FOLATE ONE-CARBON METABOLISM IN MOUSE MODELS OF NEURAL TUBE DEFECTS

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Neural tube defects (NTDs) are common and severe birth defects. These abnormalities arise early in development, likely before the mother is aware she is pregnant. It has been well established that low maternal folate status before and during pregnancy is associated with NTD risk. However, the mechanism of how folic acid supplementation prevents NTDs is yet to be elucidated. It is known that NTDs have a multifactorial etiology, involving genetic and environmental contributions. To date, impaired *de novo* thymidylate (dTMP) biosynthesis and its associated accumulation of uracil in DNA is the only known metabolic risk for folate-responsive NTDs. The goals of these studies were to explore NTD risk in multiple mouse models of impaired *de novo* dTMP synthesis including, *p53*$^{-/-}$, *Shmt1*$^{-/-}$, and *Shmt2α*$^{-/-}$. We challenged the models with folate deficiency to determine if folic acid could prevent NTDs in these genetically-sensitized mouse models of NTDs. We also investigated the effects of dietary components on NTD prevalence. We exposed the mice to oral arsenic trioxide in the drinking water, because it is a known teratogen that can cause NTDs. In another study we compared a reduced folate supplementation to folic acid in its ability to rescue arsenic-induced NTDs.

Our results revealed that *p53*$^{-/-}$-induced NTDs are not folic acid responsive and mouse embryonic fibroblasts (MEFs) still have increased uracil accumulation in DNA despite increased *de novo* dTMP synthesis. Another study determined that folic acid is not sufficient to rescue arsenic-induced NTDs although it does rescue genomic instability and uracil accumulation in DNA of MEFs. When comparing different types of folate supplementation, we found that a novel reduced form, 5-formyl, 10-formylTHF, is also unable to rescue arsenic-NTDs, but works as efficiently as folic acid supplementation in terms of rescuing other adverse reproductive outcomes. The last study found that loss of the isoform *Shmt2α*, does not cause NTDs even when presented with a folate deficient diet. In conclusion these studies shed light on gene-exposure interactions in the risk for NTDs.

BIOGRAPHICAL SKETCH

Erica Rose Lachenauer was the youngest daughter of three born to Heiderose and Donald Lachenauer on December 21, 1989 in Glen Ridge, NJ. She grew up in Nutley, NJ and graduated top ten in her class from Nutley High School in 2008. The author was a scholar athlete with captain positions on both the varsity volleyball and varsity crew teams in her senior year. The author left NJ to attend college at the University of California, Berkeley two days after beginning her relationship with her now husband, Arien Hashemi.

At UC Berkeley, Erica started working in the lab of Dr. Chris Vulpe in her sophomore year. For two years, she studied gene expression alterations after the exposure of flame-retardants using the indicator species, *Daphnia magna*. In her senior year, she was eligible to undertake an honors research thesis. She chose to shift her research model to study mice. Her honors research project investigated whether the cause of hair loss in iron deficient mice was an *in utero* or nursing effect. At the College of Natural Resources Honors Symposium, the author was awarded the Melis Medal for outstanding presentation. Erica graduated from UC Berkeley in May 2012 with a bachelors of science in molecular toxicology and a minor in chemistry.

The author was considering veterinary medicine as a possible career path, so after graduation she traveled abroad for the summer to gain experience with large animals on a dairy farm in Germany. Following her Germany experience, she returned to NJ and obtained a position as a veterinary assistant/receptionist at the Dog, Cat & Bird Clinic of Nutley. During her almost two years at the clinic, Erica decided she wanted to pursue

both veterinary medicine and research. She applied and was the single student accepted that year into the Cornell University College of Veterinary Medicine Combined Degree Program. After completing a year and a half of veterinary school, the author joined the lab of Dr. Patrick J. Stover to study gene-nutrient-exposure interactions in the development of birth defects. Erica plans after completing veterinary school to continue to make contributions to science and mentor young scientists and clinicians as a laboratory animal veterinarian.

I dedicate this work to my loving and supportive parents

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LIST OF ABBREVIATIONS

AdoHcy	S-adenosylhomocysteine
AdoMet	S-adenosylmethionine
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DIFF	5-formyl, 10-formylTHF
DMG	Dimethylglycine
dTMP	Thymidylate
dU	Deoxyuridine
dUMP	Deoxyuridylate
EX	Exencephaly
FA	Folic acid
FOCM	Folate one-carbon metabolism
GCMS	Gas chromatography mass spectrometry
GCS	Glycine cleavage system
Hcy	Homocysteine
HPLC	High-performance liquid chromatography
MEF	Mouse embryonic fibroblast
MTHFD	Methylene tetrahydrofolate reducatase
MTHFR	Methylenetetrahydrofolate reductase
MTR	Methionine synthase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NTD	Neural Tube Defect
SB	Spina Bifida
SHMT	Serine Hydroxymethyltranferase
SUMO	Small ubiquitin-like modifier
THF	Tetrahydrofolate
TK	Thymidine Kinase
TYMS	Thymidylate Synthase
UNG	Uracil-DNA glycosylases

CHAPTER 1: Analysis of Metabolic and Environmental Risk Factors Underlying Neural Tube Defects

Manuscript in preparation for publication

Introduction

Neural tube defects (NTDs) are severe birth defects that occur in 0.75-3.12 per 1,000 live-births worldwide with incidence varying depending on geographic location [1]. However, there are many limitations and challenges in estimating the overall global prevalence, which has been reviewed in [2] and [3]. These defects occur early in development when the neural tube, which forms the brain and spinal cord, fails to close. The results are herniation and exposure of the nervous tissue to surrounding amniotic fluid causing degradation and leading to paralysis or death of the newborn. Failed neural closure in the cranial region leads to exencephaly and then degeneration of the tissue becoming anencephaly, while caudal closure failure is named spina bifida. Anencephaly is more severe and not compatible with postnatal life. In contrast, advances in medicine have extended the survival rate of those with spina bifida, however, it usually results in lifelong disability and often requires multiple surgical procedures.

Neural tube closure requires the coordination and interaction of several metabolic, transcriptional, and morphogenetic signaling networks. To add to the complexity, many nutritional and environmental exposures can alter cellular processes required for neural tube closure. Thus, the pathology of NTDs is etiologically complex and includes multiple gene-nutrient-environment interactions. It is uncertain if the genetic and environmental risks for neural tube closure defects function though common pathways/networks. This review will discuss some of the known nutrient, gene, and environmental exposures that are associated with

NTD risk, the metabolic pathways associated with risk, while also examining the relevance of mouse models to human NTDs.

Neural Tube Formation

Neurulation is the embryonic process of developing the neural tube, the precursor to the brain and spinal cord. There are two main processes of neurulation, primary and secondary. In primary neurulation, the neural plate, consisting of about 50% ectoderm, thickens and elongates into columnar cells [4, 5]. The neural plate lengthens in the cranial-caudal axis and narrows to prepare for subsequent bending. The non-canonical Wnt/planar cell polarity (PCP) pathways regulate this initiation process, known as convergent extension. The PCP pathway is complex and mutations in genes of this pathway lead to impaired migration and NTDs in mouse models [6]. The next step in neural tube closure is bending of the neural plate, which occurs strongly in two areas, the medial hinge point (MHP) at the base of the neural tube and the dorsolateral hinge point (DLHP), located at the dorsal third of the neural folds. These hinge points differ along the cranial-caudal axis. The midbrain and mid-spinal region have both MHP and DLHP, while the cranial spinal region has only MHP and the caudal spine has only DLHP [7]. Lastly, the folds come together and fuse to form the complete neural tube. Secondary neural tube closure occurs in the sacral and caudal spinal regions. Tail bud cells condense and epithelialize forming a lumen through canalization. Both primary and secondary neurulation processes are conserved in mammals, although primary neurulation differs between vertebrates [8].

Closure begins at specific initiation sites along the cranial-caudal axis and proceeds to "zipper" along the axis until it meets another closed region. It is unknown what causes a specific location to be an initiation site and these sites differ in location between mammalian species.

Humans have initiation sites in the cranial spinal region, the junction of the midbrain and hindbrain, and the most cranial forebrain [9, 10]. Mice are the most common animal models used in NTD studies, however, the closure process of rabbits and pigs seem to be more similar to that of humans. Rabbits and pigs have the same initiation sites with the exception of rabbits having an addition caudal midbrain closure site [11, 12]. In contrast, mice, rats, and hamsters have a closure site in the middle of the midbrain region, but the same cranial spinal and cranial forebrain closure sites [13-15]. The major benefit of the mouse model that rabbit and pigs lack is the ability for targeted genetic mutations. It is also controversial as to whether initiation locations of different species are relevant in NTD risk and formation. Cranial closure initiation sites can differ in different mouse strains, which can be seen in NTD mouse models with variable exencephaly location [16, 17]. This translates to human documentation of anencephaly location variations [18].

History of Folic acid and NTD Prevention

Low maternal vitamin B_9, folate, nutrient status is the best-established nutritional risk factor for NTDs. This association was originally indicated by clinical observations in the 1960s and 1970s that women with recurring NTD-affected pregnancies had impaired folate metabolism and/or status [19, 20]. Following these observations, studies in the 1980s demonstrated that periconceptional folic acid supplementation reduced NTD risk [21-25]. Unfortunately, many of these studies used multivitamins and thus, the direct effect of folic acid could not be proven. It wasn't until the 1990s when two randomized controlled clinical trials provided unequivocal evidence that folic acid supplementation can reduce both NTD recurrence [26] and occurrence

[27]. The evidence was sufficient to end one clinical trial early due to ethical concerns with maintaining a control group without folic acid supplementation [26].

The results of these studies led to the U.S. Public Health Services and Institute of Medicine recommendation that women of child-bearing age take 400 μg folic acid supplement daily and 4 mg/day for those with a previous NTD pregnancy. However, these recommendations were not successful in lowering NTD rates as most pregnancies in the U.S. are unplanned and neural tube closure occurs usually before the mother is aware that she is pregnant [28]. In 1998, the U.S. Food and Drug Administration (FDA) mandated the fortification of folic acid in enriched cereal grains using its labeling authority [29]. Since the start of mandatory fortification, the NTD incidence has decreased 35-50% in the U.S. [30-33]. Fortification of the US food supply with folic acid was distinctive from previous nutrient fortification initiatives in that the target of the intervention was not to address a folate deficiency or deficiency-related disease in the population, but rather sought a medical outcome targeting women of child-bearing age who were susceptible to NTD-affected pregnancies. Identification of the genetic and other physiological factors that confer NTD risk are an area of active investigation.

Currently, less than 100 countries have mandatory folic acid fortification programs, none of which are European Union countries, though it is likely the UK will soon fortify flour [34, 35]. One of the arguments against fortification is the potential for unintended risk to the general population, although there are no known risks associated with folic acid intake below the upper level (UL) of exposure (1 mg/day) [36]. The U.S. National Toxicology Program performed a systemic report to evaluate the risk of high doses of folic acid and found no conclusive evidence for adverse effects at exposures below the UL [37].

Biomarkers of Risk

Early detection of NTDs is important for parents to have the option for termination or the chance to plan for the birth of an affected baby. An early diagnosis also allows for the opportunity for clinicians to make informed decisions about delivery such as time, route, and site. With advances in medicine, there are now certain places that offer *in utero* surgery, however, this procedure depends on fetal and maternal benefits verse risks. To date, the two primary diagnostic tests for prenatal screening of NTDs are ultrasound and maternal serum alpha-fetoprotein (MSAFP) [38]. Ultrasound is suggested if it is high quality and is performed in the second trimester, because it is a more effective method of detection than MSAFP [39]. However, ultrasound detection depends on the size and location of the defect, fetal position, amniotic fluid volume, maternal body habitus, and the skill/equipment of the sonographer. The MSAFP test also has drawbacks in that the false negative rate is 24.9% [40].

Although not a prenatal screening diagnostic, maternal folate status has shown one of the strongest association with NTD risk. Multiple studies have shown that biomarkers in the one-carbon metabolism pathway are associated with NTD risk. Decreased serum and red blood cell folate levels have been observed in mothers with NTD pregnancies [41-45]. Similarly, elevated plasma levels of homocysteine, a member of the remethylation pathway in one-carbon metabolism, are often exhibited in NTD cases as shown in multiple studies [41, 42, 44, 46-51]. Low maternal vitamin B_{12} status is also associated with NTD cases [41, 42, 46, 47]. Other studies have observed or suggested biomarkers for NTDs in methylation proteins [47, 52], microRNAs [53], fetal CSF [54], and the protein PCSK9 [55]. A recent proteomics study by Shen *et. al* 2016 [56] also discovered three additional protein biomarkers in maternal serum.

Studies identifying biomarkers associated with NTD risk are essential for developing effective, sensitive, and specific non-invasive diagnostic prenatal screening tools.

Risk Factors

Folate status and family history are some of the the strongest risk factors for NTDs. Hence, the genes encoding enzymes and other proteins associated with folate metabolic pathways have been identified as candidate genes for NTD risk. Identifying the folate-related metabolic pathways associated with NTD risk has been challenging, as the pathways are tightly interconnected, such that perturbations in one pathway simultaneously disturb all pathways in the network [57]. The network that comprises folate-mediated one-carbon metabolism (FOCM) is required for the synthesis of purine nucleotides, thymidylate, and the remethylation of homocysteine to methionine (Figure 1.1). Methionine can be subsequently converted to S-adensoylmethionine to support over 100 cellular methylation reactions. The one-carbons required for the synthesis of these compounds come from the catabolism of serine, glycine, histidine, and choline. Important downstream effects of altered FOCM include depressed rates of DNA synthesis, increased uracil incorporation into DNA leading to genome instability, and changes in chromatin methylation patterns.

To date, *de novo* thymidylate (dTMP) biosynthesis is the only FOCM pathway consistently shown to be causal in folic acid-responsive NTD pathogenesis, which accounts for up to 70% of human NTD risk [26]. Impairments of *de novo* dTMP synthesis lead to genomic instability and cell death. Decreased rates of *de novo* dTMP synthesis cause a reduction in the dTTP pool needed for DNA synthesis and an increase in the substrate dUMP. Since DNA polymerases cannot distinguish between dUTP and dTTP, with impaired *de novo* dTMP

synthesis there is an increase in misincorporation of uracil in the DNA [58, 59]. Many studies have demonstrated that folate deficiency causes genomic instability and chromosomal breakage [60-63]. The base excision repair (BER) pathway is initiated and uracil DNA glycosylases can excise uracil from the DNA. However, with the imbalanced nucleotide pools, uracil is just as likely to be reincorporated into the DNA and it is a futile cycle. Eventually single stranded breaks will occur from AP endonucleases cleaving the DNA at abasic sites. If two single-stranded DNA breaks are in close enough proximity they will lead to a double-strand break [64]. Thus, uracil accumulation in DNA can lead to cell cycle arrest and apoptosis [65]. Folic acid supplementation rescues DNA uracil levels and micronuclei frequency [58]. This mechanism may be a significant cause of folic acid-responsive NTDs and uracil an important risk factor.

The key to establishing the etiology of folic acid-responsive NTDs, folic acid-unresponsive NTDs, and other factors associated with NTDs, is understanding if they work through a common folate-related mechanism, and which pathway(s) is causal in NTD pathogenesis. The potential relationship of the risk factors to FOCM and a summary of folate genes and their association to human and mice NTDs can be found below and in Tables 1.1 and 1.2, respectively.

Folate One-Carbon Metabolism (FOCM)

Folates function in the cell as a family of cofactors that transfer one-carbon units primarily from glycine and serine to biosynthesis pathways including *de novo* dTMP and purine synthesis, as well as remethylation of homocysteine to methionine (Figure 1.1).

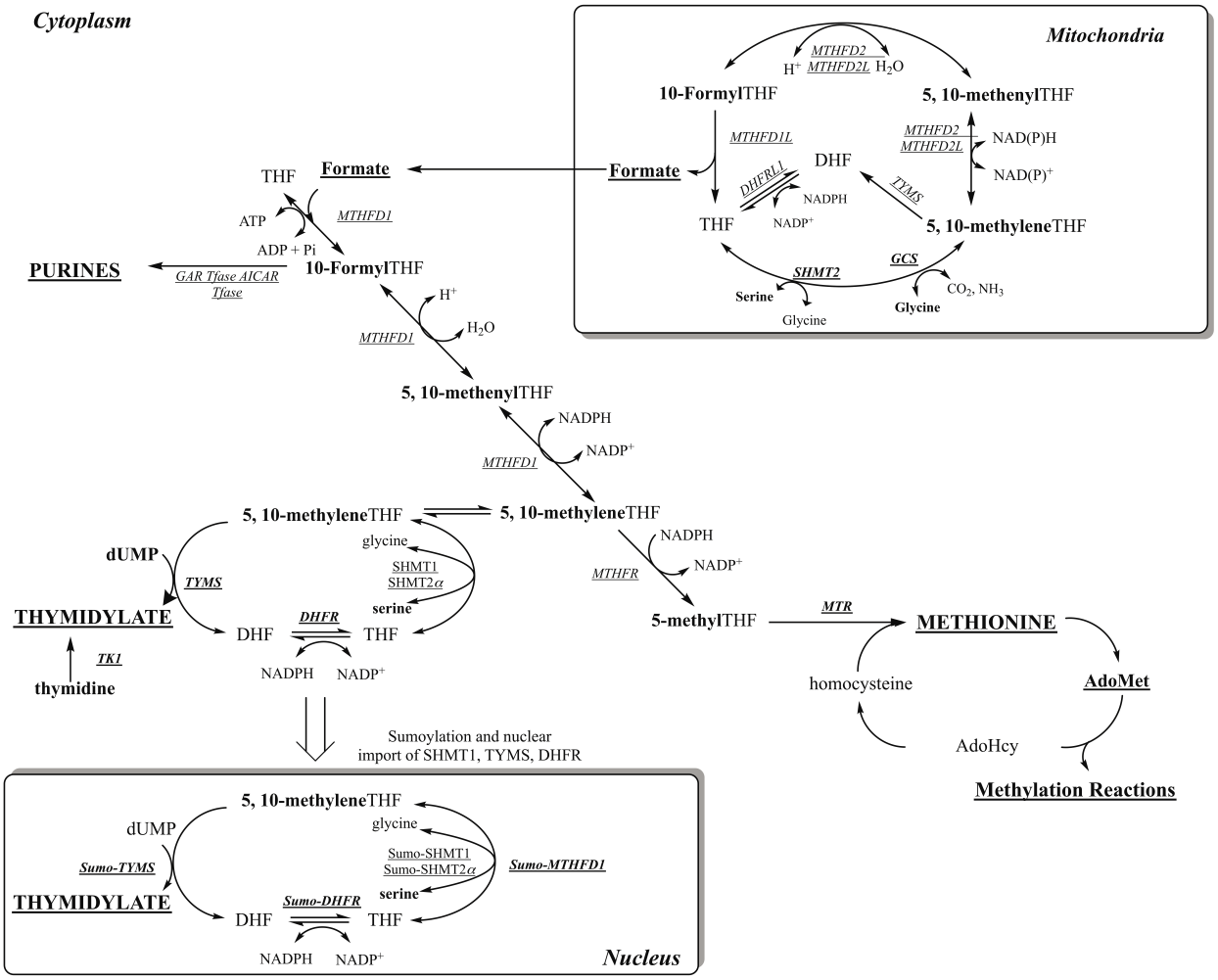

Figure 1.1: Folate one-carbon metabolism (FOCM)

FOCM is compartmentalized in the cell between the mitochondria, nucleus, and cytosol to produce formate, thymidylate, purines, and methionine. Mitochondrial enzymes are serine hydroxymethyltransferase 2 (SHMT2), methylenetetrahydrofolate dehydrogenase 2 (MTHFD2), methylenetetrahydrofolate dehydrogenase 1 like (MTHFD1L), thymidylate synthase (TYMS), dihydrofolate reductase 2 (DHFR2), glycine cleavage system (GCS), and thymidine kinase 2 (TK2). Cytosolic enzymes are methenyltetrahydrofolate synthetase (MTHFS), methylene tetrahydrofolate reductase (MTHFR), methioninesynthase (MTR), and thymidyine kinase 1 (TK1). The following enzymes are in the cytosol and upon SUMOylation are transported to the nucleus: methylenetetrahydrofolate dehydrogenase 1 (MTHFD1), serine hydroxymethyltransferase 1 (SHMT1), serine hydroxymethyltransferase 2α (SHMT2α), thymidylate synthase (TYMS), and dihydrofolate reductase (DHFR). This figure was adapted with permission from Dr. Martha Field.

