as the methyl donor for cellular methylation reactions including the methylation of chromatin. Folate deficiency and genetic disruption in folate-metabolizing enzymes impair folate-dependent anabolic reactions, thereby resulting in reduced proliferative capacity, increased DNA uracil levels, elevated homocysteine and chromatin hypomethylation (21).

Biomarkers of impaired folate status, including reduced serum folate levels and elevated plasma homocysteine, have been linked to increased risk for several pathological conditions in pregnancy and fetal development, including neural tube, cardiac, limb, and jaw defects, pre-eclampsia, placental abruption, low birth weight, pre-term delivery, intrauterine growth restriction, and spontaneous abortion (22-29). The mechanism(s) that underlie pathogenesis in these diverse conditions are unknown. Whereas a majority of studies have investigated mechanisms underlying the association between impaired folate status and NTD risk, less is known about the mechanism(s) by which impairments in folate metabolism lead to other adverse pregnancy outcomes. It has been suggested that reduced maternal and/or fetal folate status compromises cellular proliferative capacity in the developing fetus and/or placenta due to limited nucleotide availability (24).

Formate is a primary source of 1Cs for nucleotide biosynthesis , and is generated from serine, glycine or choline catabolism in the mitochondria (30).

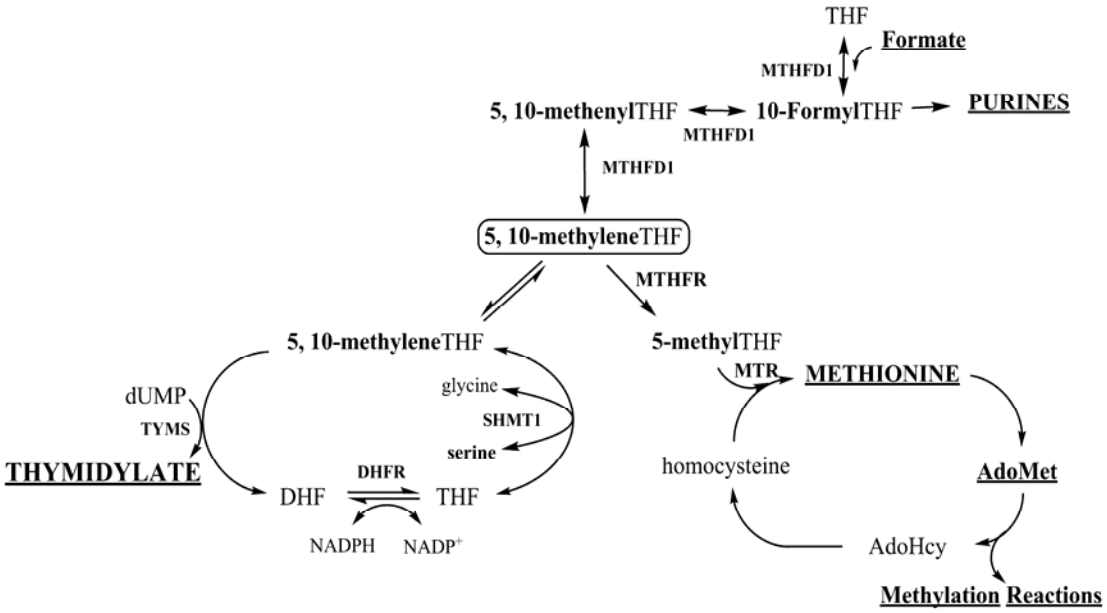


Figure 4.1. Folate-mediated one carbon metabolism
One-carbon metabolism in the cytoplasm is required for the *de novo* synthesis of purines and thymidylate, and for the remethylation of homocysteine to methionine. Formate is generated by one-carbon metabolism in mitochondria from serine, glycine, sarcosine and dimethylglycine. MTHFD1 catalyzes the ATP-dependent synthesis of 10-formylTHF, as well as the NADP-dependent reduction of 10-formylTHF to methyleneTHF. Abbreviations: THF, tetrahydrofolate; MTHFD1, 10-Formyltetrahydrofolate synthetase /Methenyltetrahydrofolate synthetase/ Methylenetetrahydrofolate dehydrogenase; MTHFR, Methylenetetrahydrofolate reductase; MTR, Methionine synthase; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; SHMT1, Cytoplasmic serine hydroxymethyltransferase; TYMS, Thymidylate synthase; DHFR, Dihydrofolate reductase; dUMP, deoxyuracil monophosphate; DHF, dihydrofolate.

Formate traverses to the cytoplasm and condenses with tetrahydrofolate (THF) to form 10-formylTHF, the cofactor required for *de novo* purine biosynthesis. This ATP-dependent reaction is catalyzed by the synthetase domain of the trifunctional enzyme, MTHFD1, which is encoded by *MTHFD1*. The NADP-dependent cyclohydrase/dehydrogenase domain of MTHFD1 catalyzes the reduction of 10-formylTHF to methyleneTHF, which is utilized for synthesis of thymidine, serine, or methionine (31) (Figure 4.1). Association studies of human gene variants that encode folate-dependent enzymes have consistently identified *MTHFD1* as a candidate gene contributing to risk for adverse pregnancy outcomes. A common variant of *MTHFD1*, 1958G>A, results in the substitution of arginine for a glutamine in the active site of the synthetase domain. *MTHFD1* G1958A has been linked to increased risk of NTDs (32-34), cleft lip and palate (35), congenital heart defects (36), placental abruption (37), unexplained second trimester pregnancy loss (38), and intrauterine growth restriction (39). The G1958A variant encodes a thermolabile enzyme associated with a 36% reduction in enzymatic activity and a 22% reduction in the incorporation of labeled formate into DNA, indicating disrupted *de novo* purine biosynthesis (36). Interestingly, adequate folate status protected against reduced protein stability associated with the G1958A polymorphism. These data suggest that impairments in *de novo* purine biosynthesis may underlie pathogenesis in response the *MTHFD1* G1958A SNP, but this has not been demonstrated experimentally.

A *Mthfd1*-deficient mouse model containing a gene trap vector insertion in the synthetase domain of the *Mthfd1* gene has recently been generated and characterized (40). Homozygous *Mthfd1* $^{gt/gt}$ mice are not viable, demonstrating the requirement for folate-activated formate during embryonic development. *Mthfd1*$^{gt/+}$ mice are viable, exhibit 50% decreased MTHFD1 protein levels and exhibit impaired cellular methylation capacity but enhanced *de novo* thymidylate biosynthesis (40). In this

study we have investigated the effect of *Mthfd1* heterozygosity as a model of the *MTHFD1* G1958A variant on embryonic development and neural tube closure. We demonstrate that maternal *Mthfd1* deficiency is a risk factor for adverse pregnancy outcomes by impairing folate status, methionine metabolism, and purine biosynthesis.

Results

$Mthfd1^{gt/gt}$ mice exhibit early embryonic lethality

Mthfd1 is an essential gene in mice (40). To determine the approximate embryonic stage at lethality, litters were harvested from intercrosses of heterozygous B6.129(P2)C57Bl/6- $Mthfd1^{gt/+}$ (N6) mice fed a standard rodent diet at various gestational time points, and the genotype distribution of embryos was determined (Table 4.1, and data not shown). There were no $Mthfd1^{gt/gt}$ embryos recovered at any time point during gestation beginning at embryonic day 9 (E9). The genotype distribution from ten litters harvested at E9 differed significantly from that expected by Mendelian inheritance (Table 4.1, X^2 analysis, $p = 3.8 \times 10^{-8}$). The data indicate that embryonic lethality in $Mthfd1^{gt/gt}$ embryos occurs prior to E9.