Tetrahydrofolate (THF) is the metabolically active form of folate, which carries the one carbon units at one of three oxidation states, formate (10-formylTHF), formaldehyde (5,10-methyleneTHF), and methanol (5-methylTHF) [66]. These biosynthesis pathways interconnect forming a network called folate one-carbon metabolism, which is compartmentalized in the cytosol, mitochondria, and nucleus.

Table 1.1: Human NTD Association Studies of Folate Genes

Gene	Positive	Negative/None
FOLR	[75-76]	[71-74]
SLC19A1		[78]
SLC46A1		[80-82]
MTR		[118-121]
MTRR	[118, 119, 121, 123]	
MTHFR	[120,121,123,128-132]	[183]
MTHFD1L	[136-139]	
TYMS	[150-152, 154]	[153 and 155]
DHFR	[159, 162]	[154,160, 163
MTHFD1	[155, 169-174]	[175,176]
SHMT1	[186]	[155,181-184]

Folate Transport

There are three key membrane proteins that are responsible for uptake of folate into mammalian cells, the folate receptor (Folr1, originally named folate receptor alpha in humans and folate binding protein 1 in mice), reduced folate carrier (RFC1 or SLC19A1), and proton-

coupled folate transporter (PCFT or SLC46A1). The proton-coupled folate transporter works at low pH and transfers folate in the intestines, while the reduced folate carrier and folate receptors work well at neural pH levels [67-69]. In contrast to the reduced folate carrier, folate receptors have high affinity binding and transport proteins by receptor-mediated endocytosis [70].

Folate receptors are encoded by three genes in humans (*FOLR1, FOLR2, and FOLR3*) and two genes in mice (*Folr1 and Folr2*). Previous epidemiological studies were not able to find an association to human *FOLR* genes and NTDs [71-74]. However, in 2010, O'Bryne *et al.* [75] was the first to discover an association with *FOLR* and NTD cohorts using a family based transmission-disequilibrium test. A recent follow-up study from the same group found twelve novel variants in *FOLR* associated with myelomeningocele [76]. Mouse models also show mixed findings. Mice null for *Folr2* have normal neural tube closure and no morphology abnormalities at birth. However, *Folr1* null mice are not viable unless supplemented with folinic acid and then about 30% exhibit exencephaly [77].

The reduced folate carrier has been well studied with respect to its possible link to NTD pathogenesis. Results have been conflicting, and one meta-analysis found there was not an association between the (A80G) *SLC19A1* gene polymorphism and risk of NTDs [78]. Null mouse models of *Slc19a1* have folinic acid-responsive NTDs, demonstrating the importance of this transporter to neural tube closure [79]. The *SLC46A1* gene, which encodes the PCFT, has been less well studied. Loss-of-function mutations in the gene have been reported in folate malabsorption, but there has been no association with NTD cases [80-82]. The mouse model of deficient *Slc45a1* displays folate malabsorption, but does not exhibit NTDs [83].

Fumonisins are mycotoxins produced by fungi that contaminate corn-based foods. Fumonisins have been implicated as NTD risk factors for populations consuming a majority of corn-based

food in their diet that may be contaminated [84-86]. The most well studied fumonisin is fumonisin B_1 (FB_1), which has been used in mouse studies to induce NTDs [87-89]. It has been proposed that fumonisin inhibits folate uptake and utilization through depletion of complex sphinginoids that are linked to the glucosylphosphotidyl inositol anchor folate-binding protein (Folbp1) in membrane rafts [87, 88]. To further demonstrate fumonisin's inhibition of folate metabolism and its link to NTDs, supplementation of folic acid was able to rescue FB_1-induced NTDs in mice [90].

Table 1.2: Mouse Model NTDs of Folate Genes

	NTD association	
Gene	**Yes**	**No**
Folr2		[77]
Folr1	Not viable, but supplemented with folinic acid and ~30% EX [77]	
Slc19a1	Folinic acid responsive [79]	
Slc45a1		Folate malabsorption, but no NTDs [83]
Cbs		[112-114]
Mtr		embryonic lethal, HETs viable without NTDs [116,117]
Mtrr		[122]
Mthfr		[125]
Amt	Lethal with 87% NTDs with methionine supplementation [139]	
Glcd	25-29% NTDs rescued with formate [140]	

Mthfd1l	100% NTDs rescued past lethality with sodium formate supplementation [142]	
Mthfd2		[143]
Tyms	Inhibitors, 5-FU and raltitrexed cause NTDs in dose dependent manner [157, 158]	Embryonic lethal and HETs maintain protein levels [156]
Dhfr	Inhibitor methotrexate causes NTDs [165-167]	Embryonic lethal but normal neural tube closure [164]
Mthfd1		Embryonic lethal [94,177]
Shmt1	Folic acid responsive EX [188,189]	

Cytosol

Formate generated mostly in mitochondria is condensed with THF to form 10-formylTHF and then 5,10-methyleneTHF through the ATP and NADPH dependent pathway utilizing the trifunctional enzyme methylenetetrahydrofolate (MTHFD1). 5,10-methyleneTHF can then be used in the *de novo* dTMP biosynthesis pathway, which is comprised of serine hydroxymethyltransferase 1 (SHMT1), 2α (SHMT2α), DHFR, and thymidylate synthase (TYMS) enzymes. During S-phase of the cell cycle, these enzymes undergo SUMOlyation and nuclear import to produce dTMP in the nucleus [91, 92]. 10-formylTHF can be utilized in *de novo* purine synthesis to produce ATP and GTP for DNA and RNA synthesis. The remethylation of homocysteine to methionine also occurs in the cytosol. Methionine synthesis relies on 5-methyTHF and the vitamin B_{12} dependent enzyme methionine synthase (MS). Methionine is adenosylated to form S-adenosylmethionine (AdoMet), a one-carbon donor for methylation reactions.

De novo purine synthesis

A recent network correlation analysis by Gao *et. al* 2018 [93], used microarray data from NTD mouse mutants and corresponding wild-type controls to identify genes with high correlation to NTDs or folic acid responsiveness. They found two candidate genes involved in *de novo* purine synthesis (*Mthfd1* and *Gart*) that demonstrated differential expression between folic acid-responsive and folic acid-resistant mutants. MTHFD1 was mentioned previously and is important in generating 10-formylTHF for *de novo* purine synthesis from formate and THF [94]. GART (phosphoribosylglycinamide formyltransferase) is also a folate-dependent trifunctional enzyme involved in three different reactions for *de novo* purine synthesis. Expression levels for both genes (*Mthfd1* and *Gart*) were reduced in folic acid-resistant mutants. Folic acid supplementation was not sufficient to reverse these expression levels. Thus, the authors conclude it is also unlikely that targeting the *de novo* purine biosynthesis pathway would rescue folic acid-resistant NTDs.

Anti-epileptic drugs (AEDs)

AEDs have been long known to be teratogenic and induce NTDs. These drugs can impair folate absorption through a competitive interaction, increased degradation of folate, or impaired folate enzyme activity, all ultimately leading to decreased plasma folate levels [95, 96]. Valproic acid (VPA) is one of the most potent AEDs and has a high association with NTD risk [97]. A recent study analyzed the metabolic profiles of mouse embryos with maternal valproate acid-induced NTDs [98]. VPA has previously been proposed to cause NTDs through oxidative stress, histone deacetylase (HDAC) inhibition, and impaired folate metabolism [99-102]. Although it is recommended that pregnant women on anti-epileptic drugs consume higher levels of folic acid,

there is controversial epidemiological data as to whether folic acid supplementation can prevent these drug-induced NTDs [100, 103-105]. In the study by Akimova *et al.* 2017, the authors found VPA significantly alters purine and pyrimidine biosynthesis. They hypothesize the interaction of VPA and folic acid metabolism is likely through the amino acids glutamate and aspartate. Both amino acids are necessary for purine and pyrimidine biosynthesis and are also both targeted by VPA for altered synthesis, degradation, and transport. The authors propose impaired nucleotide synthesis may be a cause of VPA teratogenicity, however, folic acid supplementation was not able to rescue these NTDs.

Homocysteine Remethylation

As stated previously, plasma homocysteine (Hcy) has been used as a sensitive biomarker of impaired folate metabolism as well as NTDs [41, 106-108]. It has been proposed that the preventative mechanism of folic acid supplementation in reducing NTD risk comes from the reduction in total plasma Hcy. Hyperhomocysteinemia is thought to impair the fetal cell cycle and induce apoptosis, decrease levels of AdoMet the primary methyl donor leading to inadequate gene expression, and/or increase AdoHyc, a competitive inhibitor of methyltransferases [44, 109, 110]. Studies in chicks have demonstrated exposure to Hcy and homocysteine thyolactone (HTL) can cause embryo NTDs. A recent study by Zhang *et. al* [111] suggests the cause of these NTDs from high levels of Hcy is due to histone modifications (homocysteinylation, KHcy) in general and specifically H3K79Hcy that alter expression in neural tube closure genes. However, this study used HTL concentrations of 0.5-5 mM, which are likely not within the physiological exposure range. Normal human Hcy plasma levels are between 5-15 μM, which is 100-1000 fold lower than levels used in these experiments.

In addition, there is no evidence from epidemiological studies or mouse models that elevated homocysteine causes NTDs. Cystathionine beta synthase (CBS) converts homocysteine to cystathionine as part of the transsulfuration pathway. Homozygous gene deletion of *Cbs* in mice leads to plasma total homocysteine levels 20-50 times higher than wild-type mice [112, 113]. Levels of AdoMet in these mice were decreased, while AdoHyc were not altered [114]. However, even with such elevated homocysteine plasma levels, these mice do not develop NTDs. In humans, hyperhomocyteinemia can also result from deficiency in methionine synthase (MTR), the enzyme that transfers a carbon unit from 5-methyl-THF to homocysteine to form methionine and THF [115]. Mice with a deletion in this gene are embryonically lethal and undergo resorption by embryonic day 8.5. Heterozygotes are viable, have elevated homocysteine, and have 60% MTR activity, but are not reported to develop NTDs [116, 117]. Likewise, human polymorphisms in this gene have no association with NTD case or maternal risk [118-121]. Methionine synthase reductase (MTRR) is required to regenerate active MTR. Humans with MTRR deficiency have similar symptoms to those with MTR insufficiency and mice lacking *Mtrr* are also embryonically lethal. A gene trap mouse model that has very low expression of MTRR is viable, has elevated homocysteine and methionine, but does not develop NTDs [122]. Polymorphisms in this gene are associated with maternal risk, but not with fetal cases [118, 119, 121, 123]. Loss of methylenetetrahydrofolate reductase (*Mthfr*) causes hyperhomocysteinemia and decreased methylation capacity with a decrease in AdoMet and an increase in AdoHcy [124]. Even when challenged with folate deficiency, these mice do not develop NTDs [125].

The most studied gene variant in human NTD risk is in a common polymorphism of *MTHFR*. There are more than 30 known mutations in this gene and 10 polymorphisms that

modify its enzymatic activity [126]. Decreased MTHFR activity leads to a decrease in 5-methyTHF impairing homocysteine remethylation and elevating homocysteine levels [127]. Multiple studies have implicated a role for *MTHFR* rs1801133 in NTD risk [128, 129] and follow-up meta-analyses have agreed with these findings [120, 121, 123, 130-132]. As shown previously with other examples of elevated total plasma homocysteine, there was not a causative affect leading to NTDs. In addition, a meta-analysis argues that NTD risk from MTHFR variant is due to folate impairment and not altered homocysteine remethylation [133]. Thus, the *MTHFR* variant lowers plasma folate levels, which causes the increase in NTD risk.

Mitochondria

About 75% of the one-carbon units for cytosolic FOCM are produced in the mitochondria, demonstrating its critical role in FOCM [134]. The mitochondria produces one-carbon units in the form of formate through the catabolism of serine, glycine, histidine, betaine, dimethylglycine, and sarcosine [135]. The mitochondrial enzymes methylenetetrahydrofolate dehydrogenase (MTHFD2) and 2-like (MTHFD2L) reversibly convert 5,10-methyleneTHF, 5,10-methenylTHF, and 10-formylTHF. The formyl group from 10-formylTHF is released as formate from THF by the enzyme methylenetetrahydrofolate dehydrogenase 1- like (MTHFD1L).

Polymorphisms in the *MTHFD1L* gene and genes of the glycine cleavage system have been indicated with increased risk of NTDs in human populations [136-139]. Both have also been shown to cause NTDs in mouse knockout models. Aminomethyltransferase (AMT) and glycine decarboxylase (GLDC) are part of the glycine cleavage system that catabolizes glycine to transport a one-carbon unit onto THF. Deficiency of *Amt* is embryonically lethal with 87%

occurrence of NTDs that is reduced with methionine supplementation and not folic acid [139]. Similarly, loss of *Glcd* leads to 25-29% embryos with NTDs. These NTDs can be rescued with maternal supplementation with formate [140]. Valproic acid, the AED that causes NTDs, also targets the glycine cleavage system in both rats and humans [141]. *Mthfd1l*$^{-/-}$ mice are also embryonically lethal, but with 100% penetrance of NTDs [142]. Supplementation with sodium formate is able to rescue these NTDs and allow embryos to survive past the point of embryonic lethality [142]. These human data and mouse models clearly illustrate the necessity of mitochondrial folate metabolism in neural tube closure.

A mouse model with loss of *Mthfd2* is embryonically lethal, but after neural tube closure, which develops normally [143]. The lethality is independent of neural tube closure and caused by impaired hematopoiesis. Maternal supplementation with either glycine or sodium formate did not alter embryonic lethality or embryo growth [144]. Other studies have suggested that MTHFD2L may be able to compensate for loss of MTHFD2 at the time of neural tube closure [145]. Research from Shin *et. al* 2014 [146] found evidence that could support this idea through observation that MTHFD2L expression is increased during neural tube closure.

Nucleus

The *de novo* biosynthesis of dTMP occurs in both the cytosol and the nucleus. As stated earlier, the enzymes undergo SUMOylation and translocate into the nucleus during the S-phase of the cell cycle [147]. SHMT1 and SHMT2α are isozymes that act as scaffolding proteins to anchor the *de novo* dTMP synthesis enzymes to the nuclear lamina [91, 147-149]. TYMS converts 5,10-methyleneTHF and deoxyuridine (dUMP) into dihydrofolate (DHF) and dTMP. DHFR recycles DHF back to THF. The multifunctional scaffolding enzymes SHMT1 and

SHMT2α along with MTHFD1 then reversibly convert THF to 5,10-methyleneTHF to complete the cycle.

There is increasing evidence that *de novo* dTMP biosynthesis underlies folate responsive NTDs. Two common *TYMS* polymorphisms consisting of tandem repeats in the 5' untranslated region (UTR) and a 6-bp deletion in the 3' UTR cause decreased expression of TYMS [150, 151]. Association studies of NTD risk and the tandem repeats in the 5' UTR of the TYMS gene have yielded conflicting results, with one study determining a 4-fold increase in risk for SB in U.S. non-Hispanic whites, and another study from the UK did not find an association [152, 153]. Another SNP association study found three different fetal polymorphisms, rs2847149, rs1001761, and rs502396, that were associated with a two-fold increase in NTD risk [154]. However, a following SNP study found protective polymorphisms in both maternal, rs699517 and rs502396, and fetal, rs2847153, SNPs [155].

A loss of function mouse model of TYMS was created with a N-ethyl-N-nitrosourea (ENU) mutagenesis screen. Although these embryos undergo implantation, they are embryonically lethal due to impaired gastrulation [156]. The relationship between TYMS disruption and NTD incidence could not be assessed in this model, as the $Tyms^{+/-}$ mice increase transcription from the intact allele such that TYMS protein levels are maintained in the heterozygous knockouts [156]. A recent study used the drug 5-fluorouracil (5-FU) to target and inhibit TYMS activity in wild-type C57BL/6 mice. They found increasing percentages of NTDs with increasing dose of the drug [157]. A previous similar study used raltitrexed, a specific inhibitor of TYMS, and observed increasing percentages of NTDs in live embryos with increasing dosages of the inhibitor [158].

DHFR is an important enzyme in both *de novo* dTMP biosynthesis as well as reducing folic acid into the biologically available folate form. There have been a multitude of SNP association studies analyzing *DHFR*, but they have shown mixed results with NTD risk [154, 159-163]. The 19-bp deletion in intron 1 of DHFR, rs70991108, has been the best studied and has also provided mixed results in NTD risk. Paul *et al.* 2018 [160] did not find an association with an Indian population, while Johnson *et al.* 2003 [162] discovered an association with the deletion and spina bifida mothers, but not spina bifida fathers or patients. Parle-McDermott *et al.* 2007 [163] determined a protective effect of the DHFR deletion in an Irish population. One study looked at nine different polymorphisms (not including the 19-bp deletion) and did not find an association with any to risk of spina bifida [154]. Another study not analyazing the 19 bp deletion, found an association with the rs1643649 polymorphism in DHFR that results in improper splicing [159]. Similar to the *Mthfd2*$^{-/-}$ mouse model, loss of *Dhfr* is embryonically lethal from defective hematopoiesis at E13.5-14.5, but the embryo has normal neural tube closure [164].

Pharmacological inhibitors of DHFR are used in chemotherapy treatments of cancer and rheumatoid arthritis. Methotrexate is a DHFR inhibitor that impairs folate metabolism and causes NTDs in mice [165-167]. Studies using these mice have shown that folate metabolism impairment cause copy number variations (CNVs) in DNA [166, 167]. CNVs cause alterations in the number of copies of a specific region of DNA and are formed by impairments in recombination or replication. Thus, folate dysregulation can cause DNA damage in multiple ways.

Polymorphisms in MTHFD1 have been extensively investigated. Specifically the 1958G>A polymorphism that results in decreased enzymatic stability and activity [168]. Many

studies in various populations including Chinese [169, 170], Irish [171-173], Italian [174], and U.S. [155], have shown a significant association of the *MTHFD1* polymorphism and risk for NTDs. Curiously, this association was not observed in the Dutch population [175, 176] or with mouse models. Loss of function of *Mthfd1* through a gene-trap is embryonically lethal, emphasizing its crucial role in one-carbon metabolism [94]. Even when challenged with a folate- and choline-deficient diet, neither maternal *Mthfd1*$^{gt/+}$ nor embryonic genotype influences NTD risk in mice [177]. An important explanation for this finding is that the loss of function of *Mthfd1*$^{gt/+}$ in mouse liver and *MTHFD1* in human fibroblasts increases MTHFD1 nuclear localization and rates of *de novo* thymidylate biosynthesis, at the expense of homocysteine remethylation [178-180]. In addition, even during folate deficiency, nuclear folate levels resist depletion, as compared to the cytosolic compartment [179]. These compensatory mechanisms emphasize the importance of nuclear folate metabolism in NTD pathogenesis.

There is mixed evidence for the association of *SHMT1* polymorphisms in the risk for human NTDs. The most thoroughly studied polymorphism is rs1979277, 1420C>T polymorphism. Five studies in Dutch, UK, Chinese, and Californian populations did not see any association with risk to NTDs [155, 181-184]. However, all of these studies only considered maternal or maternal and infant contributions to the associated risk of NTDs and the polymorphism. In addition, animal models have demonstrated that paternal contribution to NTD risk can be critical [185]. When K. Rebekah *et al.* 2017 [186] examined both maternal and paternal contribution to NTD risk in the fetus, they discovered significant association of the SHMT1 1420C>T polymorphism. Specifically they found that infants with TT genotypes from CT mothers and TT fathers had a significantly high risk for NTDs. Since the risk was not

observed with mothers of TT genotype and CT fathers, it suggests the T allele from the mother is the significant risk factor.

However, mouse models have shown a different story. Mice with a loss of *Shmt1* are both viable and fertile, however, they exhibit impaired *de novo* thymidylate biosynthesis and accumulation of uracil in DNA [187]. On a folate deficient diet, $Shmt1^{-/-}$ and $Shmt1^{+/-}$ mice reveal sporadic NTDs in the form of exencephaly [188, 189]. These mice are the only mouse model with impaired folate metabolism to develop folic acid-responsive NTDs. Importantly, this mouse mimics human NTD risk in that it exhibits a low penetrance of NTDs of about 13% that is rescued with folic acid supplementation [188].

Diabetes

Hyperglycemia is a known teratogenic factor with the most common embryopathy being congenital heart defects and NTDs [190-192]. Although high glucose levels are common to both type 1 and type 2 diabetes, there are disease characteristic differences including age of onset, ethnicity, obesity, and duration that can alter the teratogenic effect of diabetic embryopathy [191]. Only a handful of studies have compared the two types and the results have not been consistent [193-199]. However, all of these studies demonstrate that maternal diabetes causes embryopathy including NTDs. The most critical time point to prevent diabetes-induced structural birth defects is for maternal hyperglycemia to be controlled before and during the first trimester [200]. Because gestational diabetes mellitus usually develops in the second half of pregnancy, it is not associated with increased risk for congenital anomalies [201]. However, it does increase the risk for other adverse outcomes such as prenatal and perinatal mortality [202]. Animal models of diabetic-induced NTDs can be created by glucose-injections in pregnant dams,

demonstrating that maternal high glucose levels can cause NTDs [203-205]. The hyperglycemia is thought to induce oxidative stress and cause DNA damage and ER stress, although the exact mechanism is unknown.

Glucose monitoring and control are essential during the periconceptional period. However, about one in three women of reproductive age that have diabetes mellitus are undiagnosed [206] and additionally, like the general population, the majority of women with preexisting diabetes have unplanned pregnancies [207]. A study with buccal mucosa cells from patients with Type 1 and Type II diabetes demonstrated the metabolic disease causes DNA damage that can be rescued with folic acid supplementation in patients [208]. Animal studies have found folic acid supplementation can prevent diabetic embryopathy [209-212] and epidemiology case control studies have seen a reduction in diabetes induced NTD risk with folic acid supplementation [213, 214]. Thus, the American Diabetes Association not only supports the US Public Health recommendation of daily folic acid supplementation for women of reproductive age, but also recommends those with preexisting diabetes to increase their intake from the suggested 400 μg to 600 μg daily [28, 215]. Thus, there may be an interconnection between diabetes and folate metabolism in the etiology of NTDs.

Arsenic

Arsenic is an environmental toxin and was recorded as number one on the priority list for hazardous substances by the Agency for Toxic Substances and Disease Registry (ATSDR) in 2017 [216]. This list is used to prioritize substances based on human health concerns by examining potential human exposure, frequency, and toxicity. Arsenic is a known teratogen that has been shown to cause NTDs in animal models when it is administered during organogenesis

[217-220]. Multiple studies have examined the interactions between arsenic exposure and folate metabolism [221-223]. Arsenic methylation is required for its elimination from mammals, a pathway, which relies on folate through the availability of S-adenosylmethionine (AdoMet) produced by FOCM [224, 225]. A recent study in Bangladesh found that arsenic exposure causes a reduction in the efficiency of folic acid to prevent NTDs [226]. Another new finding, demonstrated that low doses of arsenic can target and degrade SHMT1 and MTHFD1, two important enzymes in FOCM and impair *de novo* dTMP biosynthesis [227]. These results demonstrate an important interaction between arsenic toxicity, folate-dependent dTMP biosynthesis, and NTD risk.

Not only does arsenic exposure interact and alter folate metabolism, but it has also been associated with the development of diabetes [228-230]. Four different mechanisms have been proposed on how arsenic exposure may induce diabetes. Arsenic can inhibit the glucose transporter GLUT4, induce pancreatic β-cell damage through ROS, impair insulin secretion, and stimulate liver gluconeogenesis [231]. Thus, arsenic has the ability to impair glucose homeostasis leading to a diabetic phenotype. This demonstrates an interaction network between arsenic, diabetes, FOCM, and the risk for NTDs.

Obesity

Obesity is a growing epidemic and a cause of many adverse health outcomes. It also complicates pregnancy and can cause birth defects including NTDs [232]. Two meta-analyses have shown a significant association between obese mothers and the risk of having babies with NTDs [233, 234]. An epidemiological study of an Irish population found an association between a leptin receptor SNP and NTDs, which could be a link between obesity as a risk factor for

NTDs [235]. There is also a possible confounding factor of maternal diabetes as well and the mechanism of how obesity increases NTD risk is unknown. There have been studies that found obesity decreases folate levels [236] and that folic acid supplementation has reduced efficacy in obese women [237]. Obese women also have altered hormonal levels and chemicals such as inflammatory markers that occur regardless of comorbidities such as diabetes [238, 239]. These alterations in the intrauterine environment can impair fetal development and may be the cause of NTDs.

Hyperthermia

It has been long established in multiple animal models that a significant increase in maternal body temperature can cause NTDs as well as other birth defects [240-243]. Follow-up epidemiologic studies have found associations with fever as well as hot tub or sauna use during pregnancy and a risk for NTDs [244-248]. A recent study found that folic acid supplementation could attenuate the risk for NTDs in mothers with periconceptional fever [249]. This result suggests a link between FOCM and hyperthermia with the risk for NTDs. Other factors such as genetic susceptibility, nutritional deficiencies or excesses, as well as alcohol consumption may modify the risk of hyperthermia-induced NTDs [243, 250-253].

Alcohol

Maternal alcohol consumption during pregnancy is a known teratogen to cause a wide range of birth defects that uses the umbrella term fetal alcohol spectrum disorder (FASD). One of the possible associated birth defects that have been studied is NTDs, although it has not been confirmed and is not usually on the list of characteristics for FASD [254]. Some studies have

shown a risk for NTDs with maternal alcohol consumption [255-258], while others have not [259, 260]. However, it has been clear in animal models that ethanol can cause NTDs [261-263] and alcohol consumption interacts with FOCM. Alcohol has been shown to lower blood folate levels while increasing homocysteine (Hcy) [264] as well as reducing the level of AdoMet and activity of methionine synthase (*MTR)* [265, 266]. The mechanisms of alcohol toxicity have not been fully elucidated, but have been proposed to be caused by ROS and cellular and mitochondrial DNA damage leading to intrinsic apoptosis [267-270]. More in depth analysis is reviewed in [271].

Cigarette Smoking

Cigarette smoke exposure is another well-known teratogen. A majority of studies have found cigarette smoking is a risk factor for NTDs and that includes secondhand smoking as well [272-276]. Exposure of mice or rats both *in vivo* and in *vitro* to nicotine or cigarette smoke causes oxidative stress causing developmental abnormalities [277-279]. Nicotine is considered the major teratogenic substance in tobacco smoke as it undergoes rapid placental transfer to the embryo and accumulates in the fetal blood and amniotic fluid [280-282]. Nicotine has been shown to increase intracellular calcium and reactive oxygen species (ROS) causing excessive neuroepithelial death leading to NTDs in mice [277, 283]. In-depth reviews have demonstrated oxidative stress can cause developmental abnormalities, including NTDs [284, 285].

Conclusions:

It is clear that NTDs have a complex and multifactorial etiology. It is important that researchers continue to use a broad approach utilizing genetic mouse models as well as human

epidemiological data to piece out mechanisms of failed neural tube closure. Mice have long been used as models for human NTDs because of the accessibility to targeted DNA mutagenesis. It is estimated there are over 300 genetic mutations in mice that cause NTDs [286]. Although, the etiology of NTDs remains to be elucidated, these mouse models have been imperative in deciphering and analyzing mechanisms of neural tube closure. However, with this multitude of mouse models it is important to determine the specific criteria that make for the best human NTD models.

First, human NTDs occur at a very low incidence of about 1 in 1000 births [287]. Mouse models should mimic this phenotype, as very high penetrance of NTDs is not relevant to the human population. About 70% of human NTDs are folate-responsive, however, only a handful of mouse models have been tested for folate-responsiveness. It is important we continue to assess mouse models so that it can be determined which ones represent the majority of the human population. Even though maternal folate status is highly associated with NTDs, folate deficiency alone is not sufficient to cause NTDs. Thus, there is an important genetic component and gene-environment interactions when studying NTD etiology. Thus, mouse models that only have one genetic mutation and cause NTDs without a nutrient or environmental interaction, do not reflect human NTDs. In addition, genetic modifiers can reflect multifactorial complexity of NTD inheritance, making mouse models of different backgrounds helpful in determining NTD penetrance differences and modifier genes. Lastly, it is important that these models have subtle metabolic changes. NTDs cases do not have drastic metabolic alterations in either the mother or the infant. All of these considerations need to be studied and used when interpreting the vast number of NTD mouse models.

From analysis of the FOCM pathways, mouse models, as well as human epidemiology studies in this review, the *de novo* thymidylate biosynthesis demonstrated the strongest evidence for causing folic acid responsive NTDs. Although some of the epidemiological data has shown mixed results, these may be due to genetics within specific population studies or not including paternal contributions as seen with the SHMT1 polymorphism. It is clear that *de novo* dTMP synthesis plays a crucial role in FOCM. This evidence is seen with compensation in folate deficiency where nuclear folates are preserved at the expense of the cytosolic compartment. Similarly, the *Shmt1* mouse mutant has been the only model of a folate metabolism enzyme that has low penetrance of NTDs that are folic acid responsive. The model also implicates the important risk factor of NTDs being uracil in DNA. Thus, it is important to further analyze modifiers of *de novo* dTMP synthesis, their role in uracil accumulation in DNA, as well as their link to risk of NTDs.

In this review the many different risk factors for NTDs were analyzed. All of the risk factors can cause oxidative and/or direct DNA damage revealing a common pathway and susceptibility of the neuroepithelium. This sensitivity of the neural tube is likely the reason minor alterations in nutritional, environmental, and genetics disrupt the neuroepithelium specifically, while the rest of the embryo develops normally. Likewise, it was revealed that many of the risk factors interact with each other physiologically. Arsenic exposure has been shown to cause metabolism dysregulation and diabetes while diabetic DNA damage can be rescued with folic acid and arsenic relies on FOCM for excretion at the same time as it inhibits important enzymes in FOCM. The interactions and cross talk between these risk factors likely plays an important role in the multifactorial etiology of NTDs.

In conclusion, there is still a long way to go in elucidating NTD etiology. Every study and analysis is an important piece of the puzzle that is the complex network of etiological pathways.

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CHAPTER 2: p53 disruption increases uracil accumulation in DNA and leads to folic acid non-responsive neural tube defects in mice

Manuscript under preparation for submission

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Abstract:

Neural tube defects (NTDs) are birth defects that result in herniation and exposure of nervous tissue during embryogenesis when the neural tube fails to close. It is estimated that 70% of all human NTDs are can be prevented with folic acid supplementation. The tumor suppressor protein p53 plays an important role in development. Both increased and decreased expression and/or function of p53 can lead to NTDs in mouse models. *In vitro* studies show p53 inhibits the expression of some of the folate-mediated *de novo* thymidylate (dTMP) biosynthesis pathway enzymes. Mouse models with impaired *de novo* dTMP synthesis have increased rates of NTDs that are responsive to folic acid or deoxyuridine supplementation. The aim of this study was to determine if dietary folic acid could rescue $p53^{-/-}$-induced NTDs in mice. It also sought to determine the effect of loss of p53 has on pathways in folate one-carbon metabolism. $p53^{+/-}$ females were randomized and weaned onto either a folic acid-sufficient diet or a folic acid-deficient diet. After 8 weeks on diet, the females were time-mated to $p53^{-/-}$ males. The pregnant

females were sacrificed at E12.5 and embryos were analyzed for NTDs. Primary mouse embryonic fibroblasts (MEFs) were used for cell studies. Maternal dietary folic acid intake did not reduce NTD incidence in *p53*$^{-/-}$ embryos. *p53*$^{-/-}$ MEFs had higher rates of folate-dependent *de novo* dTMP synthesis, *de novo* purine biosynthesis, uracil accumulation in DNA, and proliferation, and folate did not modify these phenotypes. These results indicate that loss of p53 causes increased growth and thus increased nucleotide synthesis to accommodate cellular needs, however not at levels that are sufficient to ameliorate uracil accumulation in DNA. This study provides additional evidence that uracil in DNA is a risk factor for NTDs.

Introduction:

When the neural tube, which is the precursor to the brain and spinal cord, fails to close during early development, structural birth defects, namely neural tube defects (NTDs) occur. Randomized controlled clinical trials have determined that up to 70% of human NTD occurrence can be rescued by folic acid supplementation before and during pregnancy [1]. However, the mechanism through which folic acid prevents human NTDs remains unknown [2]. NTDs have a multifactorial etiology and genetic and environmental factors contribute to risk. Over 300 mouse models have been established that develop one or more NTDs [3]. However, few of these models have been examined for and/or exhibit folic acid responsiveness and the vast majority do not reflect factors that contribute to human susceptibility.

To date, serine hydroxymethyltranferase (*Shmt1*) is the only folate-dependent enzyme shown to be directly involved in the etiology of folic acid-responsive NTDs in mouse models. The only phenotypes observed in the *Shmt1* mouse model (*Shmt1*$^{+/-}$ or *Shmt1*$^{-/-}$) are impaired *de novo* thymidylate (dTMP) biosynthesis and the associated accumulation of uracil in DNA [4-6].

The *de novo* dTMP pathway is folate dependent to produce dTMP from dUMP with the utilization of an enzyme complex consisting of SHMT1, dihydrofolate reductase (DHFR), and thymidylate synthase (TYMS) [7]. Decreased rates of *de novo* dTMP synthesis cause a reduction in the dTTP pool. Because DNA polymerases do not distinguish between dUTP and dTTP, reduced pools of dTTP will lead to misincorporation of uracil into the DNA [8]. Cell cycle arrest and p53-mediated apoptosis result from uracil accumulation in DNA [9]. Uracil misincorporation in DNA is cell type-and/or tissue-specific, as enrichment of uracil in DNA varies among tissues, however the biological mechanisms for these differences are likely multi-factorial [10].

The neural epithelium requires rapid and coordinated cell proliferation as well as apoptosis programs for normal neural tube closure to occur [11, 12]. Thus, many NTD mouse models exhibit impaired regulation of cell death [13]. The tumor suppressor protein p53 is an important cell cycle regulator. Both the over-expression and reduced expression of p53 in mice leads to death or developmental abnormalities [14, 15]. Embryos deficient in p53 are viable, but exhibit an incomplete penetrance of exencephaly, the cranial form of an NTD [16]. Other studies have shown that p53 represses the expression of two important enzymes in the *de novo* dTMP pathway, DHFR and TYMS, although it is unclear if the decreased expression of these enzymes impairs *de novo* dTMP synthesis [17-19]. Therefore, the purpose of this study was twofold: to determine if loss of p53 alters folate metabolism by affecting the rate of *de novo* dTMP and/or *de novo* purine synthesis in mouse embryonic fibroblasts as well as to determine whether NTDs in *p53*$^{-/-}$ embryos are folic acid-responsive.

Materials and Methods:

Animal Housing and Husbandry

All animal studies were reviewed and approved by the Cornell University Institute Animal Care and Use Committee in accordance with the guidelines in the Animal Use and Welfare Act as well as all state and federal laws. Founder colony mice were purchased from the Jackson laboratory, B6.129S2-Trp53^{tm1Tyj}/J (Jax Stock: 002101 p53 KO). At weaning, *p53*$^{+/-}$ females were weaned onto either an AIN93G diet lacking folic acid (-FA) or an AIN93G control (+FA) diet for at least 8 weeks before breeding and remained on the diet for the duration of gestation. The *p53*$^{+/-}$ females were bred to *p53*$^{-/-}$ males. Thus, the embryos exhibited *p53*$^{-/-}$ and *p53*$^{+/-}$ genotypes. Every morning females were checked for a vaginal plug and if found dams were considered pregnant at E0.5. Pregnant females were sacrificed by cervical dislocation at E12.5 to ensure appropriate time had passed since normal neural tube closure. Blood was collected by cardiac puncture and plasma and red blood cells were separated by centrifugation. The embryos were examined for NTDs and the embryonic sac of a subset of embryos was saved for genotyping. Another subset of embryos was fixed in 10% neutral buffered formalin. Characteristics such as crown-rump length, implant number, and resorption number were recorded. Genotyping for p53 was performed using established protocols [20].

Plasma Metabolite Analysis

Cardiac puncture was used to collect maternal blood in EDTA coated tubes. The blood was spun down to separate red blood cells and plasma, then immediately snap-frozen in liquid nitrogen. Folate plasma concentrations were quantified with the *Lactobacillus casei* microbiological assay as described previously [21], with n=5 dam plasma samples per diet group. Stable isotope

dilution capillary gas chromatography-mass spectrometry as described in [22] was used to measure total plasma homocysteine, cystathionine, methionine, glycine, serine, dimethylglycine (DMG), methylglycine (MG), and cysteine, n=24 mice for +FA group and n=18 mice for the –FA group.

Mouse Embryonic Fibroblasts and Cell Culture

All embryos were dissected from folic acid-sufficient (+FA diet) dams at E12.5. Only grossly developmentally normal embryos were used and each biological replicate had at least one *p53*$^{-/-}$ embryo and a *p53*$^{+/-}$ littermate. Embryos were washed with PBS and sterilely dissected in a tissue culture hood. The embryos were cut up and digested in Trypsin for 20 min at 37°C. Each genotype embryo was then plated in modified minimal essential medium (MEM) with either low (2 nM (6S)-5-formyltetrahydrofolate) folate media or high (25 nM (6S)-5-formyltetrahydrofolate) folate media. The modified MEM media consisted of alpha-MEM (Hyclone) lacking glycine, serine, methionine, pyridoxine, folate, and all nucleosides/nucleotides. This media was supplemented with 10% (vol/vol) dialyzed FBS, 200 μM methionine, 2.5 g/L sodium bicarbonate, and 1 mg/L pyridoxine.

Effect of p53 on Folate Enzyme Levels

MEFs were plated in 10 cm plates for at least three doublings. Cell cycle was blocked with hydroxyurea added at 1 mmol/L final concentration. Following 24 h. of treatment, the media was changed to release the cell cycle block for four hours. Cells were pelleted and then lysed by sonication in lysis buffer (0.15 M NaCl, 5 mM EDTA pH 8, 1% Trition X100, 10 mM Tris-Cl, 5 mM DTT, and a dilution of 1:100 Sigma PI Cocktail 8340). Proteins (45 μg/well) were

separated by a 4-20% Tris-Glycine gel (NuSep) and then transferred onto a PVD membrane at 4°C using a BioRad transblot apparatus. Membranes were blocked overnight at 4°C or for 1 hour at room temperature in 5% milk in PBS with 0.1% Tween-20. Membranes were washed with PBS with 0.1% Tween-20 and then incubated with primary antibodies for 1 hour at room temperature or overnight at 4°C. Membranes were washed again with PBS with 0.1% Tween-20 and then incubated with horseradish peroxidase conjugated secondary antibody diluted 1:50,000 for 1 hour at room temperature. The membrane was washed again before being developed with chemiluminescent substrate. The following primary antibodies were used at 1:1000 dilution; MTHFD1 (described previously [23]), SHMT1 (described previously [24]), TYMS (Cell Signaling 3766), and DHFR (Abcam ab124814), while Alpha tubulin (Active Motif 39527), and GAPDH (Novus Biologicals NB300-328) were used as loading controls.

Deoxyuridine Suppression Assay

MEFs were plated in triplicate in six well plates with media supplemented with 62.5 nM [^{3}H]-thymidine and 5 μM [^{14}C]-deoxyuridine. The incorporation of these radiolabels into DNA reports on the relative efficiency of the salvage pathway verses the *de novo* thymidylate biosynthesis pathway respectively. Nuclear DNA was isolated using a Roche DNA template kit followed by RNase A treatment and another Roche DNA product kit. Isotope incorporation into DNA was quantified with a Beckman LS6500 scintillation counter in dual disintegrations per minute (dpm) mode. Experiments were performed with three biological replicates and two technical replicates each.

Formate Suppression Assay and Separation of Purines

MEFs were plated in triplicate in six well plates with media supplemented with 1 nM [^{3}H]-hypoxanthine and 10 μM [^{14}C]-formate. The incorporation of these radiolabels into DNA reports on the relative efficiency of the salvage pathway verses the *de novo* purine synthesis pathway respectively. Nuclear DNA was isolated using a Roche DNA template kit followed by RNase A treatment and another Roche DNA product kit. Isotope incorporation into DNA was measured with a Beckman LS6500 scintillation counter in dual disintegrations per minute (dpm) mode. Experiments were performed with three biological replicates and two technical replicates each. To confirm results, nucleotides were separated and run on HPLC as described previously [25].

Uracil in DNA

A Roche kit was used to isolate nuclear DNA from MEFs that were cultured in 10 cm plates for at least three doublings. Nuclear DNA was treated with RNase at a concentration of 0.2 mg/mL for 15 min at room temperature and then a Roche DNA product kit was used for purification. Uracil concentrations were measured using gas chromatography-mass spectrometry as described in [5] with the following modifications: 2 μg of nuclear DNA and 50 pg uracil internal control (molecular mass 118.04 from Cambridge Isotope Laboratories, Inc. 3917) were used in the assay.