Maternal Mthfd1 disruption and low folate status causes FGR

The effect *Mthfd1* disruption in $Mthfd1^{gt/+}$ embryos on embryonic development and neural tube closure in response to compromised maternal folate status was examined. Congenic 129P2Ola- $Mthfd1^{gt/+}$ and $Mthfd1^{+/+}$ female littermates were weaned onto either the AIN93G (control) diet or the folate/choline deficient AIN93G (FCD) diets. At 8 weeks of age, 129P2Ola-$Mthfd1^{+/+}$ and $Mthfd1^{gt/+}$ females were mated to 129P2Ola- $Mthfd1^{gt/+}$ or $Mthfd1^{+/+}$ males, respectively, in order to discern the independent contributions of maternal and embryonic *Mthfd1* genotype to embryonic development. Litters were harvested at E11.5 or E14.5 and examined for gross morphological abnormalities. Genotype distribution in litters examined at E11.5 and

Table 4.1. Early embryonic lethality in *Mthfd1*$^{gt/gt}$ embryos. *Mthfd1*$^{gt/+}$ mice were intercrossed and 10 litters were harvested at E9.5. The expected genotype distributions were calculated based on expected Mendelian inheritance. Deviation from the expected genotype distribution was determined using a X^2 statistic. The observed genotype distribution differed significantly from the expected genotype distribution ($p = 3.8 \times 10^{-8}$)

	Observed Genotype Distribution			**Expected Genotype Distribution**		
Genotype	**Male**	**Female**	**Total**	**Male**	**Female**	**Total**
Mthfd1$^{+/+}$	11	11	22	7.25	7.25	14.5
Mthfd1$^{gt/+}$	17	19	36	14.5	14.5	29
Mthfd1$^{gt/gt}$	0	0	0	7.25	7.25	14.5
Total	28	30	58	29	29	58

E14.5 did not differ significantly from expected values (Tables 4.2, 4.3; X^2 test, p = 0.5 and p = 0.3, respectively). No NTDs were observed in any of the litters examined. However, a proportion of embryos examined at E11.5 from crosses of *Mthfd1*$^{gt/+}$ female mice or *Mthfd1*$^{+/+}$ female mice maintained on the FCD diet were either resorbing and/or severely malformed, suggesting severe fetal growth restriction (FGR, Table 4.2). Malformed embryos were characterized by an irregular and convoluted neuroepithelium, torqued body symmetry, and severe delay in heart, limb, and jaw development (Figure 4.2B-D). In many cases, affected embryos were in the process of resorbing (Figure 4.2C-D). Embryo loss and/or developmental arrest appeared to have occurred between E9.5 and E11.5, following neural tube closure. No NTDs were observed, even in embryos that were in the process of resorption.

The incidence of fetal growth restriction (FGR) in litters derived at E11.5 was not influenced by embryonic *Mthfd1* genotype (p = 0.85), as approximately equal numbers of *Mthfd1*$^{gt/+}$ and *Mthfd1*$^{+/+}$ littermates were affected by FGR (Table 4.2). FGR incidence was significantly influenced by both maternal *Mthfd1* disruption and maternal diet ($X^2 = 4.42$, p = 0.03 and $X^2 = 5.16$, p = 0.02, respectively). There was also a trend towards an effect of embryonic sex on FGR incidence ($X^2 = 3.31$, p = 0.068), with more males affected than females (14 vs. 8). There were no FGR-affected embryos uncovered in 12 litters isolated from crosses of *Mthfd1*$^{gt/+}$ males and *Mthfd1*$^{+/+}$ females fed the control diet. Approximately 10% of embryos isolated either from crosses of *Mthfd1*$^{+/+}$ males and *Mthfd1*$^{gt/+}$ females fed the control diet or crosses of *Mthfd1*$^{gt/+}$ males and *Mthfd1*$^{+/+}$ females fed the FCD diet exhibited FGR. The highest incidence of FGR occurred in litters derived from *Mthfd1*$^{gt/+}$ females fed the FCD diet, with approximately 20% of embryos exhibiting FGR. The additive effect of

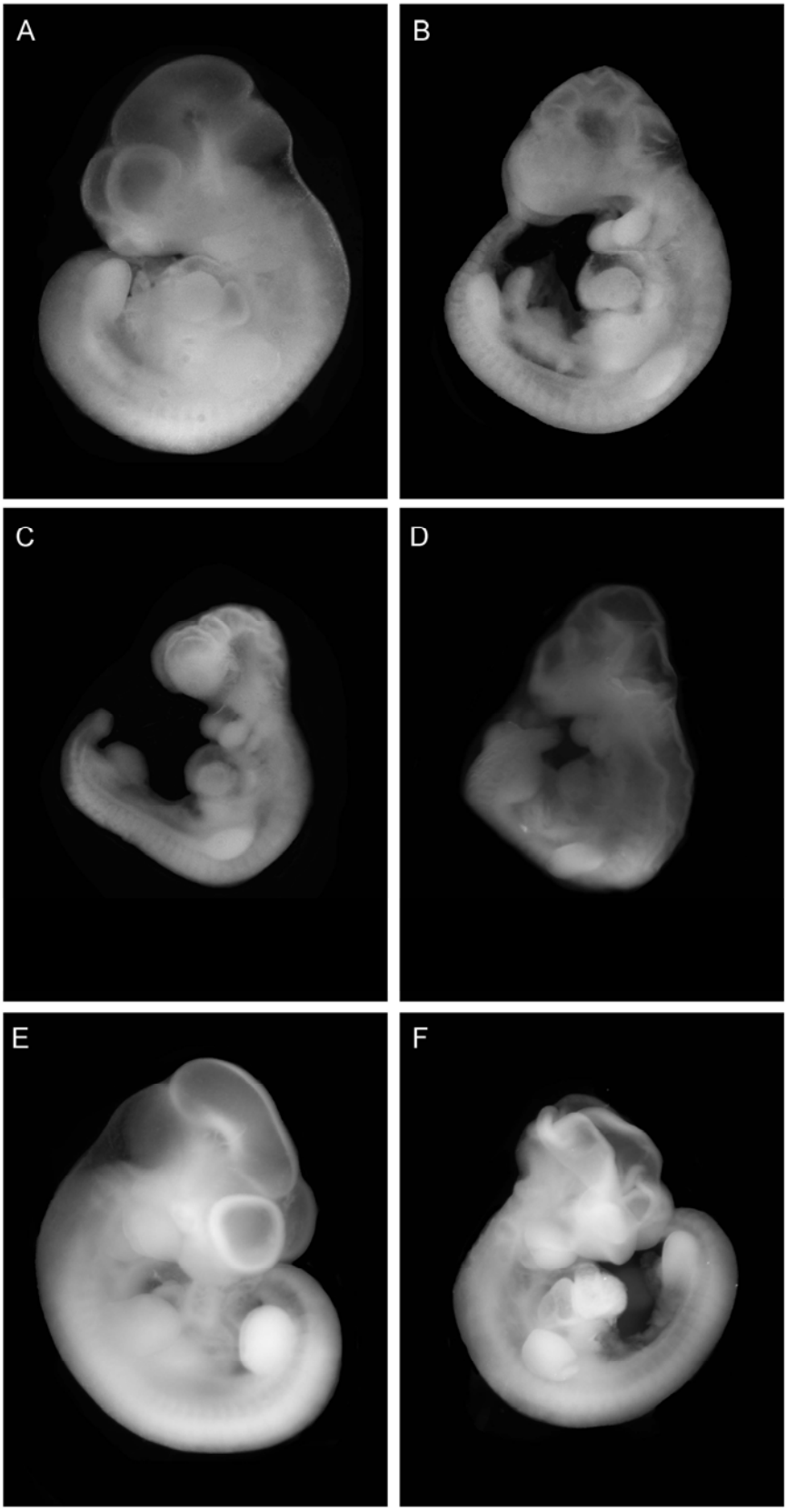
Table 4.2. Incidence of FGR in E11.5 embryos harvested from crosses of *Mthfd1*$^{gt/+}$ and *Mthfd1*$^{+/+}$ mice fed either the control or FCD diet. Main effects of diet, maternal *Mthfd1* genotype, and embryonic *Mthfd1* genotype and sex on FGR incidence were analyzed by analysis of variance in SAS. Differences between observed and expected genotype distribution by genotype and sex were analyzed using a chi-squared statistic. There were no differences between observed and expected genotype distributions (X^2 test, $p = 0.5$). Maternal genotype ($p = 0.03$) and maternal diet ($p = 0.02$) significantly influenced the number of FGR-affected embryos.

Diet	Maternal Genotype	No. of Litters	Embryonic genotype	Total Embryos	No. Female	No. Male	# Affected (sex)
Control	*Mthfd1*$^{+/+}$	12	*Mthfd1*$^{+/+}$	18	8	10	0
			Mthfd1$^{gt/+}$	22	14	8	0
	Mthfd1$^{gt/+}$	15	*Mthfd1*$^{+/+}$	28	16	12	3 (2M, 1F)
			Mthfd1$^{gt/+}$	21	10	11	2 (1M, 1F)
FCD	*Mthfd1*$^{+/+}$	15	*Mthfd1*$^{+/+}$	30	15	15	4 (3M, 1F)
			Mthfd1$^{gt/+}$	30	15	15	1 (M)
	Mthfd1$^{gt/+}$	12	*Mthfd1*$^{+/+}$	35	14	21	6 (5M, 1F)
			Mthfd1$^{gt/+}$	28	14	14	6 (2M, 4F)

Table 4.3. Incidence of FGR in E14.5 embryos harvested from crosses of *Mthfd1*$^{gt/+}$ and *Mthfd1*$^{+/+}$ mice fed either the control or FCD diet. Main effects of diet, maternal *Mthfd1* genotype, and embryonic *Mthfd1* genotype and sex on FGR incidence were analyzed by analysis of variance in SAS. Differences between observed and expected genotype distribution by genotype and sex were analyzed using a chi-squared statistic. There were no differences between observed and expected genotype distributions (X^2 test, $p = 0.3$). There was a significant interaction between maternal genotype and maternal diet ($p = 0.03$) that predicted the # of FGR-affected embryos.

Diet	Maternal Genotype	No. of Litters	Embryonic genotype	Total Embryos	No. Female	No. Male	# Affected (sex)
Control	*Mthfd1*$^{+/+}$	11	*Mthfd1*$^{+/+}$	22	11	11	0
			Mthfd1$^{gt/+}$	24	12	12	0
	Mthfd1$^{gt/+}$	17	*Mthfd1*$^{+/+}$	22	11	11	0
			Mthfd1$^{gt/+}$	28	11	17	3 (1F, 2M)
FCD	*Mthfd1*$^{+/+}$	20	*Mthfd1*$^{+/+}$	28	14	14	3 (2F, 1M)
			Mthfd1$^{gt/+}$	18	11	7	3(2F,1M)
	Mthfd1$^{gt/+}$	15	*Mthfd1*$^{+/+}$	19	10	9	0
			Mthfd1$^{gt/+}$	23	9	14	0

Figure 4.2. Maternal *Mthfd1* disruption affects embryonic development.
(A, B) Normal (A) and growth-restricted (B) *Mthfd1*$^{+/+}$ littermates isolated from a *Mthfd1*$^{gt/+}$ female fed the FCD diet.
(C) Wild-type embryo exhibiting growth restriction and in the process of resorption, isolated at E11.5 from a *Mthfd1*$^{gt/+}$ female fed the FCD diet.
(D) *Mthfd1*$^{gt/+}$ embryo exhibiting growth restriction, torqued body symmetry, and highly irregular neuroepithelial organization, isolated at E11.5 from a *Mthfd1*$^{gt/+}$ female fed the FCD diet.
(E, F) Normal *Mthfd1*$^{+/+}$ embryo (E) and *Mthfd1*$^{gt/+}$ littermates with exencephaly (F). Embryos were uncovered in a litter isolated from a *Mthfd1*$^{gt/+}$ females fed the FCD diet and supplemented with 500 μM hypoxanthine.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compromised maternal folate status and maternal *Mthfd1* disruption on FGR incidence suggests that these two factors did not interact in FGR pathogenesis.

Litters examined at E14.5 had significantly fewer FGR-affected embryos as compared to litters examined at E11.5 ($X^2 = 3.65$, $p = 0.056$; Table 4.3). FGR-affected embryos examined at E14.5 were similar in appearance to those examined at E11.5 except that developmental arrest had occurred slightly later in development. FGR incidence in E14.5 embryos was not affected by embryonic *Mthfd1* genotype. A significant interaction was observed between maternal *Mthfd1* genotype and maternal diet on the number of FGR-affected embryos at E14.5 ($X^2 = 4.34$, $p = 0.037$), indicating that the incidence of FGR was greatest in litters derived from *Mthfd1*$^{gt/+}$ females on the control diet (5%) and in litters from *Mthfd1*$^{+/+}$ females fed the FCD diet (11%). No FGR-affected embryos were uncovered at E14.5 from *Mthfd1*$^{+/+}$ females fed the control diet or *Mthfd1*$^{gt/+}$ females fed the FCD diet. The absence of FGR-affected embryos at E14.5 in litters derived from *Mthfd1*$^{gt/+}$ females fed the FCD diet indicated that affected embryos from these litters did not survive past E11.5 and were resorbed.

Hypoxanthine supplementation in Mthfd1$^{gt/+}$ *females*

The effect of maternal hypoxanthine supplementation on fetal outcomes was examined in *Mthfd1*$^{gt/+}$ females maintained on the FCD diet. Pregnant female mice were supplemented with 500 μM hypoxanthine in drinking water from the time of vaginal plug until sacrifice at E11.5. Hypoxanthine supplementation did not affect the genotype distribution of embryos derived from *Mthfd1*$^{gt/+}$ females on the FCD diet (data not shown). Hypoxanthine supplementation did result in a 50% increase in the incidence of FGR-affected embryos as compared to unsupplemented *Mthfd1*$^{gt/+}$ females fed the FCD diet, although this comparison did not reach significance (28% vs. 19%; $X^2 = 4.32$, $p = 0.11$). Although FGR was observed in both *Mthfd1*$^{+/+}$ and

Mthfd1$^{gt/+}$ embryos derived from hypoxanthine-supplemented litters, the increased incidence of FGR in these litters could be attributed to a doubling of the incidence of FGR in *Mthfd1*$^{gt/+}$ embryos as compared to *Mthfd1*$^{+/+}$ littermates (10 vs. 5 embryos; 36% vs. 19%; p = 0.08). In addition, two *Mthfd1*$^{gt/+}$ embryos exhibited exencephaly of 15 litters derived from *Mthfd1*$^{gt/+}$ females on the FCD diet supplemented with hypoxanthine (Figure 4.2E-F).

Maternal Mthfd1 disruption and impaired folate status influence embryo length at E14.5

Crown-rump length was measured in individual embryos derived from the crosses described above. Crown-rump lengths of embryos at E11.5 were not significantly affected by embryonic *Mthfd1* genotype, maternal *Mthfd1* genotype or maternal diet (data not shown). Crown-rump lengths of E14.5 embryos were also unaffected by embryonic *Mthfd1* genotype. However, there was a trend towards a significant interaction of maternal *Mthfd1* genotype and diet (F= 2.9, p = 0.09) on crown-rump lengths of embryos at E14.5. Embryos derived from *Mthfd1*$^{gt/+}$ females on the FCD diet had decreased crown-rump lengths as compared to embryos derived from *Mthfd1*$^{+/+}$ females on the control diet at E14.5 (10.9 mm vs. 11.6mm, p = 0.05, Figure 4.3).

Effect of maternal Mthfd1 disruption and low folate status on fertility

Total implants and resorptions were examined in litters isolated at E11.5 and E14.5 from *Mthfd1*$^{gt/+}$ and *Mthfd1*$^{+/+}$ females maintained on the control or FCD diets (Tables 4.4, 4.5). Analysis of total litter implants included viable embryos, FGR-affected embryos that were either viable or in the process of being resorbed, and complete resorptions. Analysis of resorptions included complete resorptions only; FGR-affected embryos that were in the process of resorbing were excluded from these

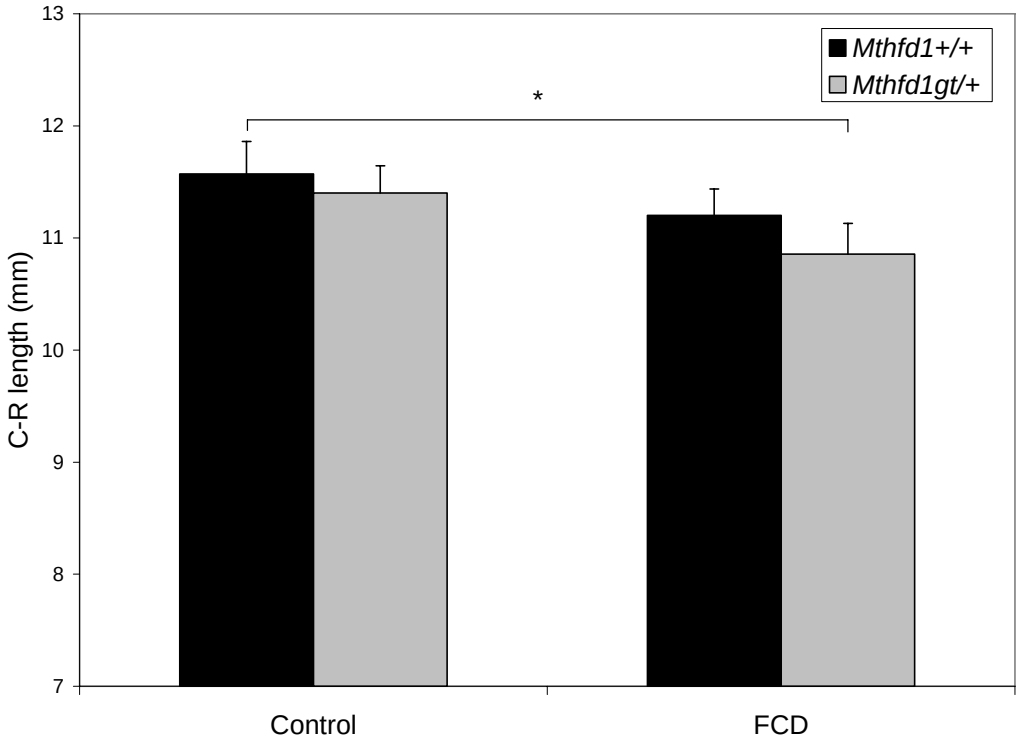

Figure 4.3 Crown-rump length in embryos isolated at E14.5 as a function of maternal *Mthfd1* genotype and diet. Main effects of embryonic genotype, maternal genotype, and maternal diet and relevant interactions were analyzed by analysis of variance in SAS. Data represent mean ± SEM. There was a trend towards a significant interaction effect of maternal genotype x maternal diet on CR-length ($p = 0.09$).
***** Embryos derived from *Mthfd1*$^{gt/+}$ females on the FCD diet had decreased crown-rump lengths as compared to embryos derived from *Mthfd1*$^{+/+}$ females on the control diet at E14.5 ($p = 0.05$).