MTT Proliferation Assay

MEFs were plated in multiple 96 well plates with 8 technical replicates. Cells were counted with trypan blue stain and plated at 1000 cells per well density. A plate was read at the 0 time point which was 24 h. after the cells were seeded. At the 0 time point and each additional time point, a

plate was treated with 25 mg/mL MTT final concentration for 4 h. The media was removed and 100 μL of DMSO was added. The plate was read with a BioTek Gen5 plate reader at 570 nm. Fold change was calculated by diving the measurement of that day by the measurement at time 0.

Statistics

The comparisons of proportions of NTDs were calculated using the "N-1" Chi-squared test. The western quantification comparisons were calculated with a student's t-test. The plasma metabolites were compared with a student's t-test. All MEF data was analyzed with a multi-level model because each dam was measured over several embryos and every embryo was repeatedly measured over two plates with different media. The fixed effect of media and genotype was tested and if the interaction term was not significant it was removed and reanalyzed. The MTT assay was analyzed on day 3 and day 5 with fixed effect model of genotype and media.

Results:

Loss of p53 induces NTDs that are not rescued by dietary folic acid

p53$^{-/-}$ embryos exhibited exencephaly (EX) as well as spina bifida (SB), as shown in lateral images in **Figure 2.1A**. NTDs were analyzed at the litter level because folic acid-exposure results from a given dam's dietary exposure. Litters born to dams consuming +FA diet had a similar percentage of NTDs as those born to dams consuming a -FA diet (20/46 (43%) and 14/35 (40%) respectively, $p>0.05$) (Figure 2.1B). When NTDs are separated into litters that had embryos with exencephaly only (17/46 (37%) +FA, 9/35 (25.7%) -FA), spina bifida only (0/46 (0%) +FA, 2/35 (5.7%) –FA), or both exencephaly and spina bifida (3/46 (6.5%) +FA, 3/35 (8.6%) –FA), there was no significant difference between litters from dams with or without FA

in their diet. The number of embryos per litter and resorptions per litter were also not different between diet-exposure groups ($p>0.05$). A subset of embryos was genotyped to see whether differences would arise between diets when separated by genotype. A total of 43 litters, 173 embryos from +FA dams and 131 embryos from –FA dams were genotyped. Of the embryos genotyped, no $p53^{+/-}$ embryos exhibited NTDs and there was no difference in NTD occurrence ($p>0.5$) between diet groups for the $p53^{-/-}$ embryos (11/83 (13%) +FA, 9/68 (13%) –FA) **Table 2.1**.

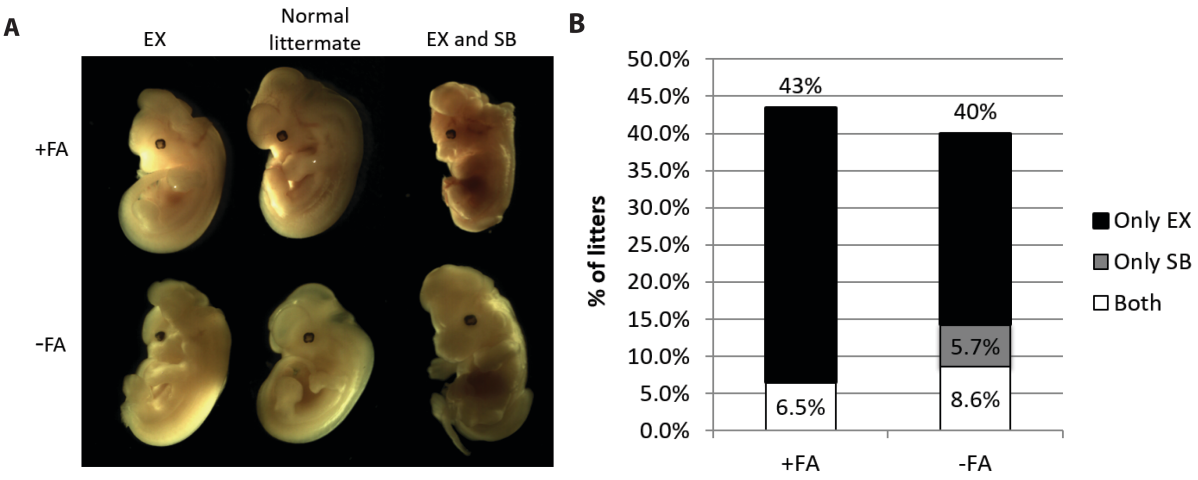

Figure 2.1: Embryos with a loss of p53 have an incomplete penetrance of NTDs that are folic acid non-responsive

A total of 81 litters and 566 embryos were analyzed. 46 litters with a total of 321 embryos were born from dams consuming the +FA diet and 35 litters with a total of 245 embryos were born from dams consuming the –FA diet. **A.** Lateral views of $p53^{-/-}$ embryos from both folate sufficient (+FA) and folate deficient (-FA) dams. (Exencephaly (EX) and spina bifida (SB) embryo images are not from the same litter). **B.** Percentage of litters with NTDs separated by EX only, SB only, or EX and SB (Both).

Table 2.1: Average embryonic crown-rump length and percentage of exencephaly, spina bifida, or any NTD as a function of dam diet and embryonic genotype

Embryo analysis	+FA		-FA	
	p53$^{-/-}$	*p53*$^{+/-}$	*p53*$^{-/-}$	*p53*$^{+/-}$
Embryo crown-rump length	8.57 ± 0.19^{a}	8.73 ± 0.18^{b}	8.63 ± 0.25^{ab}	8.71 ± 0.25^{ab}
Exencephaly (%)	11 (12)	0 (0)	7 (13)	0 (0)
Spina Bifida (%)	0 (0)	0 (0)	2 (4)	0 (0)
Any NTD (%)	11 (12)	0 (0)	7 (13)	0 (0)
Total embryos	90	90	54	43

Embryo crown-rump length is listed as least squares means ± standard error and measured in mm. Only viable embryos at E12.5 were analyzed for crown-rump length measurements. Diets sufficient in folic acid (+FA) and diets deficient in folic acid (-FA). Different letters are significant from each other, $p<0.05$.

Loss of p53 inhibits growth only when dams are fed a folate-sufficient diet.

Crown-rump length measurements did not show a significant interaction between embryo genotype and diet (Table 2.1, p=0.43). However, there was a significant difference between genotypes (Table 2.1, p=0.016). *p53*$^{-/-}$ embryos were smaller than the *p53*$^{+/-}$ embryos. Upon further analysis, crown-rump length differences between genotypes was only significant for embryos from dams fed the +FA diet (Table 2.1, p=0.0072) and not significant from dams consuming the –FA diet (Table 2.1, p>0.1).

Plasma analysis confirms folate deficiency in dams with FA deplete diets

Dams consuming the folic acid-deficient diets had significantly lower plasma folate levels as compared to dams consuming folic acid-sufficient diets (**Table 2.2**, 53.3 fmol/μL vs 130

fmol/μL respectively, p=0.002). Plasma metabolites including total plasma homocysteine, cystathionine, methionine, glycine, serine, DMG, MG, and cysteine were measured using GCMS (Table 2.2**)**. As expected, elevated homocysteine, cystathionine, and serine levels were found in dams consuming folic acid-deficient diets as compared to those consuming folic acid-replete diets ($p<0.0001$, $p<0.05$, and $p<0.005$ respectively).

Table 2.2: Plasma metabolites of dams

Metabolite	Folic Acid	No Folate	p-value
Homocysteine (μM)	12.41 ± 4.03	23.45 ± 7.77	<0.0001
Cystathionine (nM)	1657.2 ± 546.6	1989.9 ± 458.7	<0.05
Cysteine (μM)	153.4 ± 29.4	173.9 ± 18.4	NS
Methionine (μM)	49.45 ± 11.74	53.87 ± 11.49	NS
Gycine (μM)	115.3 ± 37.7	103.2 ± 31.8	NS
Serine (μM)	134.71 ± 21.9	160.5 ± 31.8	<0.005
DMG (μM)	9.497 ± 0.909	9.722 ± 0.847	NS
MG (μM)	27.214 ± 8.934	25.312 ± 9.512	NS
Folate (fmol/μl)	130.09 ± 8.23	53.29 ± 15.34	<0.005

All plasma metabolites excluding folate were measured by stable isotope dilution capillary gas chromatography-mass spectrometry. The table displays the mean ± standard error of the mean. n=24 mice for folic acid sufficient (+FA) group and n=18 mice for the folic acid deficient (–FA) group. Folate was measured using the *L. casei* assay with n=5 samples in each diet group.

p53$^{-/-}$ MEFs exhibit increased protein expression in folate metabolism enzymes

Protein levels for the *de novo* thymidylate biosynthesis enzymes, MTHFD1, TYMS, DHFR, and SHMT1, were analyzed by western blot. Four different biological replicates of each genotype of

MEFs were analyzed. All MEFs were grown in folate replete media and cell cycle blocked. MTHFD1 enzyme levels were significantly increased in *p53*$^{-/-}$ MEFs (**Figure 2.2**, p= 0.02). DHFR also showed significantly higher protein levels in the *p53*$^{-/-}$ MEFs (Figure 2.2, p= 0.05). Neither TYMS nor SHMT1 showed a significant difference in protein levels between *p53*$^{-/-}$ and *p53*$^{+/-}$ MEFs.

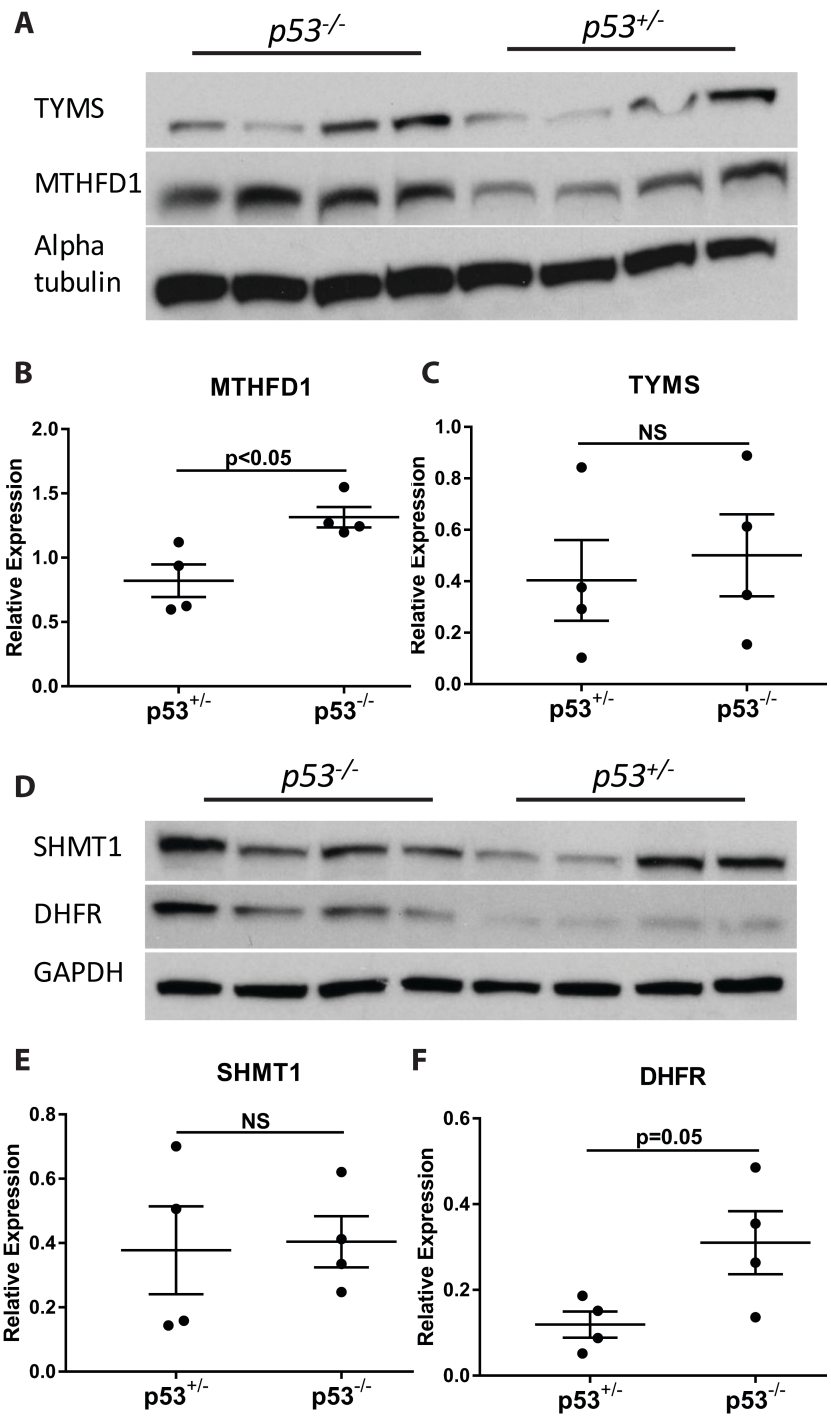

Figure 2.2: *p53*$^{-/-}$ MEFs have higher protein expression of *de novo* enzymes

Western blots of MEFs cultured in folate replete media (25 nM). Each lane is a biological replicate with n=4 per genotype. **A**. Blot of TYMS and MTHFD1 with Alpha tubulin as the loading control **B., C.** Quantifications of the blot in A compared to loading control Alpha tubulin **D.** Blot of SHMT1 and DHFR with GAPDH as the loading control **E., F.** Quantifications of the blot in D compared to loading control GAPDH

p53$^{-/-}$ MEFs have significant alterations in folate metabolism compared to *p53*$^{+/-}$ MEFs

Because p53 has been shown to repress *de novo* thymidylate synthesis pathway enzymes *in vitro*, the deoxyuridine suppression assay was performed to identify any differences in *de novo*

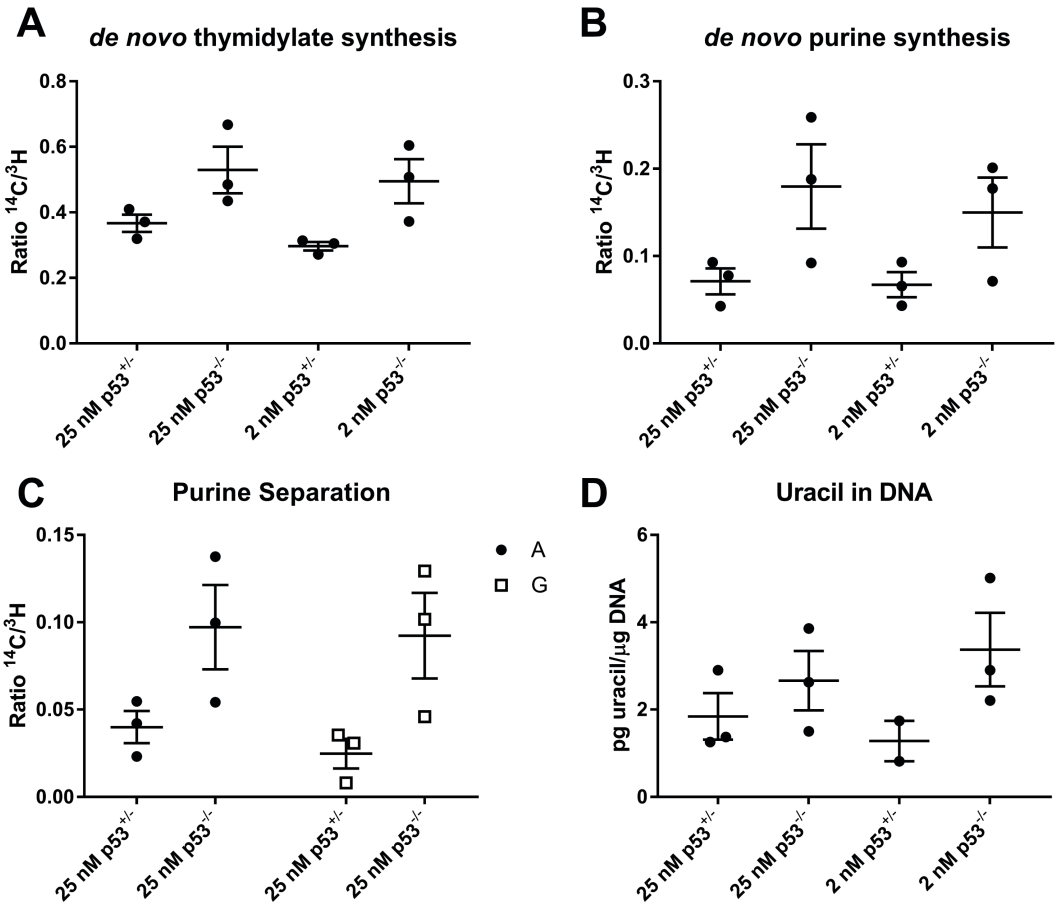

Figure 2.3: Folate dependent nucleotide *de novo* synthesis and uracil in DNA, is increased in *p53*$^{-/-}$ MEFs

MEFs were isolated from grossly normal embryos from folate sufficient *p53*$^{+/-}$ dams. They were cultured in modMEM media lacking folate and nucleotides. The media was supplemented with (6S)-5-formyltetrahydrofolate at a low (2 nM) or high (25 nM) concentration. Each point represents a biological replicate. **A.** Rate of *de novo* thymidylate synthesis **B.** Rate of *de novo* purine synthesis **C.** Purine nucleotides separated by HPLC **D.** Uracil accumulation in DNA

thymidylate synthesis between the genotypes and diet groups. The *p53*$^{-/-}$ MEFs in both low and high folate media exhibited significantly higher rates of *de novo* thymidylate synthesis than *p53*$^{+/-}$ MEFs (**Figure 2.3A**). The fixed effect of genotype was significant (p= 0.0016). There were no significant differences between high and low folate for each genotype and the interaction term between genotype and folate was not significant. *De novo* purine synthesis capacity was analyzed to determine if metabolic alterations in other one-carbon metabolism pathways were occurring. There was no significant interaction term between genotype and folate for this outcome (Figure 2.3B). Although there was more than a two fold increase in *de novo* purine synthesis in the *p53*$^{-/-}$ MEFs, the difference between genotypes was not statistically significant. To confirm that this assay was reporting on purine synthesis and was not affected by incorporation of formate into thymidylate, DNA was digested, and nucleotides were separated by HPLC (Figure 2.3C). Again, there was no significant difference between genotypes.

p53$^{-/-}$ MEFs accumulate uracil in DNA

Uracil enrichment in DNA can occur due to impaired *de novo* thymidylate biosynthesis. Although *de novo* thymidylate synthesis was elevated in the *p53*$^{-/-}$ MEFs compared to the *p53*$^{+/-}$ MEFs, there was a genotype effect of increased incorporation of uracil in DNA (p=0.05), with about a two-fold increase in the *p53*$^{-/-}$ MEFs (Figure 2.3D). This effect was not modified by folate status and there was no significant interaction term between genotype and folate status.

p53$^{-/-}$ MEFs exhibit accelerated proliferation compared to *p53*$^{+/-}$ MEFs

Proliferation of the MEFs was assessed using the MTT colorimetric assay. The assay measured growth over multiple days and found that *p53*$^{-/-}$ MEFs had increased rates of growth (**Figure 2.4A**). There was no significant difference in growth between folate status of the culture media. At day 3 the *p53*$^{-/-}$ MEFs averaged a 3.6 fold increase while the *p53*$^{+/-}$ MEFs had a 2.6 fold increase (genotype significance of $p<0.0001$, Figure 2.4B). By day 5, the *p53*$^{-/-}$ MEFs still had a significantly higher fold increase than the *p53*$^{+/-}$ MEFs (4.7, 3.6 respectively, $p<0.005$, Figure 2.4C).