Table 4.4. Resorptions and implants in litters derived at E11.5 from *Mthfd1*$^{gt/+}$ and *Mthfd1*$^{+/+}$ dams fed the control or FCD diets. Values represent mean ± SEM. Main effects of *Mthfd1* genotype, diet, and the interaction of genotype and diet on implantation-rate and log-transformed resorption rates were analyzed by analysis of variance in SAS. There was a significant main effect of diet on the total number of implants ($F = 9.59$, $p = 0.003$). There was also a trend towards a main effect of *Mthfd1* genotype on total implants ($F = 2.82$, $p = 0.099$). The interaction between *Mthfd1* genotype and diet was not significant.

Diet	Maternal Genotype	No. of Litters	Total Implants	No. implants/ litter	Total Resorp.	No. resorp/ litter
Control	*Mthfd1*$^{+/+}$	10	34	3.4 ± 0.6	1	0.1 ± 0.1
	Mthfd1$^{gt/+}$	15	56	3.7 ± 0.6	7	0.47 ± 0.23
FCD	*Mthfd1*$^{+/+}$	16	74	4.4 ± 0.5	10	0.63 ± 0.24
	Mthfd1$^{gt/+}$	13	82	6.3 ± 0.7	10	0.77 ± 0.28
p value, diet effect				$p < 0.005$		NS
p value, genotype effect				NS		NS
p value, diet X genotype effect				NS		NS

Table 4.5. Resorptions and implants in litters derived at E14.5 from *Mthfd1*$^{gt/+}$ and *Mthfd1*$^{+/+}$ dams fed the control or FCD diet. Values represent mean ± standard error. Main effects of *Mthfd1* genotype, diet, and the interaction of genotype and diet on implantation-rate and log-transformed resorption rates were analyzed by analysis of variance in SAS. There was a trend towards a main effect of maternal diet on total implants (F= 3.06, p = 0.08). *Mthfd1* genotype did not significantly influence any outcomes.

Diet	Maternal Genotype	No. of Litters	Total Implants	No. implants/ litter	Total Resorp.	No. resorp/ litter
Control	*Mthfd1*$^{+/+}$	11	43	3.9 ± 0.6	6	0.55 ± 0.25
	Mthfd1$^{gt/+}$	17	55	3.2 ± 0.3	14	0.82 ± 0.18
FCD	*Mthfd1*$^{+/+}$	22	55	2.5 ± 0.3	17	0.77 ± 0.25
	Mthfd1$^{gt/+}$	12	30	2.5 ± 0.4	11	0.91 ± 0.40
p value, diet effect				NS		NS
p value, genotype effect				NS		NS
p value, diet X genotype effect				NS		NS

analyses. Maternal diet significantly affected the total number of fetal implants in litters derived at E11.5; there were significantly more implants in litters derived from females maintained on the FCD diet as compared to the control diet (5.4 vs. 3.6; F = 9.59, p = 0.003). Total fetal implants were also slightly higher in *Mthfd1*$^{gt/+}$ dams as compared to *Mthfd1*$^{+/+}$ dams (5.0 vs. 4.0; F = 2.82, p = 0.099). Total fetal implants in litters isolated from *Mthfd1*$^{gt/+}$ females fed the FCD diet and supplemented with 500 μM hypoxanthine were significantly lower than unsupplemented *Mthfd1*$^{gt/+}$ females on the FCD diet (3.8 vs. 6.3, p < 0.01), and were similar to litters of *Mthfd1*$^{gt/+}$ females maintained on the control diet (3.8 vs. 3.7 implants). Litters derived at E14.5 from females maintained on the FCD diet had slightly fewer fetal implants as compared to litters derived from females fed the control diet (3.3 vs. 4.3; F= 3.06, p = 0.08; Table 4.5). There was no independent effect of maternal *Mthfd1* genotype on the number of fetal implants in litters derived at E14.5.

Examination of total resorptions in litters derived at E11.5 revealed a trend towards a significant effect of maternal diet (F = 2.89, p = 0.087). More resorptions occurred in response to the FCD diet as compared to the control diet (0.7 vs. 0.28 resorptions/litter). Maternal *Mthfd1* genotype had no effect on total resorptions in litters derived at E11.5. However, only 1 resorption was found in 10 litters derived from *Mthfd1*$^{+/+}$ females on the control diet, whereas almost 50% of litters isolated from crosses of *Mthfd1*$^{gt/+}$ females on the FCD diet included at least one resorption. There was no effect of either maternal *Mthfd1* genotype or maternal diet on total resorptions in litters derived at E14.5. Hypoxanthine supplementation in *Mthfd1*$^{gt/+}$ females on the FCD diet was associated with a moderate reduction in the number of resorptions per litter as compared to unsupplemented *Mthfd1*$^{gt/+}$ females on the FCD diet (0.37 vs. 0.77), but this comparison was not statistically significance (p = 0.1, one-tailed t-test).

Maternal Mthfd1 disruption and low folate status influence markers of folate metabolism

The FCD diet was associated with a significant decline in maternal RBC folate levels measured at E11.5 (Figure 4.4; $F = 234$, $p < 0.0001$). RBC folate levels were reduced by approximately 65% on the FCD diet relative to the control diet (8 vs. 23 fmol/μg protein). *Mthfd1* disruption was also associated with a significant reduction in RBC folate levels in pregnant dams maintained on both diets ($F = 46.7$, $p < 0.0001$). However, *Mthfd1* genotype influenced RBC folate levels to a greater extent in mice maintained on the control diet than on the FCD diet ($F = 31.25$, $p = 0.01$). RBC folate levels in *Mthfd1*$^{gt/+}$ females were reduced approximately 35% compared to *Mthfd1*$^{+/+}$ females on either diet, but the magnitude of the difference was greater in females maintained on the control diet (18.4 vs. 27.9 fmol/μg protein; $p < 0.0001$) as compared to the FCD diet (6.5 vs. 10.3 fmol/μg protein; $p = 0.01$).

The effects of maternal *Mthfd1* disruption and maternal folate/choline status on plasma metabolites associated with homocysteine remethylation and metabolism were determined in plasma samples taken from pregnant female mice at E11.5 (Table 4.6). The FCD diet significantly influenced several markers of homocysteine metabolism. Plasma homocysteine was elevated approximately three-fold in all dams maintained on the FCD diet, regardless of *Mthfd1* genotype (5.3 vs. 14.2 μM, $p = 0.0008$). The FCD diet was also associated with significantly elevated cystathionine and serine levels (Table 4.6), and significantly decreased levels of dimethylglycine in all mice. *Mthfd1* genotype did not affect the levels of any of the metabolites measured. However, there was a trend towards a significant interaction effect between maternal *Mthfd1* genotype and diet on plasma methionine levels ($F = 3.24$, $p = 0.08$). Plasma methionine in *Mthfd1*$^{gt/+}$ females on the control diet were decreased relative to

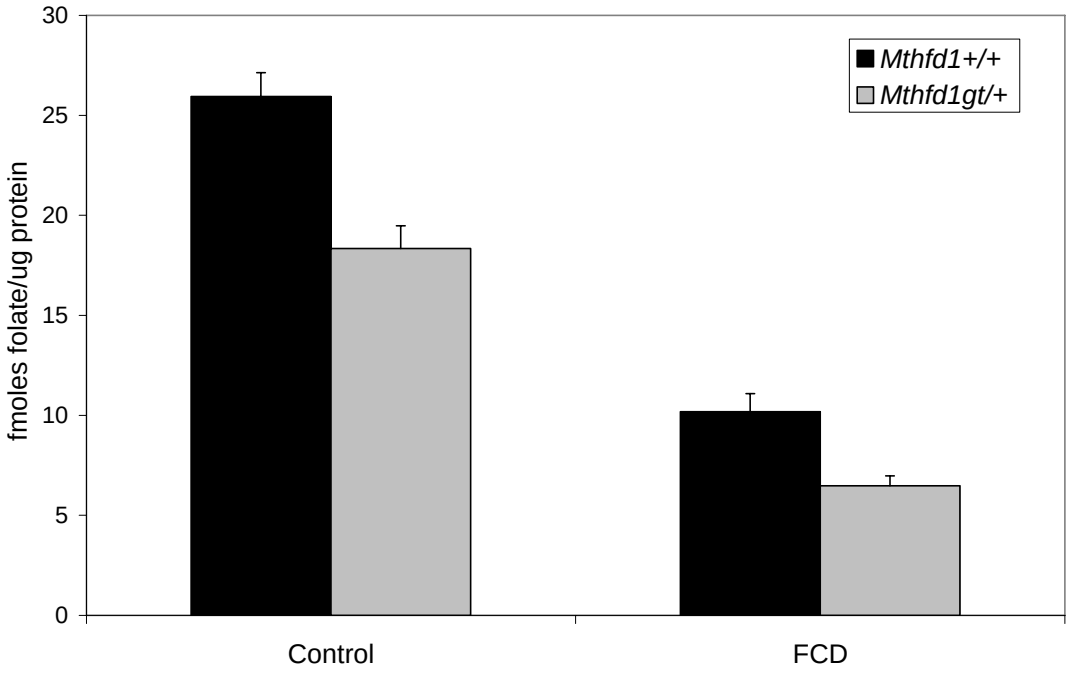

Figure 4.4. Red blood cell folate levels in *Mthfd1*$^{gt/+}$ and *Mthfd1*$^{+/+}$ mice. Both diet and *Mthfd1* genotype influenced RBC folate levels in dams. Significant main effects of diet, genotype, and diet x genotype interaction were analyzed by analysis of variance in SAS (Cary, NC). Data represent mean ± SD. A significant diet X genotype interaction ($F = 9.06$, $p = 0.01$) reflected a larger effect of genotype associated with the control diet.

Table 4.6. Plasma metabolites in pregnant *Mthfd1*$^{gt/+}$ and *Mthfd1*$^{+/+}$ dams. Values represent mean ± SEM. Independent effects of Mthfd1 genotype, diet, and the interaction between Mthfd1 genotype and diet were determined by analysis of variance. A, Mthfd1gt/+ fed the control is significantly different than Mthfd1gt/+ fed the FCD diet (p= 0.03).

Metabolite	Diet				Diet effect	Genotype effect	Diet X genotype effect
	Control		FCD				
	Mthfd1+/+	Mthfd1gt/+	Mthfd1+/+	Mthfd1gt/+			
Homocysteine (μM)	4.9 ± 0.9	5.7 ± 1.3	15.5 ± 3.1	12.8 ± 2.9	p = 0.0008	ns	ns
Cysteine (μM)	183 ± 15	183 ± 39	192 ± 12	184 ± 17	ns	ns	ns
Cystathionine (μM)	997 ± 490	694 ± 101	1211 ± 129	1204 ± 214	p = 0.03	ns	ns
α - Aminobutyric Acid (μM)	5.3 ± 0.8	4.9 ± 1.1	5.7 ± 0.5	5.1 ± 1.1	ns	ns	ns
Methionine (μM)	54.0 ± 8.6	33.5 ± 3.4	51.4 ± 3.6	56.8 ±10.6	ns	ns	p = 0.08[A]
Glycine (μM)	93.3 ± 12.1	103 ± 13	91.0 ± 14.0	80.2 ± 11.6	ns	ns	ns
Serine (μM)	115 ± 9	92 ± 7	130 ± 10	127 ± 17	p = 0.04	ns	ns
Dimethylglycine (μM)	7.25 ± 0.67	8.11 ± 0.53	4.64 ± 0.51	3.61 ± 0.44	P < 0.0001	ns	ns
Methylglycine (μM)	0.54 ± 0.05	0.56 ± 0.06	0.50 ± 0.05	0.45 ± 0.03	ns	ns	ns

Mthfd1$^{+/+}$ females fed the control diet (33.5 μM vs. 54.0 μM, p = 0.05) and *Mthfd1*$^{+/+}$ females fed the FCD diet (33.5 μM vs. 51.4 μM , p = 0.03).

Comparison of plasma metabolite profiles of hypoxanthine-supplemented and unsupplemented *Mthfd1*$^{gt/+}$ females fed the FCD diet revealed that hypoxanthine supplementation influenced glycine and methylglycine levels (data not shown). Glycine levels were significantly higher in hypoxanthine-supplemented females as compared to unsupplemented *Mthfd1*$^{gt/+}$ females fed the FCD diet (140 vs. 80 μM; p = 0.04, 1-tailed T test). Similarly, methylglycine levels were significantly elevated in hypoxanthine supplemented females as compared to unsupplemented females (0.63 vs. 0.45 μM, p = 0.01, 1 tailed t-test). Both glycine and methylglycine levels in hypoxanthine-supplemented females were also elevated in comparison to *Mthfd1*$^{gt/+}$ females fed the control diet, although these comparisons did not reach statistical significance.

Folate-mediated OCM is not disrupted in Mthfd1$^{gt/+}$ *MEFs*

The effects of *Mthfd1* disruption on *de novo* purine biosynthesis and cellular methylation potential was investigated in four independent *Mthfd1*$^{gt/+}$ and *Mthfd1*$^{+/+}$ MEF cell lines derived from crosses of C57Bl/6J females and 129.Ola- *Mthfd1*$^{gt/+}$ males. Rates of *de novo* purine biosynthesis were similar in *Mthfd1*$^{gt/+}$ and *Mthfd1*$^{+/+}$ MEF cell lines (Figure 4.5), as measured by the relative ratio of incorporation of [^{14}C]-formate to [^{3}H]-hypoxanthine into nuclear DNA. Similarly, there was no effect of *Mthfd1* disruption on the AdoMet/AdoHcy ratio, an index of cellular methylation capacity. *Mthfd1*$^{gt/+}$ MEF cell lines did not exhibit statistically significant differences in AdoMet, AdoHcy, or the AdoMet/AdoHcy ratio as compared to *Mthfd1*$^{+/+}$ cell lines (Table 4.7).

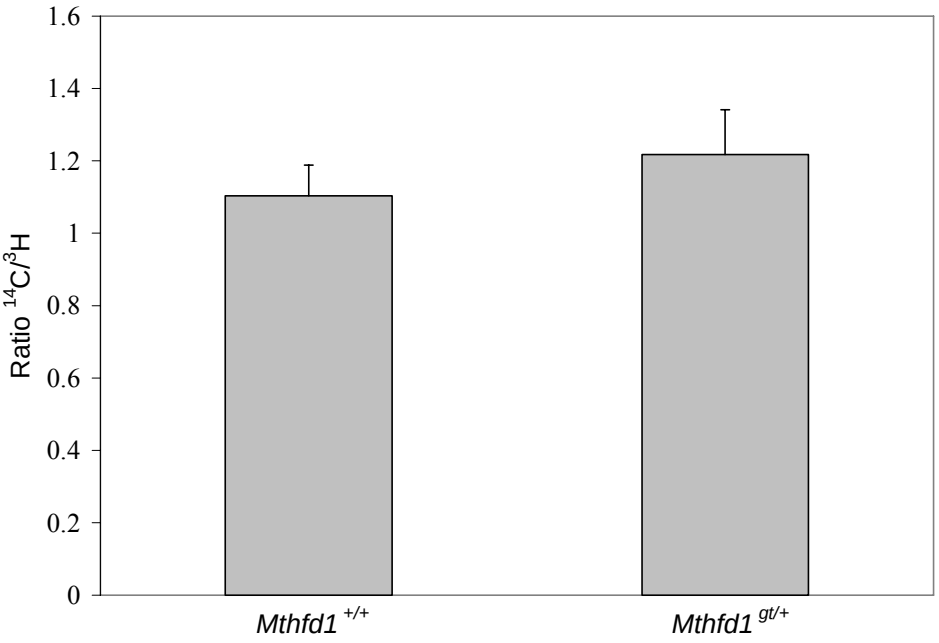

Figure 4.5. *De novo* purine biosynthesis is not altered in *Mthfd1*$^{gt/+}$ MEFs. Efficiency of *de novo* purine biosynthesis was determined by the relative enrichment of ^{14}C-formate (20 μM) / ^{3}H-hypoxanthine (2 nm) in nuclear DNA. All values are the average of triplicate measures of three different cell lines per genotype. Data represent mean ± SEM. A one-tailed unpaired T-test was used to determine genotype differences.