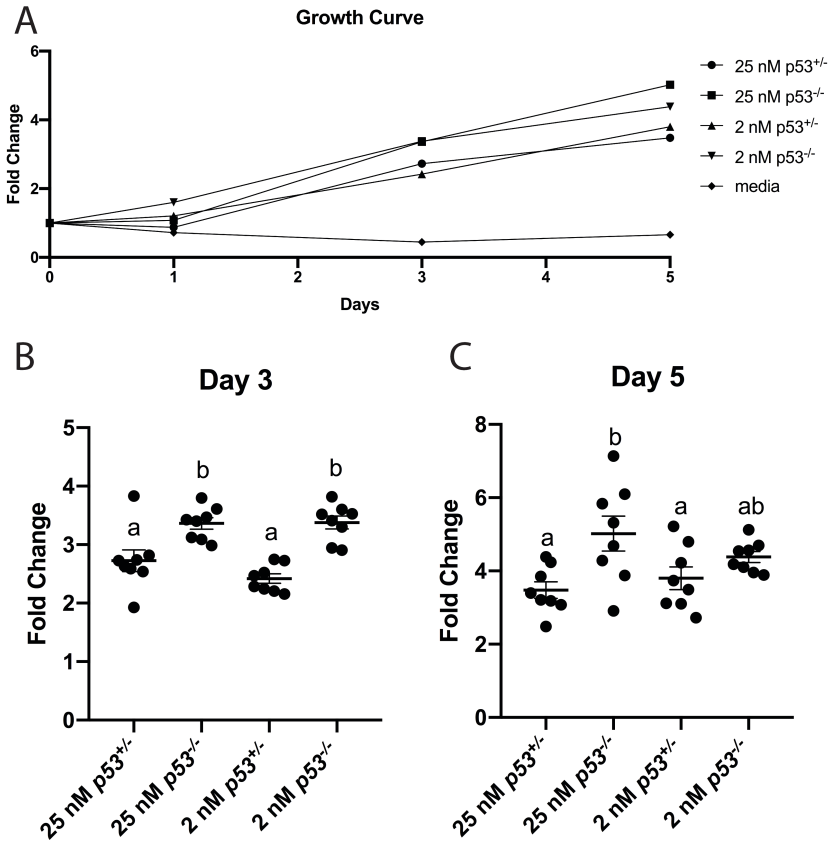

Figure 2.4: Proliferation is increased in *p53*$^{-/-}$ MEFs

MEFs were isolated from grossly normal embryos from folate sufficient *p53*$^{+/-}$ dams. They were cultured in modMEM media lacking folic acid and nucleotides. The media was supplemented with (6S)-5-

formyltetrahydrofolate at a low (2 nM) or high (25 nM) concentration. The MTT colormetric assay was used to measure growth. Fold change was calculated by dividing by day 0. An LS means Student T-test was used to determine significance. **A.** Growth curve shown by fold change of absorbance over time **B.** Quantification of Day 3 results **C.** Quantification of Day 5 results

Discussion**:**

This study demonstrated that *p53*$^{-/-}$-induced NTDs are not folic acid-responsive. The results were irrespective of NTD type and at both the litter and embryo levels. However, there was a slight increase in the occurrence of caudal NTDs when dams are on a folic acid-deficient diet. The percentage of litters with spina bifida-only NTD occurrence increased from 0% in dams consuming the control diet to 5.7% in dams consuming the folic acid-deficient diet. Although there was not a statistically significant difference, it may be a small predilection that would be significant with a larger study number (125-150 per group). Genetic NTD models tend to show NTD location bias, however, diet has not been shown to impact the preference of NTD location [26, 27]. Whether folic acid supplementation can affect NTD location occurrence warrants further study.

Embryo crown-rump length can be an indicator of adverse pregnancy outcomes [28], and often embryos with birth defects will have decreased crown-rump length. Loss of p53 caused growth retardation in embryos compared to their heterozygous littermates. This result is expected because NTDs were observed only in the *p53*$^{-/-}$ and not *p53*$^{+/-}$ embryos. However, when analyzed further, the only significant difference between genotypes was in the folic acid replete group. Since folic acid deficiency has been shown to stunt growth [4, 29], it seems folate deficiency stunts the *p53*$^{+/-}$ embryos growth so that the difference between genotypes is lost. These results also demonstrate that folic acid supplementation is not able to rescue *p53*$^{-/-}$ crown-rump length phenotype.

Neurulation requires precise and coordinated cell division and cell death. Multiple mouse models of impaired apoptosis develop an overgrowth phenotype and NTDs [30-33]. These models demonstrate that the disruptions in cell death are tissue and cell type specific. Thus, embryos can have an overgrowth phenotype specifically in the brain while the rest of the embryo size is normal or smaller as observed in the *CPP32*$^{-/-}$ mice [31]. Therefore, even though the *p53*$^{-/-}$ mice exhibit decreased crown-rump length, it does not exclude them from the possibility of an overgrowth phenotype in the neural tube.

Loss of p53 expression, as observed in MEFs in this study, results in elevated rates of nucleotide synthesis, which was more pronounced for *de novo* dTMP synthesis (Figure 2.3A). Although the increase in *de novo* purine biosynthesis did not reach significance, the p-value was approaching significance (p=0.07, Figure 2.4B). This difference would likely be more pronounced comparing the *p53*$^{-/-}$ embryos to wildtype embryos, as discussed below. The increased nucleotide synthesis correlates with the excessive proliferation in *p53*$^{-/-}$ MEFs (Figure 2.5). With a high demand for cell division and DNA replication, nucleotide synthesis is increased. Loss of p53 leads to elevated rates of folate-dependent *de novo* dTMP synthesis, as protein levels of folate-dependent *de novo* dTMP synthesis enzymes are increased when p53 is absent (Figure 2.3A). However, the increase in dTMP synthesis may not be sufficient to meet the demands for dTTP synthesis in *p53*$^{-/-}$ MEFs, and thereby cannot prevent uracil misincorporation in DNA (Figure 2.3D) [34].

Shmt1$^{-/-}$ mice exhibit impaired folate metabolism and folic-acid responsive NTDs. This model exhibits impaired *de novo* dTMP synthesis and increased uracil in DNA, but no other severe metabolic or other phenotypes [4-6]. The NTDs and uracil accumulation in DNA are rescued by dietary folic acid. Thus, if the *p53*$^{-/-}$ NTDs are a result of increased uracil in DNA, as

in the *Shmt1*$^{-/-}$ model, they would be predicted to be folic acid-responsive. However, this study found that neither *p53*$^{-/-}$-induced NTDs nor uracil accumulation in DNA is rescued by folic acid. The accumulation of uracil in nuclear DNA resulting from p53 loss-of-function, despite increased activity of *de novo* dTMP synthesis, suggests that p53 regulates other pathways that contribute to uracil accumulation in DNA, including DNA repair pathways. This study provides additional evidence that uracil in nuclear DNA is a risk factor for NTDs. This study identified two possible mechanisms responsible for p53-associated NTDs: 1) increased uracil accumulation in DNA or 2) increased proliferation and overgrowth in the neuroepithelium.

This study has limitations. For breeding purposes, *p53*$^{+/-}$ dams were used to generate *p53*$^{-/-}$ embryos because *p53*$^{-/-}$ mice are poor breeders and can be susceptible to cancerous tumors as early as 3 months of age. Thus, in order to reduce animal numbers, the study only included *p53*$^{+/-}$ and *p53*$^{-/-}$ genotypes. It is very likely that there would be much larger differences if the *p53*$^{+/+}$ genotype was included and compared to the *p53*$^{-/-}$. Another limitation to our study is that the use of MEFs may not be representative of the neural epithelium. However, MEFs do play an important role in cellular migration, thus any alterations could affect neural tube closure.

In conclusion, this study indicates that the *p53*$^{-/-}$ mouse model of NTDs exhibit altered *de novo* thymidylate biosynthesis and elevated uracil in DNA, but are not folic acid-responsive. Similar to the *Shmt1*$^{-/-}$ and *Shmt1*$^{+/-}$ mouse model, uracil in DNA may be a risk factor for NTDs. Whether uracil accumulation in DNA and/or increased proliferation in the neuroepithelium are playing causative or correlative roles in NTD occurrence in the *p53*$^{-/-}$ mouse model, requires further investigation.

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CHAPTER 3: Arsenic trioxide causes neural tube defects by targeting folate metabolism and genome stability

Manuscript in review for publication- ERL generated all or partial data for Figures 3.2, 3.3, 3.4, 3.5, and 3.6

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Abstract:

Neural tube defects (NTDs) are a class of common congenital malformations arising from multifactorial interactions between genetic and environmental factors. Low maternal folate status is a strong determinant of NTD risk, which interacts with gene variants and other environmental factors to confer NTD risk. The *Shmt1*$^{-/-}$ and *Shmt*$^{+/-}$ mouse model of impaired thymidylate biosynthesis exhibits folic-acid responsive NTDs. Arsenic trioxide (As_2O_3) is a potent inhibitor

of thymidylate biosynthesis, and induces DNA damage in a dose-dependent manner in cell culture models. The objective of this study was to determine the impact of dietary folic acid in preventing NTDs in wild-type and *Shmt1*$^{-/-}$ dams exposed to arsenic-contaminated drinking water. *Shmt1*$^{-/-}$ and *Shmt1*$^{+/+}$ female mice were randomized to either AIN93G or AIN93G lacking folic acid diets for a minimum of eight weeks and then time-mated to males with the same genotype. From one week before mating and through sacrifice at E12.5, dams were exposed to either 0, 8, or 25 ppm arsenic-contaminated drinking water. Embryos were examined for the presence of NTDs at E12.5. As_2O_3-induced DNA damage was quantified in mouse embryonic fibroblasts (MEFs) as a function of *Shmt1* genotype. Maternal exposure to As_2O_3 increases NTD occurrence in embryos and DNA damage in mouse embryonic fibroblasts (MEFs) in dose-dependent manner. Arsenic-induced NTDs were not folic acid-responsive, whereas arsenic-induced effects on DNA damage were prevented by folic acid in culture medium. Reduced folate-dependent *de novo* thymidylate synthesis, a pathway which is inhibited by As_2O_3 at low concentrations, causes NTDs in mice, which are insensitive to dietary folic acid. These findings establish As_2O_3 as a causative factor in the etiology of folic acid-resistant NTDs.

Introduction:

Neural tube defects (NTDs), including anencephaly, spina bifida and encephalocele, are a class of congenital malformations of the central nervous system caused by failure of the neural tube to close during the fourth week of gestation in humans (1). The incidence of NTDs worldwide is estimated to be between 0.5 to 60 per 10,000 births, and thus NTDs present a significant public health burden (2, 3). Risk for folic acid-responsive NTD-affected pregnancy involves interacting genetic, nutritional, and other environmental factors, but the causal pathways

have yet to be established (2, 4, 5). Trials have shown that approximately 70% of human NTDs can be prevented by maternal folic acid intake (6, 7), which lead to folic acid food fortification initiatives that have reduced NTD incidence globally (7, 8). Numerous mouse models of impaired one-carbon metabolism have been generated to elucidate the metabolic pathways whose disruption leads to NTDs (2). To date, other than disruption of folate transporters (9), disruption of serine hydroxymethyltransferase 1 (*Shmt1)* in mice is the only mouse model of impaired folate metabolism to develop folic acid-responsive NTDs (10-12).

Disruption of *Shmt1* in mice impairs folate-dependent *de novo* thymidylate (dTMP) biosynthesis (11, 13). *De novo* dTMP biosynthesis is catalyzed by the enzymes SHMT1 and SHMT2α (the cytosolic and nuclear isoform of SHMT2), methylenetetrahydrofolate dehydrogenase 1 (MTHFD1), thymidylate synthase (TYMS), and dihydrofolate reductase (DHFR). These enzymes are present in the cytosol during G1 phase and undergo SUMOylation, leading to their nuclear import and multienzyme complex assembly at the nuclear lamina during S and G2/M phases (14-18). In the *de novo* dTMP synthesis cycle, SHMT and MTHFD1 generate an activated one-carbon unit in the form of 5,10-methyleneterahydrofolate (5,10-methylene-THF), from serine and formate, respectively (**Figure 3.1**).

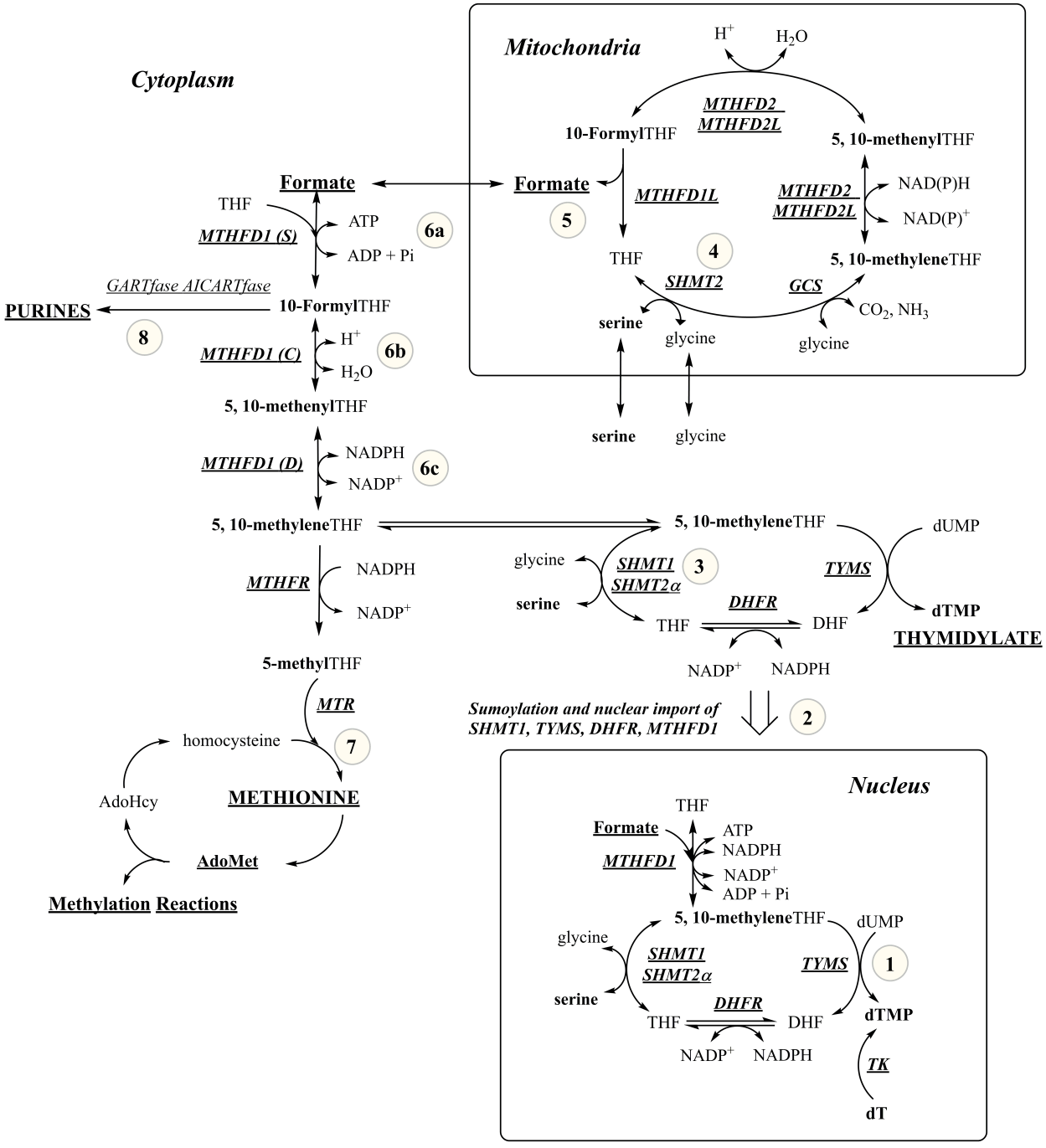


Figure 3.1: Compartmentalization of folate-dependent one-carbon metabolism

Nuclear *de novo* dTMP biosynthesis requires TYMS, DHFR, MTHFD1, SHMT1 and SHMT2α **(1)**. These enzymes are SUMOylated and imported into the nucleus at the onset of S phase **(2)**. The *Shmt2* gene is expressed as two transcripts: one that generates SHMT2 for mitochondrial one-carbon metabolism **(4)**, and SHMT2α, which functions in the cytosol and nucleus **(3)**. The hydroxymethyl group of serine is a primary source of one-carbon units. Serine enters the pool of activated one-carbon units through the SHMT-catalyzed reaction in the cytosol **(3)** and mitochondria **(4).** In mitochondria, serine and glycine are converted to formate **(4, 5)**, which traverses to the cytosol

and nucleus, where it is condensed with THF by MTHFD1 **(6a)**. MTHFD1 is a trifunctional enzyme possessing formylTHF synthetase (S) **(6a)**, methenylTHF cyclohydrolase (C)**(6b)**, and methyleneTHF dehydrogenase (D)**(6c)** activities. 5,10-methyleneTHF is synthesized by SHMT **(3,4)** or MTHFD1 **(6)** and used for *de novo* dTMP synthesis **(1)**. One-carbon metabolism in the cytosol includes the remethylation of homocysteine to methionine **(7)** and the *de novo* synthesis of purines **(8)**. Folate-mediated one-carbon metabolism is thus compartmentalized within the cell to allow efficient metabolic flux of THF-activated one carbon units to biosynthetic reactions and cycling of THF. AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; AICAR Tfase, aminoimidazolecarboxamide ribonucleotide transformylase; GAR Tfase, glycinamide ribonucleotide tranformylase; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase.

TYMS catalyzes the conversion of 5,10-methyleneTHF and deoxyuridylate (dUMP) to dTMP and dihydrofolate. DHFR catalyzes the NADPH-dependent conversion of DHF to THF. MTHFD1 is the primary source of folate-activated one-carbon units for *de novo* dTMP synthesis, whereas the SHMT isozymes are scaffolds that connect the *de novo* dTMP synthesis pathway to the nuclear lamina at sites of DNA synthesis (15, 19). Disruption of *Shmt1* expression prevents formation of the nuclear multi-enzyme complex and reduces rates of *de novo* dTMP biosynthesis leading to elevated rates of uracil misincorporation into DNA, genome instability, and folic acid-responsive NTDs in mice (11, 20). The SHMT1 C1420T variant, which results in an L474F amino acid substitution that impairs nuclear import of SHMT1 (19) and disrupts the multi-enzyme complex and has recently been associated with increased NTD risk in humans (21).

Arsenic is a common environmental toxin. More than 140 million people worldwide are exposed to arsenic-contaminated drinking water at levels above the recommended safe concentration of 10 μg/L and in foods grown or produced in arsenic-contaminated conditions (22-25). Arsenic exposure through drinking water has been associated with excess adult mortality (26-30) and adverse pregnancy outcomes including preterm delivery, stillbirth, spontaneous abortion, and low birth weight (31-37). Chronic exposure to arsenic in populations has been linked to increased risk of cancer, diabetes, cardiovascular disease, kidney disease,

anemia, skin disease, and neurotoxicity (24, 38-52). Arsenic was also shown to reduce the effect of folic acid in the prevention of myelomeningocele, a specific form of NTD, in Bangladesh (53, 54). Intraperitoneal injection of arsenate in folate transport-compromised mice indicated no interactions among folate transport, arsenate, and NTDs (55).

Recently, *de novo* dTMP synthesis has been shown to be a sensitive target of low-dose arsenic trioxide (As_2O_3) exposure (56). As_2O_3 increases turnover of MTHFD1 and SHMT1, leading to depressed rates of *de novo* dTMP biosynthesis, increased incorporation of uracil in DNA, and increased DNA damage in cell culture models (56). To understand the role of arsenic in NTD etiology, we investigated the ability of folic acid to prevent arsenic-induced NTDs in wild-type and *Shmt1*$^{-/-}$ mouse models.

Materials and Methods:

Study animals

All animal experiments and procedures were approved by the Cornell Institutional Animal Care and Use Committee (Cornell University, Ithaca, NY) according to the guidelines of the Animal Welfare Act and all applicable federal and state laws. Mice were maintained on a 12-h light/dark cycle in a temperature-controlled room. Generation of *Shmt1*$^{-/-}$ mice was previously described (9). *Shmt1*$^{+/-}$ females were bred to *Shmt1*$^{+/-}$ males, generating *Shmt1*$^{+/+}$, *Shmt1*$^{+/-}$, and *Shmt1*$^{-/-}$ males and females. At weaning, *Shmt1*$^{+/+}$ and *Shmt1*$^{-/-}$ females were randomly assigned to defined FD (folic acid deficient) or FC (folic acid control) diets for at least 8 weeks prior to mating and throughout the breeding period and gestation. After eight weeks (at age 11 weeks), females from the FD and FC groups were further randomized to 0, 8, or 25 ppm As_2O_3 administered *ad libitum* in drinking water for a week prior to mating and for the duration of

gestation. Study *Shmt1*$^{+/+}$ or *Shmt1*$^{-/-}$ females were housed overnight with *Shmt1*$^{+/+}$ or *Shmt1*$^{-/-}$ males, respectively, and vaginal plugs identified the next morning were considered embryonic day E0.5. Pregnant females were sacrificed by cervical dislocation at E12.5, and blood was collected by cardiac puncture. Gravid uteri were removed, and all implants and resorption sites were recorded. Embryos were examined for presence of NTDs and measured for crown-rump length. Embryos examined for morphologic abnormalities were derived at E12.5 and fixed in 10% neutral buffered formalin.