Table 4.7. AdoMet and AdoHcy concentrations in *Mthfd1*-deficient MEFs. Concentration of AdoMet and AdoHcy were determined by HLPC analysis. One-tailed t-tests were used to determine genotype differences.

Genotype	Metabolite		
	AdoMet (pmoles/ug protein)	AdoHcy (pmoles/ug protein)	Ratio (AdoMet:AdoHcy)
Mthfd1+/+	0.04 ± 0.008	0.11 ± 0.056	0.82 ± 0.43
Mthfd1gt/+	0.04 ± 0.005	0.04 ± 0.005	0.75 ± 0.12
p value	ns	ns	ns

Discussion

Mthfd1$^{gt/+}$ mice exhibited a 50% decrease in formylTHF synthetase activity, similar to the reduction of activity observed for the human formylTHF synthase protein in the *MTHFD1* G1958A variant (40). In this study, the metabolic disruption associated with *Mthfd1*$^{gt/+}$ mice adversely affected fertility and embryonic development, and recapitulated reproductive outcomes associated with the human G1958A polymorphism including delayed heart and jaw development and pregnancy loss. Maternal *Mthfd1* disruption and/or low folate status induced fetal growth restriction, whereas embryonic *Mthfd1* disruption had no effect on fetal growth. Complete loss of *Mthfd1* expression resulted in very early fetal loss prior to neurulation, consistent with human studies demonstrating that the G1958A SNP is not in Hardy-Weinberg equilibrium, with the 1958A genotype being underrepresented relative to expectations (32). Together, these data confirm that the generation of 1Cs in the cytoplasm by C1THF synthase is essential for early embryonic development and that partial loss of synthetase activity at the maternal level is sufficient to affect fetal growth and development.

In the absence of nutrient supplementation, neither maternal nor embryonic *Mthfd1* disruption caused NTDs. However, hypoxanthine supplementation of *Mthfd1*$^{gt/+}$ pregnant females fed the FCD diet, the group that exhibited the greatest degree of impairment in 1C metabolism, resulted in 2 of 28 *Mthfd1*$^{gt/+}$ embryos exhibiting exencephaly. Hypoxanthine supplementation also resulted in an increase in the incidence of FGR in the same litters. The observed increase was due to a two-fold increase in the incidence of FGR in *Mthfd1*$^{gt/+}$ embryos. The increase in FGR-affected embryos and incidence of NTDs in litters derived from hypoxanthine-supplemented *Mthfd1*$^{gt/+}$ females fed the FCD diet indicates either that hypoxanthine

supplementation negatively impacted fetal growth or that hypoxanthine supplementation rescued earlier fetal loss in *Mthfd1*$^{gt/+}$ embryos. The latter explanation is supported by the observed moderate decrease in the number of resorptions in litters derived from hypoxanthine supplemented *Mthfd1*$^{gt/+}$ females as compared to unsupplemented *Mthfd1*$^{gt/+}$ females. Rescue of early fetal loss by hypoxanthine supplementation indicates impaired purine biosynthesis as an underlying cause of impaired fertility and fetal development in response to *Mthfd1* disruption. Furthermore, the occurrence of NTDs in *Mthfd1*$^{gt/+}$ embryos also implicates impaired nucleotide biosynthesis in NTD pathogenesis. Although purine biosynthesis was not impaired in *Mthfd1*$^{gt/+}$ MEFs in this study, Christensen et al (41) have previously demonstrated purine auxotrophy in immortalized MEF lines in which *Mthfd1* has been inactivated. Furthermore, the effect of *Mthfd1* disruption on adverse fetal outcomes was most strongly associated with maternal genotype. Similarly, the NTD risk associated with the human *MTHFD1* G1958A polymorphism is strongly linked to maternal genotype (32,38). Therefore, maternal *de novo* purine biosynthesis capacity may be more important for fetal development than embryonic *de novo* purine biosynthesis.

In a previous study, plasma folate levels were unaffected in *Mthfd1*$^{gt/+}$ male mice (40). In this study, we observed a significant reduction in RBC folate levels in pregnant *Mthfd1*$^{gt/+}$ females. Although AdoMet and AdoHcy levels were unaffected in *Mthfd1*$^{gt/+}$ MEFs, *Mthfd1*$^{gt/+}$dams maintained on the control diet exhibited reduced plasma methionine levels, suggesting that *Mthfd1* disruption also influenced the availability of 5-methylTHF for methionine biosynthesis. Similarly, reduced liver AdoMet levels were reported previously in *Mthfd1*$^{gt/+}$ male mice maintained on the control, but not the FCD diet (40). Thus, maternal folate deficiency and/or impairments in purine and/or methionine biosynthesis may have contributed to poor

reproductive outcomes in *Mthfd1*$^{gt/+}$ females.

Maternal hypoxanthine supplementation rescued early fetal loss, but did not ameliorate fetal growth restriction associated with combined maternal *Mthfd1* disruption and dietary maternal folate and choline deficiency. Furthermore, maternal hypoxanthine supplementation induced NTDs in *Mthfd1*$^{gt/+}$ dams consuming the FCD diet. These data indicate that the underlying cause of FGR in neurulation-stage embryos may be distinct from that of earlier fetal loss, or that maternally-derived free nucleotides may be more available to the fetus prior to formation of the definitive placenta. The timing of FGR in affected embryos coincided with the development of the definitive placenta in mice. Elevated homocysteine in response to the FCD diet may have also contributed to impaired placental function in *Mthfd1*$^{+/+}$ females, as homocysteine is an established risk factor for vasculopathy (42-45). In any event, these data indicate that maternal hypoxanthine supplementation increases risk for NTDs in folate-deficient *Mthfd1*$^{gt/+}$ dams, potentially by rescuing early fetal loss.

Experimental Procedures

Experimental animals and diets

All animal experiments were approved by the Cornell Institutional Animal Care and Use Committee according to the guidelines of the Animal Welfare Act and all applicable federal and state laws. Mice were maintained on a 12 hour light/dark cycle in a temperature controlled room. For timed pregnancies, virgin female mice aged 70-120 days were housed overnight with males. The following morning, females were examined for the presence of a vaginal plug. Nine o'clock A.M. the day of the plug was designated as embryonic day 0.5 (E0.5). Pregnant females were sacrificed by carbon dioxide-induced asphyxiation and blood was collected by cardiac puncture. Embryos were harvested at E11.5 or E14.5. Gravid uteri were removed and all

implants and resorptions sites were recorded. Embryos were dissected free of extra embryonic membranes under a dissecting microscope, and examined for presence of NTDs. All yolk sacs were collected for subsequent genotyping. Embryos were fixed for 24 hours in 10% neutral buffered formalin, dehydrated in an ethanol series, and stored in 70% ethanol.

The generation and characterization of mice with a gene trap insertion in the *Mthfd1* gene (*Mthfd1*$^{gt/+}$) has been previously described (40). To determine the developmental stage of embryonic lethality in *Mthfd1*$^{gt/gt}$ mice, embryos were examined from crosses of heterozygote B6.129P2-*Mthfd1*^{gt} mice. Diet studies were conducted using *Mthfd1*$^{gt/+}$and *Mthfd1*$^{+/+}$ mice derived from crosses of 129P2/OlaHsd females to heterozygote 129P2/OlaHsd-*Mthfd1*$^{gt/+}$ male mice. Female mice were randomly assigned to either an experimental AIN93G diet lacking folate and choline (FCD) or an AIN93G (Control) diet (Dyets, Bethlehem, PA) at weaning. Choline was excluded from the experimental diet in addition to folate to increase stress on the homocysteine remethylation cycle. Choline synthesis requires methyl groups in the form of AdoMet and it provides a source of one-carbon units for homocysteine remethylation through its catabolism (46). Females were maintained on diet from weaning throughout the breeding period and for the duration of gestation, until killed. For studies involving hypoxanthine supplementation, hypoxanthine (Sigma) was dissolved in nanopure water at a concentration of 10 mM and diluted with acidified drinking water to a final concentration of 500 μM. The final concentration of hypoxanthine in drinking water was verified by uv spectroscopy (ε =10.5 mM at λ= 250 nm).

Genotype analysis

Genotyping of the *Mthfd1*^{gt} allele was performed as recently described (40). Embryonic sex was determined using a genotyping protocol described elsewhere (47).

Analysis of red blood cell folate levels

Folate levels in red blood cell samples was quantified using a *Lactobacillus casei* microbiological assay as previously described (48).

Determination of plasma metabolites

Plasma levels of homocysteine, cystathionine, cysteine, α - aminobutyric acid, methionine, glycine, serine, N,N-dimethylglycine and N-methylglycine were determined by stable isotope dilution capillary gas chromatography mass spectrometry as previously described (49,50)

Generation of mouse embryonic fibroblast cell lines

Mouse embryonic fibroblast (MEF) cell lines were generated from embryos isolated 10-14 days post-coitus from crosses of C57Bl6/J females and 129P2.OlaHsd-*Mthfd1*$^{gt/+}$ males. Genotyping of MEF lines was completed both at the time of generation using nuclear DNA isolated from embryo heads, as well as from cell pellets, using the same genotyping protocols described above. MEFs were incubated at 37° C in Defined Minimal Essential Medium, alpha modification (Hyclone, Inc) with 10% fetal bovine serum.

Formate suppression assay

MEFs were plated in triplicate in 6-well plates at a density of 1 X10^4 cells per well. A formate suppression assay, which measures the relative efficiency of *de novo* purine nucleotide synthesis relative to synthesis from the purine nucleotide salvage pathway, was performed as previously described (51).

Determination of AdoMet and AdoHcy concentrations

Total levels of AdoMet and AdoHcy were measured in MEF cell pellets using an established HPLC procedure (52,53).

Statistical Analysis

Analyses of FGR incidence and embryonic crown-rump length were conducted using

repeated-measured analysis of variance (PROC MIXED or PROC GENMOD, SAS, Cary, NC) in which independent variables included maternal and embryonic *Mthfd1* genotype, maternal diet ,and embryo sex, and litter was considered as a repeated measure. Relevant interaction terms were included unless assumptions of variance were violated. Analysis of total litter resorptions and implants was analyzed by analysis of variance in which litter was considered as the unit of analysis and independent variables included maternal *Mthfd1* genotype and diet. Resorption rate was calculated as the ratio of resorptions/ total implants per litter and log-transformation was applied to normalize the data. Analysis of total red blood cell folate levels and metabolite levels was accomplished by analysis of variance in which maternal *Mthfd1* genotype and diet were considered as independent variables. Specific comparisons within main effects were assessed by comparisons of least-squared means. X^2 analyses were used to assess any deviation from expected genotype ratios based on Mendelian inheritance. For cell culture assays, genotype differences were assessed using a one-tailed t-test.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Scope of Problem

Neural tube closure defects (NTDs) arise from complex interactions of genetic and environmental risk factors. Low maternal folate status is a strong environmental determinant of NTD risk (1). Furthermore, it has been clearly demonstrated from both observational and randomized controlled trials that maternal folic acid supplementation substantially reduces both occurrence and recurrence of NTDs in the general population (2-6). Unfortunately, promotion of maternal vitamin use for NTD prevention has proved largely ineffective as a majority of pregnancies are unplanned and the process of neural tube closure occurs within the first three week of human gestation, often before most women know they are pregnant. As such, many countries today either fortify or are considering fortification of their food supply with folic acid for the prevention of NTDs (7-10). Although the protection against NTDs afforded by maternal folic acid supplementation is so convincing as to drive public policy, the mechanism(s) by which folic acid supplementation prevents NTDs remain unknown. This unusual predicament results in fortification of entire populations for the protection of a relatively small subset of the population (e.g. women of childbearing age) with very little known regarding potential consequences for other population groups (e.g. aging individuals, cancer patients). Therefore, identifying the direct mechanism(s) underlying the association between disrupted folate metabolism and NTD risk is critical for better targeting of preventative measures and minimization of potential unintended consequences.

In parallel to identifying the mechanism(s) by which impaired folate metabolism affects neural tube closure, the identification of candidate genes that

mediate elevated NTD risk in humans is necessary in order to identify and target genetically susceptible individuals. There is a strong genetic component to NTD risk (11); although genetic variants have been identified within folate-related genes that contribute to elevated NTD risk (12), the penetrance of these variants does not account for the prevalence of NTDs in the general population (13). Furthermore, investigation of current folate-related gene candidates has not yielded any further information as to underlying mechanism of NTD pathogenesis, and genetic disruption of these same candidate genes in mice does not yield NTDs (14), preventing the use of these models to further investigate metabolic mechanisms and suggesting that these genes may not directly contribute to NTD risk.

Folate-mediated one carbon metabolism (OCM) is essential for nucleotide biosynthesis, the remethylation of homocysteine to methionine, and S-adenosylmethionine (AdoMet)-dependent cellular methylation. OCM can be impaired by nutrient deficiency, either of folate or other micronutrients that influence folate status, or by variation in genes that accumulate or metabolize folate. Impairments in OCM result in elevated homocysteine, misincorporation of uracil into DNA, reduced nucleotide availability, and chromatin hypomethylation (15). All of these metabolic sequelae have the potential to disrupt neural tube closure by impinging on the cellular proliferation, survival, differentiation or migration processes within the embryonic neuroepithelium that are required for neural tube closure. Although limited evidence from human genetic and epidemiological studies has implicated homocysteine accumulation and/or impaired cellular methylation in NTD pathogenesis (16-18), direct evidence from human or animals studies to support this hypothesis is lacking.

Summary: Deletion of Shmt1 Causes NTDs by Impairing De Novo Thymidylate Biosynthesis

The data presented herein identify both the mechanism and the candidate gene responsible for pathogenesis in folate-responsive NTDs. These data demonstrate that deletion of *Shmt1* in mice induces NTDs by impairing *de novo* thymidylate biosynthesis. This is the first demonstration of an NTD associated with deletion of a folate-metabolizing gene in mice and therefore provides a novel model with which to address metabolic mechanisms underlying folate-responsive NTDs. NTDs in response to *Shmt1* deficiency mimic the profile of human folate-responsive NTDs because they are partially penetrant, folate-responsive, and both maternal and fetal genotypes contribute to NTD risk This study also identifies impaired thymidylate biosynthesis as an underlying cause of folate-responsive NTDs. Our lab has demonstrated previously that *Shmt1* regulates the partitioning of folate-mediated OCM by prioritizing *de novo* thymidylate biosynthesis at the expense of the homocysteine remethylation cycle. Deletion of *Shmt1* causes NTDs and results in impaired *de novo* thymidylate biosynthesis in fibroblast cell lines and increased DNA uracil content in mice, whereas homocysteine levels are unaffected. Impaired thymidylate biosynthesis has been identified previously as a mechanism underlying NTD pathogenesis in response to the *splotch* mutation within the gene encoding *Pax3*. In this study we confirmed impaired thymidylate biosynthesis in *splotch* MEF cell lines and demonstrated that *Shmt1* and *Pax3* interacted in NTD pathogenesis, with *Shmt1* deficiency resulting in exacerbated lesions in $Pax3^{sp/sp}$ embryos and occasional NTDs in $Pax3^{sp/+}$ and $Pax3^{+/+}$ embryos isolated from compound *Shmt1*, *Pax3* mutant crosses. The interaction between *Pax3* and *Shmt1* occurred at both the fetal and maternal genotype levels, and was also sensitive to maternal folate status. Furthermore, *Pax3* and *Shmt1* were both shown to influence thymidylate synthase (TYMS) protein levels

as well as be mutually regulated. The demonstrated genetic interaction between *Shmt1* and *Pax3* further confirms the role of thymidylate biosynthesis in NTD pathogenesis. Lastly, these data also indicate that impairments in purine biosynthesis may also contribute to NTD pathogenesis. Collectively, these data provide additional evidence implicating impaired nucleotide biosynthesis as an underlying cause of NTDs in these two folate-responsive NTD models.

Summary: Maternal Mthfd1 Disruption Impairs Fertility, Neural Tube Closure, and Fetal Growth

A common human polymorphism in *MTHFD1* (A1298C) has been identified in human genetic screens as a maternal risk factor for NTDs, as well as adverse pregnancy outcomes related to fertility and overall embryonic development. Disruption of *Mthfd1* by insertion of a gene trap vector mimics the loss of formyl synthetase activity associated with the *MTHFD1* A1298C human polymorphism. This study confirms the maternal effect of *Mthfd1* disruption on embryonic development. Both low maternal folate status and maternal *Mthfd1* disruption resulted in fetal growth restriction, with severe delay in heart, limb, and jaw development, similar to the anomalies associated with the *Mthfd1* polymorphism in humans. Rescue of very early fetal loss with hypoxanthine supplementation revealed occasional NTDs in *Mthfd1*$^{gt/+}$ embryos, confirming a role of *Mthfd1* in neural tube closure. *Mthfd1* disruption in pregnant dams was associated with reduced folate status and impaired methionine metabolism. Together, these data indicate that disruption of *Mthfd1* affects embryonic growth and neural tube closure by affecting folate status, methionine metabolism, and purine biosynthesis, although different mechanisms my contribute uniquely to pathogenesis in different conditions.