Metabolite quantification

Maternal blood was collected by cardiac puncture in EDTA-coated tubes on ice at the time of embryo scoring, separated into RBC and plasma and immediately snap-frozen in liquid nitrogen and stored at -80°C. Maternal liver, kidney, and spleen were collected, rinsed in 1X PBS, snap-frozen in liquid nitrogen and stored at -80°C. Plasma total homocysteine, cystathionine, total cysteine, methionine, serine, glycine, and α-aminobutyric acid were assayed by stable isotope dilution capillary gas chromatography-mass spectrometry as previously described (17), n=10 mice per group. Folate concentrations were quantified using a *Lactobacillus casei* microbiological assay as previously described (57). Plasma formate was quantified as described previously (58). Non-targeted metabolic profiling of plasma and multivariate data analysis was modified from the protocol previously described (59). Plasma extraction was performed in duplicates and pentanedioic-d^6-acid (C/D/N isotopes Inc., Quebec, Canada) was used as an internal standard. For derivatization, dried samples were dissolved in 30 µl pyridine, containing 20 mg/ml methoxyamine hydrochloride and 30 µl N-methyl-N-trimetyhlsilyl-triflouroacetamide (MSTFA) and 1 µl of derivatized sample was injected in a split ratio of 1:5. The gas

chromatograph was equipped with a 20 m DB-5MS capillary column. The method applied had an overall run time of 16 min with the following temperature profile: the initial oven temperature was set to 90°C for 0.5 min. Then, the oven temperature was increased to 220°C at 13°C/min and after reaching a temperature of 220°C further increased to 325°C at 100°C/min and finally held at 325°C for 3.5 min.

Statistical analysis

Statistical analyses were performed using JMP software and SAS software packages (SAS Institute Inc., Cary, NC). The Cochran-Armitage Trend Test was used to assess the dose-response relationship between NTD incidence and As_2O_3 exposure. A Fisher's exact test for independence with a Bonferroni correction (n=3) was used to assess individual contributions of genotype, dietary folic acid, and arsenic exposure to NTD frequency. The following comparisons of NTD incidence were made: (*1*) embryos from dams fed an FD diet compared with embryos fed a control diet, (*2*) *Shmt1*$^{-/-}$ embryos from dams on FD and control diets compared with *Shmt1*$^{+/+}$ embryos from dams on FD and control diets, and (*3*) *Shmt1*$^{+/+}$ and *Shmt1*$^{-/-}$ embryos from dams not exposed to arsenic compared to embryos from dams exposed to 8 or 25 ppm As_2O_3 in drinking water. To determine interactions among embryonic genotype, dietary folic acid, and arsenic exposure a generalized linear regression model was fit to the NTD incidence (litter and embryo as unit of analysis) and sum of NTD per litter (binomial distribution, logit link function) for the following parameters: genotype, arsenic (0 or 25 ppm), diet, arsenic x diet, arsenic x genotype, diet x genotype. Odds ratios for the NTD incidence were determined using SAS. Embryonic crown-rump length and maternal weight at dissection were analyzed using a one-way ANOVA with post-hoc Tukey HSD (Honestly Significant Difference) test. To assess

the interaction between these factors, a standard least squares model was fit to the embryonic crown-rump data for the following parameters: genotype, diet, arsenic, genotype x diet, genotype x arsenic, diet x arsenic, diet x arsenic x genotype. Maternal plasma metabolites were analyzed by two-way ANOVA with post-hoc Tukey HSD (Honestly Significant Difference) test. The heatmap presents significantly altered (ANOVA, $p<0.05$), z-Score normalized metabolite data and was generated with the R package "pheatmap" (R version 3.3.1). For selected metabolites (1,5-Anhydroglucitol, 3-Hydroxybutyric Acid, Palmitic Acid and 2-Picolinic Acid), multi-factor ANOVA was applied. Multi-factor ANOVA was also used for the analysis of fluorescence intensity means (FIMs) and % cells in high γH2AX or $H_3K_4me_2$ signal.

Mouse Embryonic Fibroblast (MEF) Isolation and Cell Culture

Shmt1$^{+/+}$ and *Shmt1*$^{-/-}$ females were mated to *Shmt1*$^{+/+}$ and *Shmt1*$^{-/-}$ males, respectively. Pregnant dams were sacrificed and embryos harvested at E12.5 as described previously (11) and references therein. MEFs were maintained in MEM, α-modification (α-MEM; HyClone) supplemented with 10% (vol/vol) FBS, and 1X penicillin/streptomycin. Where indicated, "modified MEM" consisted of defined α-MEM (HyClone) that lacked glycine, serine, methionine, pyridoxine, folate, and all nucleosides/nucleotides but was supplemented with 10% (vol/vol) dialyzed FBS, 1X penicillin/streptomycin, 200 μM methionine, 2.5 g/L sodium bicarbonate, 1 mg/L pyridoxine, and 25 nM (*6S*)5-formyltetrahydrofolate where indicated.

Quantitative γH2AX and H3K4me2 Measurements using Flow Cytometry

MEFs were isolated and cultured in indicated media for 5 to 12 days. Where indicated, cells were treated with As_2O_3 for 24 h prior to quantification of γH2AX signal. γH2AX staining and

analysis was performed as described (56, 60) with minor modifications. Cells were washed with PBS, trypsinized, pelleted, and resuspended in 50 µl of PBS on ice. Anti-γH2AX FITC conjugate antibody (Millipore) was diluted to 0.6 µg/ml and Vybrant Dye Cycle Violet DNA Stain (Invitrogen) was diluted to 0.5 mM in sterile staining buffer (PBS supplemented with 1 g/L BSA, 8% (vol/vol) mouse serum, 0.1 g/L RNase A, 10 mM NaF, 1 mM Na_2MoO_4, 1 mM $NaVO_3$, 0.25 g/L of herring sperm DNA, 0.1 % (vol/vol) Triton X-100, 5 mM EDTA, 0.05 % (wt/vol) NaN_3). 150 µL of the staining solution was added to each cell suspension, vortexed, and incubated for 3h, then immediately analyzed on BD FACS Aria Cell Sorter using lasers of wavelengths of 405, 480, and 633 nm. FCS Express (De Novo Software) was used for histogram construction and analysis. High γH2AX was defined by a threshold cutoff at the mean top 2.5% of cells across triplicates in the control condition stained for total γH2AX as previously described (60). Where indicated, the staining solution contained anti-H3K4me2-Alexa-Fluor-594 antibody (Abcam). DNA stain allowed for stratification of γH2AX signal by cell cycle stage to which the Kolmogorov-Smirnov (KS) test (FCS Express, De Novo Software) was applied (61).

Results:

Arsenic induced NTDs in dose-dependent manner

NTD occurrence was determined in mice as a function of *Shmt1* genotype, dietary folic acid, and As_2O_3 exposure at 8 and 25 ppm in drinking water. Previously, *Shmt1*$^{+/-}$ and *Shmt1*$^{-/-}$ embryos have been shown to develop low-penetrance exencephaly when dams are fed diets lacking folic acid (FD diet) (10-12) **(Figure 3.2A)**. In the absence of arsenic exposure and consistent with previous reports, NTD incidence (primarily exencephaly, Figure 3.2A, E) was observed in 14.1% of litters (4.8% *Shmt1*$^{-/-}$ embryos) from dams consuming the FD diet. Litters from *Shmt1*$^{+/+}$ dams

fed the FC diet and *Shmt1*$^{+/+}$ dams fed FD diet and not exposed to As_2O_3 did not develop NTDs (Figure 3.2E, F, groups 1-4). In the generalized linear regression model that quantified individual risk factors in isolation, exposure to 25 ppm As_2O_3 was associated with higher NTD risk compared to NTD risk in embryos from dams not exposed to As_2O_3 (χ^2=6.44, p=0.011), the FD diet was associated with higher NTD risk compared to FC diet (χ^2=11.1, p=0.0009), and embryonic *Shmt1*$^{-/-}$ gene disruption was associated with higher NTD risk compared to *Shmt1*$^{+/+}$ genotype (χ^2=14.4, p=0.0001).

The effect of arsenic on NTD incidence was analyzed in dams consuming either the FC or FD diet and exposed to As_2O_3 at 8 ppm and 25 ppm. Across all diet and genotype groups, As_2O_3 administered through drinking water to dams exhibited a dose-dependent effect on NTD incidence (Figure 3.2B, 1.1, 2.2, 5.3% affected embryos in 0, 8, and 25 ppm arsenic groups, respectively, p<0.001). For *Shmt1*$^{-/-}$ dams fed the FD diet in the absence of As_2O_3 exposure, only one or two NTD-affected embryos are observed per affected litter, whereas up to 5 NTD-affected embryos per litter of 5 (100% penetrance) were observed in the 25 ppm As_2O_3 exposure group (Figure 3.2C), with an increased NTD severity, including the presence of craniorachischisis (Figure 3.2A, CR), which is not typically observed in the *Shmt1* mouse model.

The effects of maternal dietary folic acid on preventing arsenic-induced NTDs were determined for both *Shmt1*$^{+/+}$ and *Shmt1*$^{-/-}$ embryos. Across all genotype and arsenic groups, embryos from dams fed the FD diet had a significantly higher frequency of NTDs than embryos from dams fed the FC diet (5.0% vs 0.3%, respectively, p<0.001). *Shmt1*$^{-/-}$ embryos had significantly higher rates of NTDs than *Shmt1*$^{+/+}$ embryos (4.4% vs 0.4%, respectively, p<0.001) across all arsenic and diet groups. There was a trend toward reduced efficacy of folic acid in the presence of 25 ppm arsenic. There was a 16-fold reduction (from 4.8 to 0.3%) in NTD rate in

Shmt1$^{-/-}$ embryos not exposed to As_2O_3 compared to an 11-fold reduction (from 13.1 to 1.2%) in *Shmt1*$^{-/-}$ embryos exposed to As_2O_3 (Figure 3.2F). However, the pairwise term interaction between the main effects reached significance in the linear regression only when severity of presentation was included in the model (see below). Overall, the FC diet was not able to prevent arsenic-induced NTDs.

The effects of embryonic genotype on risk of arsenic-induced NTDs were determined for dams fed the FC and FD diets (Figure 3.2E). Litters from dams fed the FD diet had a significantly higher frequency of NTDs than litters from dams fed the FC diet (13.3% vs 1.6 %, respectively, $p<0.001$). Litters from *Shmt1*$^{-/-}$ dams had significantly higher rates of NTDs than litters from *Shmt1*$^{+/+}$ dams (12.3% vs 1.3%, respectively, $p<0.001$). As_2O_3 administered through drinking water to dams had a significant effect on the frequency of NTD-affected litters (4.0%, 7.7%, and 14.0% NTD rate in 0, 8, and 25 ppm As_2O_3 exposure groups, respectively, $p=0.001$). In the generalized linear regression model several conclusions can be drawn: 1) maternal exposure to 25 ppm As_2O_3 was associated with a higher NTD risk compared to no As_2O_3 exposure ($\chi^2=5.79$, $p=0.016$), 2) maternal FD diet associated with a higher NTD risk compared to control diet ($\chi^2=6.58$, $p<0.0089$), and 3) maternal *Shmt1*$^{-/-}$ genotype associated with a higher NTD risk compared to *Shmt1*$^{+/+}$ genotype ($\chi^2=12.95$, $p<0.003$). There was a trend towards reduced efficacy of folic acid in the presence of 25 ppm arsenic, as seen in the folic acid-dependent, 10.85-fold reduction (from 14.1 to 1.3%) in NTD rate in *Shmt1*$^{-/-}$ litters not exposed to As_2O_3 compared to only a 4.37-fold reduction (from 29.3 to 6.7%) in *Shmt1*$^{-/-}$ embryos exposed to As_2O_3 (Figure 3.2E), but the pairwise term interaction between the main effects reached significance only when severity was included in the linear regression model.

Severity of presentation was incorporated into the model by presenting NTD rate as a sum of affected cases per litter in addition to the total number of embryos per litter in a binomial

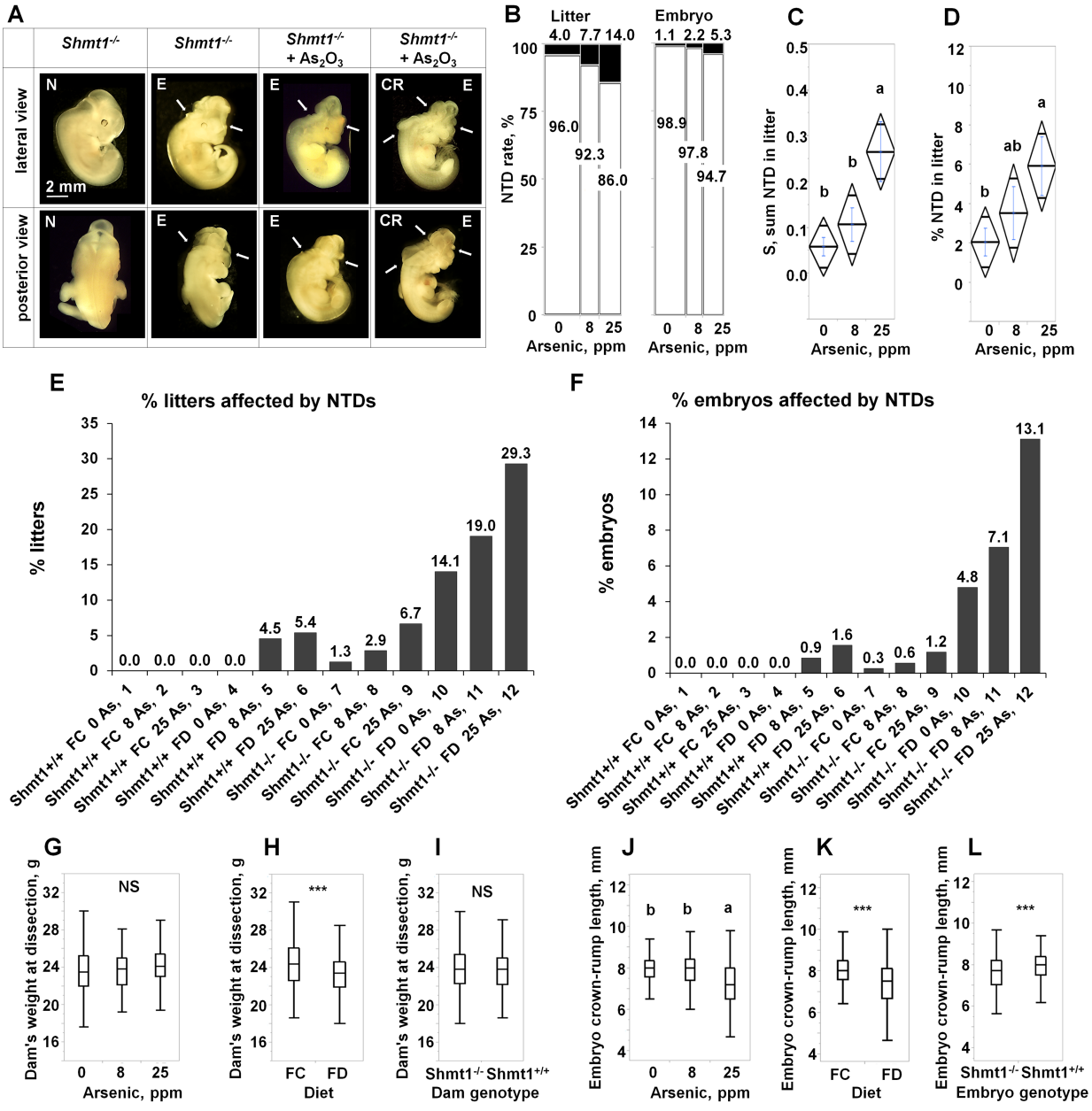


Figure 3.2: Oral maternal As_2O_3 exposure in drinking water caused NTDs and decreased crown-rump length in embryos in a dose-dependent manner.

A. Lateral and posterior views of a normal (N) embryo, an embryo with exencephaly (E), and an embryo with exencephaly and partial craniorachischisis (CR). **B**. Contingency table for overall NTD rate (black bars) by arsenic exposure scored as affected litters (best fit y = 0.3954x + 4.2173, R^2 = 0.9969, Cochran-Armitage Trend Test,

p=0.0003) or affected embryos (best fit y = 0.1215x + 0.8305, R^2 = 0.9966, Cochran-Armitage Trend Test, p<0.0001). **C**. Severity of presentation denoted as mean of sum of embryos presenting with NTDs per litter. Dose response best fit y = 0.0085x + 0.0517, R^2 = 0.9911 **D**. Severity of presentation denoted as mean % of embryos presenting with NTDs per litter. Dose response best fit y = 0.1524x + 2.1699, R^2 = 0.9954. Groups with distinct letter designations are significantly different using the Tukey-Kramer HSD, 0 vs 25 ppm, p=0.0263. **E**. Rate of NTDs presented as % affected litters per study group. **F**. Rate of NTDs presented as % affected embryos per study group. **G**. Effect of arsenic on dams' weight at dissection. **H**. Effect of diet on dams' weight at dissection. **I**. Effect of genotype on dams' weight at dissection. NS, nonsignificant. **J**. Effect of arsenic on embryonic crown-rump length. **K**. Effect of diet on embryonic crown-rump length. **L**. Effect of genotype on embryonic crown-rump length.

distribution (Figure 3.2C). In the generalized linear model incorporating main effects of genotype, diet, and As_2O_3 exposure and their interactions, the higher risk for NTD response was associated with embryonic *Shmt1*$^{-/-}$ genotype compared to *Shmt1*$^{+/+}$ genotype (χ^2=11.36, p=0.0008), with the FD diet compared to the FC diet (χ^2=6.64, p=0.01), and with As_2O_3 exposure at 25 ppm compared to no As_2O_3 exposure (χ^2=5.84, p=0.015). There were no significant interactions among genotype, diet, and arsenic exposure in the generalized linear regression model. Across all genotypes and diets, the odds of an NTD event were 3.4 times more likely for arsenic exposure at 25 ppm compared to no arsenic exposure (OR 3.4, 95% CI 1.1, 12.8). The interaction between embryonic genotype and dietary folic acid indicated that *Shmt1*$^{-/-}$ genotype was a modifier of the effect of folic acid on the risk for NTD incidence in the presence of As_2O_3 exposure (χ^2=7.6, p=0.0058). The lack of an interaction between embryonic genotype and As_2O_3 exposure indicates that As_2O_3 exposure affected NTD risk independently of genotype (χ^2=3.2, p>0.05). The interaction between diet and As_2O_3 exposure indicates that As_2O_3 exposure reduced the efficacy of folic acid in rescuing NTD incidence (χ^2=4.09, p=0.04). Embryos of all genotypes fed FC diet had a lower NTD rate than embryos all genotypes fed the FD diet when exposed to arsenic (0.2, 0.3, and 0.7% in 0, 8, 25 ppm As_2O_3 exposure groups in FC diet group, vs 2.3, 4.4, 8.3% in 0, 8, 25 ppm As_2O_3 exposure in FD diet group). The presence of NTDs in the

FC group and the As_2O_3-diet interaction in the generalized linear regression model indicate that As_2O_3 causes folic acid-resistant NTDs at the concentrations of As_2O_3 used in this study.

Effect of genotype, diet, and arsenic exposure on maternal weight gain

The effect of arsenic on maternal toxicity was determined by comparing dams' weight at embryonic day 12.5. There was no significant difference in dams' weight as a result of arsenic exposure (Figure 3.2G, $p>0.05$). Dams fed the FD diet weighed less than dams fed the FC diet, 23.3 ± 2.11 vs 24.5 ± 2.49 g (Figure 3.2H, $p<0.0001$). There were no differences in weight based on dams' genotype, 23.93 ± 2.38 vs 23.73 ± 2.29 g for *Shmt1*$^{-/-}$ vs *Shmt1*$^{+/+}$ dams, respectively (Figure 3.2I, $p>0.05$).

The effect of genotype, diet, and arsenic exposure on fetal growth

Maternal exposure to 25 ppm As_2O_3 in drinking water inhibited embryonic growth. Crown-rump length was significantly lower in embryos from dams exposed to 25 ppm As_2O_3 compared to either the unexposed group or 8 ppm exposed groups (Figure 3.2J, 8.01 ± 0.94, 7.92 ± 1.02, 7.24 ± 1.37 mm in 0, 8, and 25 ppm As_2O_3 exposure groups, respectively, $p<0.0001$). Embryos from dams fed the FD diet had significantly lower crown-rump length compared to embryos from dams fed the FC diet (Figure 3.2K, 7.44 ± 1.26 vs 8.04 ± 0.97 mm, respectively, $p<0.0001$). *Shmt1*$^{-/-}$ embryos had significantly lower crown-rump length than *Shmt1*$^{+/+}$ embryos (Figure 3.2L, 7.63 ± 1.22 mm vs 7.94 ± 1.02 mm, respectively, $p<0.0001$), consistent with previous findings (11). In a least squares model of interactions, *Shmt1*$^{-/-}$ embryos were smaller than *Shmt1*$^{+/+}$ embryos (least squares mean 7.55 ± 0.04 vs 7.82 ± 0.05 mm, respectively, $p<0.0001$), embryos from dams fed the FD diet were significantly smaller than embryos from dams fed the

FC diet (least squares mean 7.39 ± 0.04 vs 7.98 ± 0.04 mm, respectively, $p<0.0001$), embryos from dams exposed to 25 ppm As_2O_3 were significantly smaller that embryos from dams not exposed to As_2O_3 (least squares mean 7.38 ± 0.05 vs 8.00 ± 0.04 mm, respectively, $p<0.0001$). There was an interaction between As_2O_3 exposure and maternal dietary folic acid on embryonic crown-rump length. The reduction in crown-rump length resulting from As_2O_3 exposure was greater in embryos from dams fed FD diet, decreasing from 7.94 ± 0.06 to 6.85 ± 0.07 mm, compared to reduction in crown-rump length in embryos from dams fed FC diet, from 8.06 ± 0.05 to 7.9 ± 0.08, (Supplemental Table 3.1, $p<0.0001$). This indicates that folic acid depletion exacerbates As_2O_3-induced embryonic growth impairment. Although maternal diet and genotype interacted to affect embryonic growth (Supplemental Table 3.1, $p<0.0001$), there was no interaction between As_2O_3 and genotype (Supplemental Table 3.1, $p>0.05$), indicating that As_2O_3 affects embryonic crown-rump length of all genotypes tested.

Arsenic exposure and folate depletion induced genome instability in mouse embryonic fibroblasts (MEFs)

Folate depletion and As_2O_3 exposure independently induced phosphorylated histone H2AX (γH2AX) localization to nuclear DNA (**Figure 3.3 and 3.4**), which is a marker of double-strand DNA breaks and stalled replication forks (62). To determine if folate can protect against the As_2O_3-induced γH2AX response, *Shmt*$^{+/+}$ and *Shmt*$^{-/-}$ MEFs were exposed to increasing concentrations of As_2O_3 and γH2AX was quantified using flow cytometry (Figure 3.3). Two parameters were used to assess the γH2AX response: % cells in "high γH2AX" (as defined in Materials and Methods) (Figure 3.3A, C-E) and mean γH2AX per-cell fluorescence intensity

(Figure 3.3B, F-I). Representative histograms for cells in all stages of cell cycle (G_1, S, and G_2 combined, or "all cells") as well as cell-cycle-stratified cells (G_1, S, G_2) based on DNA

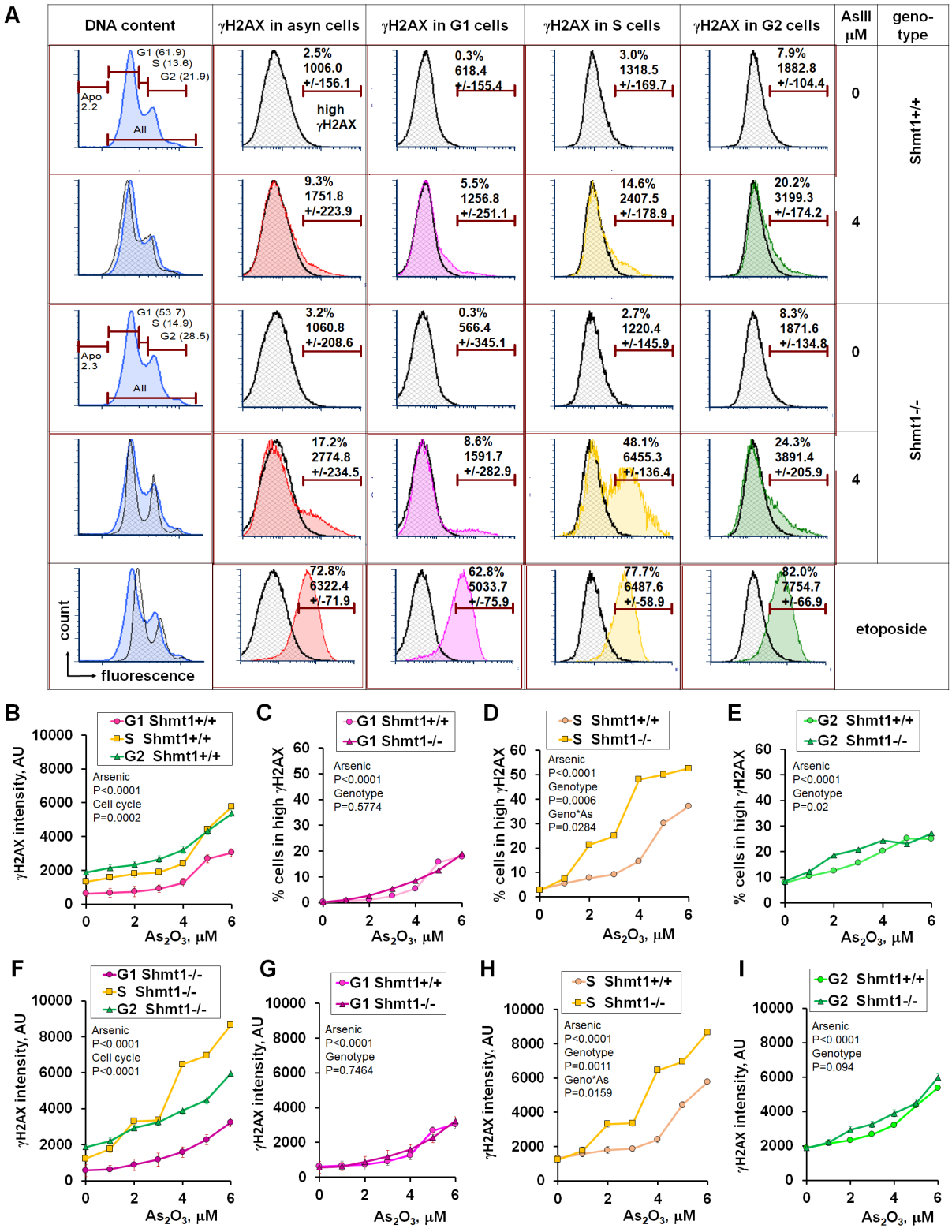


Figure 3.3. *Shmt1* genotype modifies arsenic-induced genome instability in MEFs.

MEFs were isolated from morphologically normal *Shmt1*$^{+/+}$ or *Shmt1*$^{-/-}$ embryos from dams fed the FC diet, cultured in Fol media (modMEM lacking folic acid and ribo- and deoxyribonucleosides supplemented 25 nM (*6S*)-5-formyltetrahydrofolate for 9 days, treated with indicated concentrations of As_2O_3 for 24 h prior to cell collection and analysis by flow cytometry. **A**. Representative flow cytometry histograms are shown. Gray histograms: fluorescence signal in control *Shmt1*$^{+/+}$ (row 1 and 2) or *Shmt1*$^{-/-}$ MEFs (row 3-5) without As_2O_3 exposure; colored histograms: fluorescence γH2AX signal in *Shmt1*$^{+/+}$or *Shmt1*$^{-/-}$ MEFs treated with 4 μM As_2O_3 for 24 h. First column (DNA content, gray and blue). Second column (γH2AX in asynchronous cells, gray and red): γH2AX in *Shmt1*$^{+/+}$or *Shmt1*$^{-/-}$ MEFs in all stages of cell cycle. Third column (gray and purple): γH2AX in G1-phase cells. Fourth column (gray and yellow): γH2AX in S-phase cells. Fifth column (gray and green): γH2AX in G2-phase cells. **B**. Cell-cycle-stratified mean per-cell γH2AX fluorescence intensity (FIM) +/- CV (coefficient of variation) in *Shmt1*$^{+/+}$ MEFs in response to As_2O_3. **C-E**. Genotype-stratified % cells in high γH2AX (high γH2AX was defined by a threshold cutoff at the mean top 2.5% of cells across triplicates in the control condition stained for total γH2AX) in response to As_2O_3 for: G1-phase cells (**C**), S-phase cells (**D**), G2-phase cells (**E**). **F.** Mean per-cell γH2AX fluorescence intensity (FIM)+/-CV (coefficient of variation) in cell-cycle-stratified *Shmt1*$^{-/-}$ MEFs. **G-I**. Genotype-stratified mean γH2AX fluorescence intensity (FIM) +/- CV in response to As_2O_3 in: G1-phase cells (**G**), S-phase cells (**H**), G2-phase cells (**I**) in response to As_2O_3. Multi-factor ANOVA analysis was applied to derive P values for the effect of arsenic, cell cycle, and genotype.

content are shown (Figure 3.3A). Both parameters of γH2AX activation strongly correlated in all experiments (comparing γH2AX response in Figure 3.3C to G, D to H, E to I). As_2O_3 exposure caused a dose-dependent increase in γH2AX signal in both *Shmt1*$^{+/+}$ and *Shmt1*$^{-/-}$ MEFs (Figure 3.3B-I, $p<0.0001$). *Shmt1*$^{-/-}$ genotype exacerbated the γH2AX response to As_2O_3 in S and G2 phases of cell cycle (Figure 3.3D and E, effect of genotype $p=0.0006$ and $p=0.02$, respectively), as analyzed using best-fit exponential models. *Shmt1*$^{-/-}$ MEFs exhibited higher modeled baseline γH2AX levels and responses to As_2O_3 than *Shmt1*$^{+/+}$ MEFs (Supplemental Table 3.2, 1019 vs 848 AU, respectively, with correlation formulas for *Shmt1*$^{+/+}$ MEFs in all cells $\gamma H2AX=847.71e^{0.2321[As2O3]}$, $R^2=0.87$, and for *Shmt1*$^{-/-}$ cells $\gamma H2AX= 1018.6e^{0.247[As2O3]}$, $R^2=0.99$). The γH2AX response to As_2O_3 varied depending on the stage of the cell cycle, with

the highest γH2AX levels displayed by S-phase *Shmt1*$^{-/-}$ MEFs and S- and G_2-phase *Shmt1*$^{+/+}$ MEFs. The lowest γH2AX levels were displayed by cells in G_1 phase in both genotypes (Figure 3.3B,G). *Shmt1* null status conferred greater sensitivity to As_2O_3 at low (<5 μM) concentrations (Supplemental Table 3.3, p values for γH2AX in all cells are significant at 1 μM or greater As_2O_3 concentrations in *Shmt1*$^{-/-}$ MEFs, while p values for γH2AX in all cells are significant at 5 μM or greater As_2O_3 concentrations in *Shmt1*$^{+/+}$ MEFs compared to no As_2O_3 treatment in respective genotypes), consistent with previous reports showing that *Shmt1* deficiency perturbs dTMP biosynthesis capacity (11, 13) and confers greater sensitivity to As_2O_3 (56). The cell-cycle-dependent pattern of the γH2AX response to As_2O_3 differed from γH2AX response to etoposide, a DNA-damaging agent that inhibits topoisomerase. Etoposide-treated MEF cells exhibited an equally strong increase in γH2AX signal in cells in G1 (from 0.3 to 62.8 %), S (from 2.7 to 77.7 %), and G2 (from 8.3 to 82.0 %), indicating that MEFs possess the capacity to mount a robust γH2AX signal in response to DNA damage in all stages of the cell cycle (Figure 3.3A), and that the relatively low levels of γH2AX induction in G1 MEFs treated with As_2O_3 are due to impaired dTMP synthesis during DNA replication.

The effect of folate or folic acid in culture media on baseline and As_2O_3-induced γH2AX signal was examined in *Shmt1*$^{-/-}$ MEFs by flow cytometry (Figure 3.4). *Shmt1*$^{-/-}$ MEFs were cultured in αMEM containing 1 mg/L (2.27 μM) folic acid and ribo- and deoxyribonucleosides, or in modMEM lacking folic acid and ribo- and deoxyribonucleosides with (Fol) or without (noFol) supplementation with 25 nM (*6S*)-5-formyltetrahydrofolate. Cells grown in all three types of media displayed an increase in γH2AX in response to As_2O_3 in a dose-dependent manner (Figure 3.4A). MEFs cultured in noFol media displayed the greatest level of γH2AX intensity in the absence of As_2O_3 exposure, as well as a greater increase in γH2AX intensity in

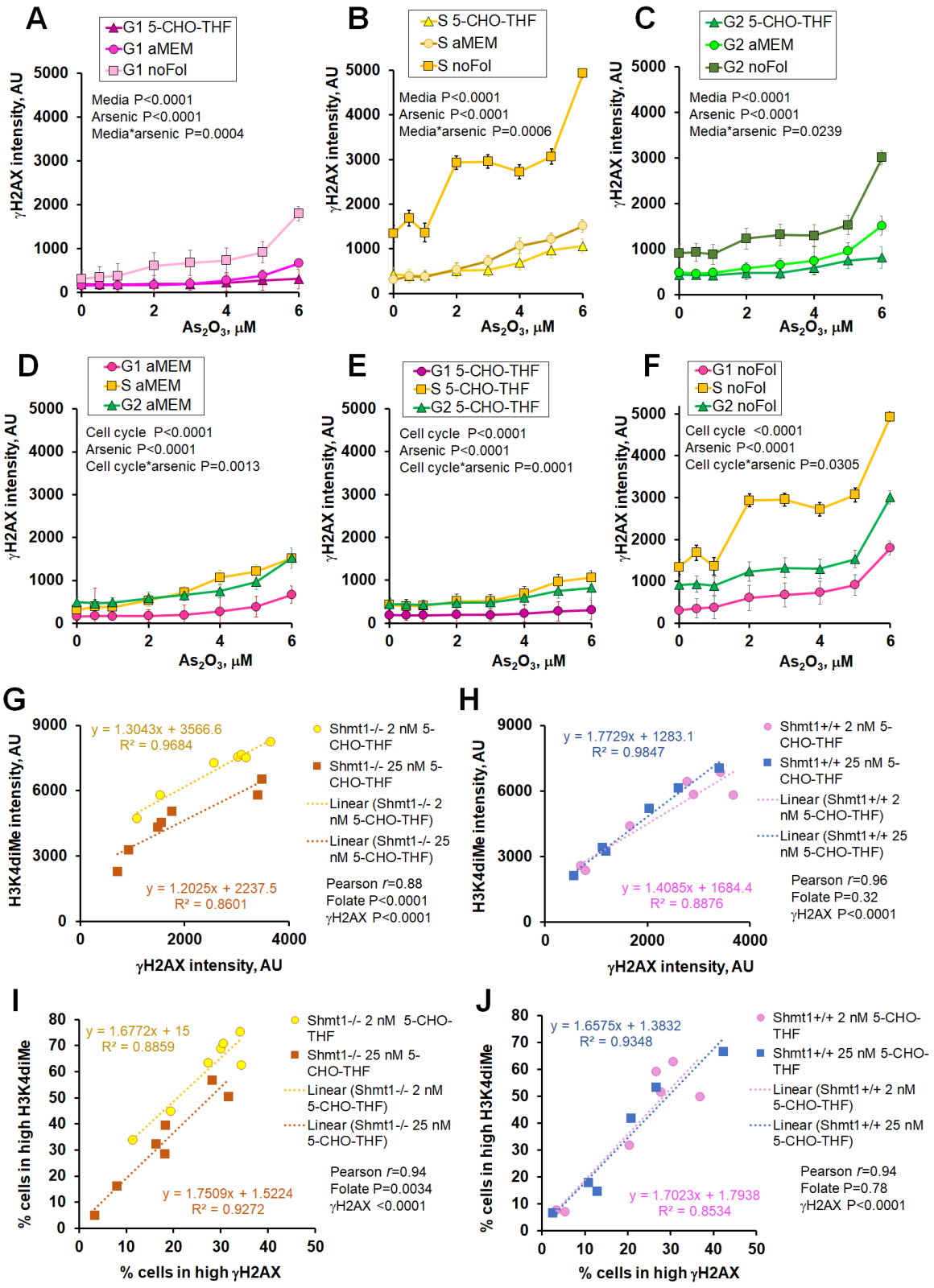

A
G1 5-CHO-THF
G1 aMEM
G1 noFol
Media P<0.0001
Arsenic P<0.0001
Media*arsenic P=0.0004
γH2AX intensity, AU
As2O3, μM
B
S 5-CHO-THF
S aMEM
S noFol
Media P<0.0001
Arsenic P<0.0001
Media*arsenic P=0.0006
C
G2 5-CHO-THF
G2 aMEM
G2 noFol
Media P<0.0001
Arsenic P<0.0001
Media*arsenic P=0.0239
D
G1 aMEM
S aMEM
G2 aMEM
Cell cycle P<0.0001
Arsenic P<0.0001
Cell cycle*arsenic P=0.0013
E
G1 5-CHO-THF
S 5-CHO-THF
G2 5-CHO-THF
Cell cycle P<0.0001
Arsenic P<0.0001
Cell cycle*arsenic P=0.0001
F
G1 noFol
S noFol
G2 noFol
Cell cycle <0.0001
Arsenic P<0.0001
Cell cycle*arsenic P=0.0305
G
y = 1.3043x + 3566.6
R² = 0.9684
y = 1.2025x + 2237.5
R² = 0.8601
Shmt1-/- 2 nM 5-CHO-THF
Shmt1-/- 25 nM 5-CHO-THF
Linear (Shmt1-/- 2 nM 5-CHO-THF)
Linear (Shmt1-/- 25 nM 5-CHO-THF)
Pearson r=0.88
Folate P<0.0001
γH2AX P<0.0001
H3K4diMe intensity, AU
γH2AX intensity, AU
H
y = 1.7729x + 1283.1
R² = 0.9847
y = 1.4085x + 1684.4
R² = 0.8876
Shmt1+/+ 2 nM 5-CHO-THF
Shmt1+/+ 25 nM 5-CHO-THF
Linear (Shmt1+/+ 2 nM 5-CHO-THF)
Linear (Shmt1+/+ 25 nM 5-CHO-THF)
Pearson r=0.96
Folate P=0.32
γH2AX P<0.0001
I
y = 1.6772x + 15
R² = 0.8859
y = 1.7509x + 1.5224
R² = 0.9272
Shmt1-/- 2 nM 5-CHO-THF
Shmt1-/- 25 nM 5-CHO-THF
Linear (Shmt1-/- 2 nM 5-CHO-THF)
Linear (Shmt1-/- 25 nM 5-CHO-THF)
Pearson r=0.94
Folate P=0.0034
γH2AX <0.0001
% cells in high H3K4diMe
% cells in high γH2AX
J
y = 1.6575x + 1.3832
R² = 0.9348
y = 1.7023x + 1.7938
R² = 0.8534
Shmt1+/+ 2 nM 5-CHO-THF
Shmt1+/+ 25 nM 5-CHO-THF
Linear (Shmt1+/+ 2 nM 5-CHO-THF)
Linear (Shmt1+/+ 25 nM 5-CHO-THF)
Pearson r=0.94
Folate P=0.78
γH2AX P<0.0001

Figure 3.4. Arsenic-induced epigenetic changes in histone phosphorylation (γH2AX) and methylation (H3K4me2) are modified by *Shmt1* genotype and folate depletion.