Implications

The data presented herein have major implications for understanding the mechanism by which impaired folate metabolism contributes to NTD pathogenesis. Previously, appropriate mouse models for addressing metabolic mechanisms underlying NTD pathogenesis were inadequate. Mouse mutants within the OCM network did not develop NTDs and therefore provided limited opportunity to study mechanism. Furthermore, the degree to which other folate-responsive NTD mouse models (e.g. *splotch*) informed pathogenesis of human NTDs was questionable because the associated genes were not implicated in human genetic screens (e.g. *Pax3*) (19). Here we describe two new models, *Shmt1*+/- and *Mthfd1*gt/+ mice, which can be used to gain further insight into pathogenesis of folate-responsive NTDs. Each of these mutants closely models NTD pathogenesis in humans; NTDs in these models are partially penetrant, folate-responsive, and reflect the relative contributions of embryonic and maternal genotype as observed in humans. These models can now be used not only to address metabolic disruption underlying NTD pathogenesis, but to link specific impairments with genomic responses and cellular responses that ultimately result in failure of neural tube closure.

The current aim of research within the field of folate-responsive NTDs is to disentangle gene-nutrient interactions in NTD pathogenesis. The data presented here provides novel insight into how nutrient status interacts with genetic disruption of folate utilization to confer NTD risk. Impaired nucleotide biosynthesis was implicated as an underlying cause of NTD pathogenesis in both *Shmt1* and *Mthfd1* models. However, subtle differences between these two models highlight the interaction between folate status and folate utilization (Chapter 2). Deletion of *Shmt1* impaired thymidylate biosynthesis but did not affect folate status; furthermore, embryonic *Shmt1* deletion was required for NTD pathogenesis. In contrast, disruption of *Mthfd1*

affected maternal folate status, which may have contributed to NTD incidence. Furthermore, the robust maternal effect of *Mthfd1* disruption suggests that maternally-derived purines may be essential for early embryonic development, suggesting that maternal nutrient status might affect some folate-dependent pathways more than others. Thus, NTDs in response to *Shmt1* deletion result from impairment of cellular folate utilization, whereas *Mthfd1* disruption may confer NTD risk by disrupting both folate accumulation and folate utilization. Disentangling the relative contributions of maternal and fetal genotype in terms of metabolic impairments will provide further insight into the interaction of maternal and fetal genotype in human NTD pathogenesis.

A robust human gene candidate for folate-responsive NTDs has yet to be identified. Although human genetic studies have implicated genes with the homocysteine remethylation cycle, these studies have been limited by their inability to identify causal mechanisms. The present data indicate that elevated homocysteine in NTD-affected pregnancies is merely a biomarker of low folate status and/or impaired folate metabolism, as both *Mthfd1* disruption and *Shmt1* deficiency resulted in NTDs in the absence of changes in homocysteine levels. Thus, these data provide further support for the hypothesis that gene candidates within the homocysteine remethylation pathway confer NTD risk by affecting folate status, *not* by impairing metabolism. Moreover, *Shmt1* haploinsufficiency and partial disruption of *Mthfd1* was sufficient to induce NTDs, indicating that relatively small changes in expression of folate-related genes contribute to NTD pathogenesis. These data imply that the failure of human genetic screens to identify promising human NTD candidate genes, including *Shmt1*, may have been due to the inability of these screens to identify alterations in expression levels, which may represent more penetrant risk factors. Lastly, these data suggest that future studies of human candidate NTD genes should focus on genes regulating

nucleotide biosynthesis instead of genes involved in the homocysteine remethylation cycle.

Future Directions

In addition to providing novel insight into metabolic mechanisms underlying folate-responsive developmental anomalies, the present data offer several avenues for future research. To begin, the experimental diet used in these studies was deficient in both folate and choline. Although our goal in using choline deficiency was to increase the demand for folate-activated 1Cs and thereby increase the demand for folate, human epidemiological studies have recently suggested that disrupted choline metabolism may be an independent risk factor for NTDs (20,21). These data warrant investigation of the independent contribution of folate and choline deficiency to NTD risk in *Shmt1*-deficient mice. If indeed choline deficiency is found to interact with *Shmt1* deficiency in NTD pathogenesis, then the metabolic mechanism underlying this interaction should be explored further.

The effects of *Mthfd1* disruption on embryonic development were sensitive to hypoxanthine supplementation, but impairments in *de novo* nucleotide biosynthesis were not directly demonstrated in this study. Further investigation of impairments in nucleotide biosynthesis including thymidylate biosynthesis, either *in vivo* or in a cell culture model, in response to *Mthfd1* disruption is warranted. In particular, investigation of nucleotide biosynthesis in a context of folate deficiency may be more informative. As *Mthfd1* disruption may confer NTD risk by both impairing both folate utilization and folate status, it is possible that impairments in purine biosynthesis in response to partial disruption of *Mthfd1* (e.g. in *Mthfd1*$^{gt/+}$ mice) will be more apparent under folate-deficient conditions.

The finding of impaired *de novo* purine biosynthesis in the *Pax3*-deficient

splotch mutant is novel and implicates impaired purine biosynthesis in NTD pathogenesis. It has been previously demonstrated that thymidine supplementation can prevent NTDs in *splotch* embryos (22,23). It would be interesting to determine if hypoxanthine supplementation could similarly rescue NTDs in *splotch* embryos, and if spina bifida or exencephaly would be more sensitive to supplementation with hypoxanthine, thereby confirming the hypothesis that impairments in purine and thymidine biosynthesis contribute to pathogenesis in spina bifida and exencephaly, respectively.

Methionine supplementation exacerbates NTDs in the *splotch* mutant, which exhibit impairments in *de novo* thymidylate biosynthesis. This effect is not mediated alterations in homocysteine levels or cellular methylation potential (24), which implies that methionine influences NTD pathogenesis by affecting thymidylate biosynthesis. Exploration of the effects of methionine supplementation on thymidylate biosynthesis in a cell culture model would shed light on this hypothesis. In addition, it would be of interest to determine whether methionine supplementation would also exacerbates NTDs in *Shmt1*-deficient mice.

Pax3 and *Shmt1* were shown to influence TYMS protein levels and interact in NTD pathogenesis; however, *Shmt1* and *TYMS* message levels were no altered in response to the *splotch* mutation. Investigation of the molecular mechanisms underlying the interaction of *Pax3* and *Shmt1* in relation to TYMS and thymidylate biosynthesis is warranted. One potential clue arises from the finding that combined *Pax3* and *Shmt1 d*eficiency resulted in embryos with craniorachiscisis in response to dietary folate deficiency. This extensive defect has been linked exclusively to mutations within the planar cell polarity (PCP) pathway (25,26) (27). As *Pax3* has not been previously linked to the planar cell polarity pathway, it is possible that *Shmt1* may fall in this pathway and if so, that *Shmt1* may either contribute to neural tube

development beyond its metabolic capacity or that thymidylate biosynthesis is involved in the PCP pathway. Indeed, the expression pattern of *Shmt1*1 across development suggests that it may play a role in developmental patterning. To explore this possibility, one could utilize the cochlea as a model to study alterations in planar cell polarity in *Shmt1*1-deficient mice. Disruption of the PCP pathway lead to defects in the orientation of the stereociliary bundles at the apex of each hair cell within the cochlea (28). Investigation of cochlear organization in *Shmt1*1-deficient mice would reveal any disruption in the PCP pathway. One could then explore alterations in expression patterns of PCP-related genes within the neural tube to understand how *Shmt1* disruption affects the PCP pathway and ultimately results in NTDs.

The ultimate goal in identifying metabolic mechanisms underlying NTD pathogenesis is to understand how these metabolic impairments translate into failure of neural tube closure. To this end, the mouse models described herein should be used to investigating underlying genomic and cellular changes that ultimately result in NTDs. Specifically, investigation of uracil misincorporation, genome stability and damage, and well as changes in proliferation rates, apoptosis and cell survival, and cell differentiation will shed light on the mechanisms by which specific metabolic impairments affect neural tube closure.

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