Shmt1$^{-/-}$ MEFs were isolated, cultured in aMEM, Fol, or noFol media (modMEM lacking folic acid and ribo- and deoxyribonucleosides with (Fol) or without (noFol) supplementation with 25 nM (*6S*)-5-formyltetrahydrofolate for 10 days, treated with indicated concentrations of As_2O_3 for 24 h prior to cell collection and analyzed by flow cytometry. A. Left, mean per-cell γH2AX fluorescence intensity +/- CV in response to As_2O_3 for cells in all stages of cell cycle (all cells). Middle, % cells in high γH2AX in response to As_2O_3 for all cells. B. Mean per-cell γH2AX fluorescence intensity +/- CV in response to As_2O_3 for cells in G1 (left), S (middle), and G2 (right) stages of cell cycle. C. % cells in high γH2AX gate in response to As_2O_3 for cells in G1 (left), S (middle), and G2 (right) stages of cell cycle. D-F. Mean per-cell γH2AX fluorescence intensity +/-CV in response to As_2O_3 for cells grown in aMEM (D), (*6S*)-5-formyltetrahydrofolate (E), or noFol (F) media. G. Correlation plot for mean intensity of H3K4me2 (also known as H3K4diMe) and γH2AX signal for *Shmt1*$^{-/-}$ MEFs grown in 2 or 25 nM (*6S*)-5-formyltetrahydrofolate-supplemented media and treated with As_2O_3 for 24 h. H. Correlation plot for mean intensity of H3K4me2 and γH2AX signal for *Shmt1*$^{+/+}$ MEFs grown in 2 or 25 nM (*6S*)-5-formyltetrahydrofolate-supplemented media and treated with As_2O_3 for 24 h. I. Correlation plot for % cells with high H3K4me2 and γH2AX signal for *Shmt1*$^{-/-}$ MEFs grown in 2 or 25 nM (*6S*)-5-formyltetrahydrofolate-supplemented media and treated with As_2O_3 for 24 h. J. Correlation plot for % cells with high H3K4me2 and γH2AX signal for *Shmt1*$^{+/+}$ MEFs grown in 2 or 25 nM (*6S*)-5-formyltetrahydrofolate-supplemented media and treated with As_2O_3 for 24 h.

response to As_2O_3 compared to MEFs cultured in αMEM or Fol media (Figure 3.4A, D-F, effect of arsenic $p<0.0001$, Supplemental Figure 3.4A, effect of arsenic $p<0.0001$, effect of media, $p<0.0001$). Cells cultured in noFol media displayed a greater percentage of cells with high γH2AX, with and without As_2O_3 compared to cells cultured in medium containing folate or folic acid (Figure 3.4A-C, effect of culture medium $p<0.0001$). In all culture conditions, γH2AX intensity increased in response to As_2O_3 when cells were stratified by G1, S, and G2 stages of cell cycle, with the greatest increase observed in cells cultured in noFol media (Figure 3.4B). The percentage of cells with high γH2AX stratified by stages of the cell cycle followed the pattern of mean γH2AX intensity, with a higher percentage of cells with high γH2AX displayed by cells grown in noFol media, followed by cells in αMEM and Fol media (Figure 3.4F, effect of cell cycle $p<0.0001$, effect of arsenic $p<0.0001$). When γH2AX intensity in response to As_2O_3 was

analyzed in cells stratified by stages of the cell cycle, cells in G1 showed the lowest γH2AX levels, while cells in S and G2 phases showed comparably high γH2AX levels in αMEM and Fol media, consistent with DNA damage during DNA replication being a pronounced outcome of As_2O_3 exposure (Figure 3.4D, left and middle). In cells grown in noFol media, S-phase γH2AX intensity levels were higher than that in G1 or G2, and continued to increase further in response to As_2O_3 (Figure 3.4F, effect of cell cycle $p<0.0001$, effect of arsenic $p<0.001$). Similarly, the percentage of cells in S phase with high γH2AX showed the greatest increase in response to As_2O_3 in all types of media (Suppl. Figure 3.1F-H). Thus, folate depletion contributes to γH2AX response to As_2O_3, with folate-depleted, S-phase cells displaying highest levels of DNA damage, consistent with a mechanism where *de novo* dTMP biosynthesis during DNA replication is a sensitive target of As_2O_3 at low doses, and where this effect is exacerbated by the lack of SHMT1 enzyme as well as by folate depletion (Figure 3.3D, effect of genotype $p<0.0001$; Figure 3.4B, effect of media, $p<0.0001$).

The effect of folate in culture media on As_2O_3-induced global H3K4me2 signal was examined by flow cytometry in *Shmt1*$^{+/+}$ and *Shmt1*$^{-/-}$ MEFs cultured in 2 or 25 nM (*6S*)-5-formyltetrahydrofolate (Figure 3.4G-J, Supplemental Figure 3.2). H3K4me2 is a histone methylation mark for open chromatin (63, 64) and associates with H3K4me3, a histone methylation mark for active gene promoters and transcriptional activation (65). H3K4me2 has been shown to associate with double stranded DNA breaks (63, 66) and to increase in response to arsenic treatment (67). H3K4me2 associated with γH2AX response to arsenic in both culture conditions (Figure 3.4G, Pearson r=0.88, linear correlation R^2=0.86, P<0.0001, H, Pearson r=0.96, linear correlation R^2=0.89, P<0.0001, I, Pearson r=0.94, linear correlation R^2=0.92, P<0.0001, J, Pearson r=0.94, linear correlation R^2=0.85, p<0.0001). In *Shmt1*$^{-/-}$ MEFs, both

γH2AX and H3K4me2 levels were higher in cells cultured in 2 nM (*6S*)-5-formyltetrahydrofolate containing culture media with and without arsenic treatment compared to cells cultured in medium containing 25 nM (*6S*)-5-formyltetrahydrofolate (Figure 3.4G, effect of folate P<0.0001, I, effect of folate P=0.003). There was no effect of folate levels in culture medium on the correlation between H3K4me2 and γH2AX in response to arsenic in *Shmt1*$^{+/+}$ MEFs (Figure 3.4H, effect of folate P=0.32, J, effect of folate P=0.78). An increase in H3K4me2 signal was observed in etoposide-treated cells (Suppl. Figure 3.2), indicating that the H3K4me2 correlation with γH2AX occurs in response to DNA damage and is not limited to the effects of arsenic. Both H3 acetylation and H3K4me3 are known to be induced by double-stranded DNA breaks and play a role in recruiting DNA repair enzymes to sites of DNA damage (68-71).

Arsenic affects biomarkers of folate metabolism in mice

The effects of 25 ppm As_2O_3 exposure in drinking water, dietary folic acid, and *Shmt1* genotype (singly and in combination) on blood biomarkers of folate-dependent one-carbon metabolism was determined in dams at E12.5 (**Figure 3.5** and Supplemental Table 3.3). Levels of plasma folate, homocysteine, cystathionine, cysteine, α-amino-butyrate, and the glycine/serine ratio responded to arsenic exposure. Plasma folate levels in arsenic-exposed dams consuming the FD diet were 50% lower than in arsenic-exposed dams consuming the FC diet (Supplemental Table 3.3, 97.6 ± 11 nmol/L in FC arsenic non-exposed dams vs 56.0 ± 6.0 nmol/L in FC arsenic-exposed dams, p=0.0097). Across all diet and genotype groups, As_2O_3 exposure elevated plasma homocysteine levels from 14.6 ± 1.65 to 22.2 ± 2.87 μM, (p=0.0003). The increase in homocysteine resulting from As_2O_3 exposure was more pronounced in *Shmt1*$^{+/+}$ dams (14.67 ±

1.99 to 27.35 ± 4.96) compared to *Shmt1*$^{-/-}$ dams (14.43 ± 2.71 to 17.06 ± 2.51). The greatest increase in homocysteine level was

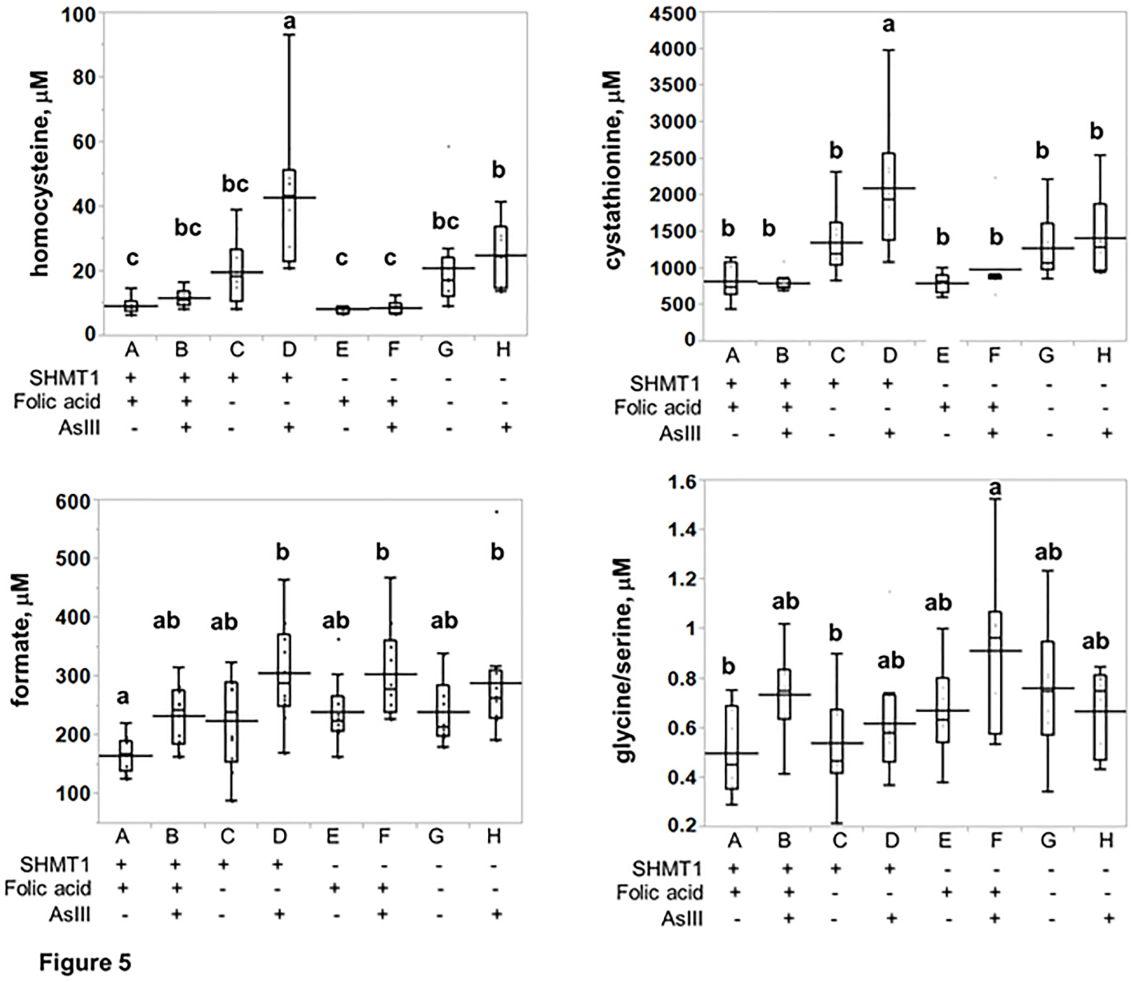


Figure 3.5. Arsenic exposure modifies biomarkers of folate metabolism in pregnant dams.

Maternal plasma metabolites (n=10 per group) were analyzed by two-way ANOVA with post-hoc Tukey HSD (Honestly Significant Difference) test, displayed as box and whisker (the line within the box represents the mean, the longer horizontal lines represents the median). Groups not connected by the same letter are significantly different.

observed in *Shmt1*$^{+/+}$ dams consuming FD diet and exposed to As_2O_3 compared to dams consuming the FC diet not exposed to As_2O_3 (Figure 3.5 upper left panel, compare D to A, 42.99 ± 6.99 vs 9.45 ± 0.77, respectively, $p<0.0001$). As expected, plasma levels of cystathionine

reflected changes in homocysteine and formate levels. Plasma glycine and serine levels were also affected by As_2O_3 exposure, as demonstrated by an elevation of the glycine to serine ratio (gly/ser) in folate-replete *Shmt1*$^{-/-}$ dams exposed to As_2O_3 compared to non-exposed *Shmt1*$^{+/+}$ dams (Figure 3.5, mean gly/ser 0.5 vs 0.91, respectively, or a 82% increase). In the two-way ANOVA analysis, both genotype and As_2O_3 exposure affected the plasma gly/ser ratio (Supplemental Table 3.3, p=0.00015 and 0.014, respectively), indicating that As_2O_3 exposure affects glycine and serine production and/or utilization, consistent with recent studies showing that As_2O_3 decreases SHMT1 and MTHFD1 enzyme levels (56). Similarly, plasma formate levels also responded to As_2O_3 exposure. Across all dietary and genotype groups, As_2O_3 exposure elevated plasma formate levels from 218 ± 10 to 284 ± 14 μM, p<0.0001). The increase in plasma formate was independent of dietary folic acid (Supplemental Table 3.3, arsenic x diet interaction, p>0.05). This observation is also consistent with As_2O_3-mediated degradation of MTHFD1 protein (72) and decreased capacity to condense formate with THF, leading to formate export from the cell into circulation.

Arsenic modifies biomarkers of altered glucose homeostasis in mice

Non-targeted, discovery-based metabolomics profiling was used to determine if other metabolic pathways were affected by As_2O_3 exposure in pregnant dams at E12.5 (**Figure 3.6**). The heatmap (Supplemental Figure 3.3) provides an overview of plasma metabolite levels significantly altered by either As_2O_3, genotype, or diet. We observed significant changes in plasma anhydroglucitol which was decreased due to As_2O_3 exposure (Figure 3.6, p<0.001 for effect of As_2O_3). Interestingly, folate status modified the effect of arsenic on hyperglycemia, as the arsenic-induced decrease in plasma anhydroglucitol was more pronounced in dams

consuming the FC diet compared to dams consuming the FD diet (Figure 3.6, Supplemental. Table 3.3; p=0.000211 for the As_2O_3 x diet interaction). Decreased plasma anhydroglucitol is associated with episodes of hyperglycemia (glucose ≥250 mg/dL in mice (73)) during which excess blood glucose remains in the glomerular

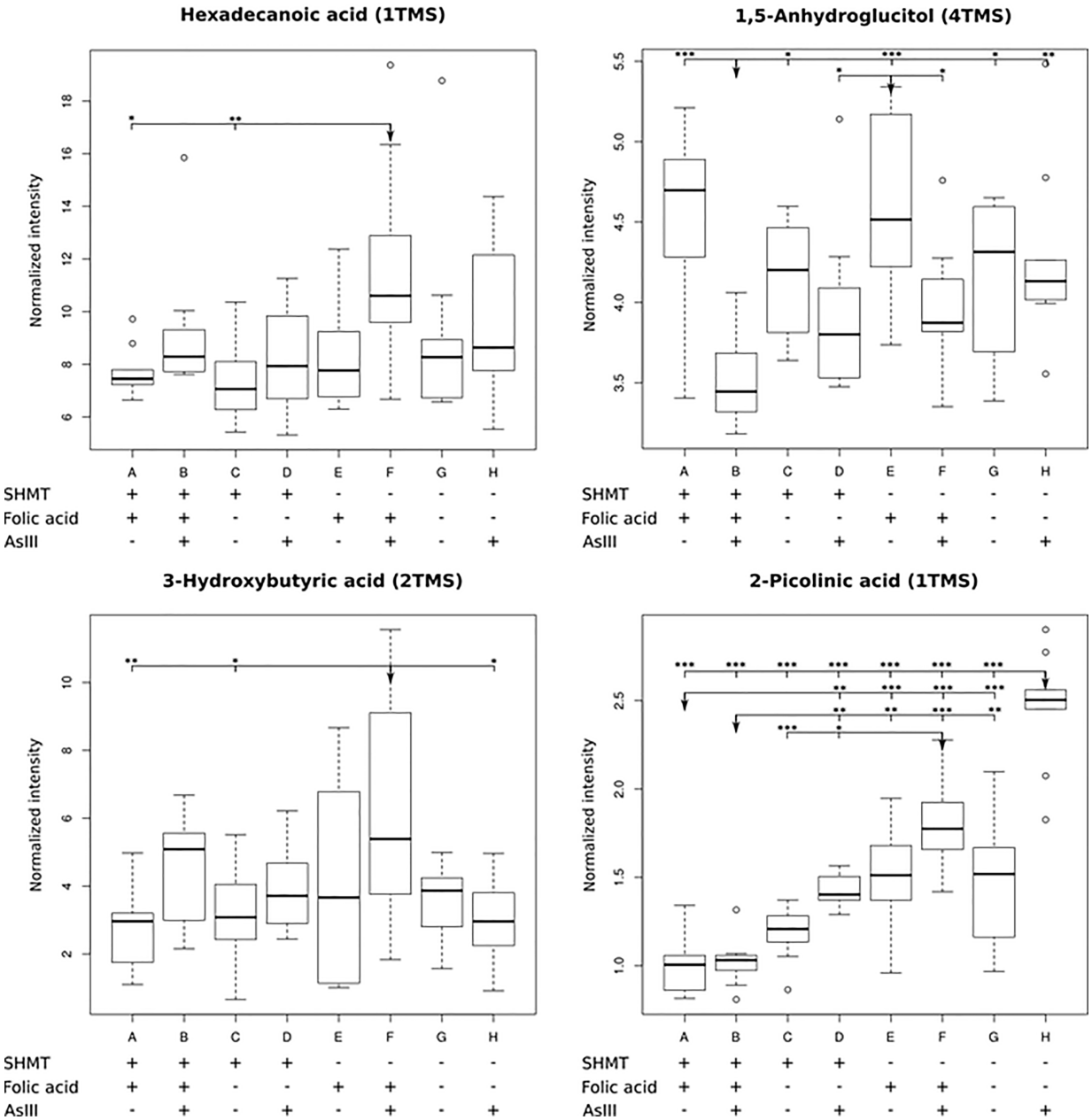


Figure 3.6. Arsenic exposure modifies biomarkers of glucose metabolism in pregnant dams.
Non-targeted metabolomics identifies changes in dams' plasma metabolites that indicate arsenic-induced hyperglycemia, mild ketosis, and dyslipidemia, consistent with metabolic footprint of diabetes. Blue dots represent individual data points, the lines within the box indicate the median, and the vertical lines outside of boxes represent the highest and lowest ends of the distribution, excluding outliers. A Tukey's Honest Significance Test was used to determine which comparisons different from the condition indicated with an arrow, * indicates $p<0.05$.

filtrate during urine production (glucosuria) and competitively inhibits reuptake of anhydroglucitol during renal filtration and reabsorption, resulting in reduced anhydroglucitol blood levels. Increased 3-hydroxybutytate (BHBA) indicates mild ketosis, a characteristic of hyperglycemia. Increased palmitic (hexadecanoic) free fatty acid is consistent with diabetic dyslipidemia. These changes in metabolites are consistent with a metabolic footprint of diabetes; they indicate episodes of arsenic-induced hyperglycemia, mild ketosis, and dyslipidemia.

As_2O_3 exposure increased plasma levels of the ketone 3-hydroxybutyrate ($p=0.03$ for effect of As_2O_3), and palmitate ($p=0.01$ for effect of As_2O_3) (Figure 3.6, upper panel). Interestingly, we also observed genotype-, diet-, and arsenic-specific increases in plasma picolinic acid (Figure 3.6, lower panel). The effect of As_2O_3 on plasma picolinic acid depended on both *Shmt1* genotype and diet and was strongest in *Shmt1*$^{-/-}$ dams fed the FD diet (Supplemental Table 3.5). The highest serum picolinic acid levels were observed in *Shmt1*$^{-/-}$ dams consuming the FD diet and exposed to As_2O_3 (Figure 3.6). Picolinic acid is a product of the kynurenine pathway and functions in inflammatory responses (74). Deregulation of the kynurenine pathway has been associated with metabolic syndrome (75) and an increase in kynurenine has been recently included as part of the diabetic footprint (76). Picolinic acid has also been shown to chelate heavy metals including Ni, Zn, Cd, Pb, Cu, and Fe (74, 77).

Discussion

De novo thymidylate biosynthesis has been recently demonstrated to be a sensitive target of As_2O_3 exposure, leading to genome instability (56). Impaired *de novo* thymidylate biosynthesis also underlies folate-responsive NTDs in mice (11). This study reports on the effects of maternal As_2O_3 exposure on embryonic development in a mouse model and demonstrates that As_2O_3 causes NTDs that are unresponsive to folic acid (Figure 3.2).

The interactions among genotype, folate, and As_2O_3 exposure varied among outcomes and model systems in this study. In mice, risk factors for NTD incidence included the *Shmt1* genotype, maternal dietary folic acid, and maternal As_2O_3 exposure, yet no interaction between maternal dietary folic acid and As_2O_3 exposure was observed. Statistical modeling indicates that both folate deficiency and As_2O_3 exposure are associated with higher NTD risk, and that As_2O_3-induced NTDs are folic-acid resistant. In contrast, As_2O_3-induced DNA damage is folate-responsive in cultured cells. DNA damage is responsive to the concentration of folate in the culture medium, *Shmt1* genotype, and As_2O_3 dose, and these modifiers interact to affect γH2AX intensity (Figure 3.3D, genotype x arsenic interaction, p=0.0284, Figure 3.4B, arsenic x media interaction, p=0.0006) with the greatest effects seen in S-phase during dTMP and DNA synthesis (Figures 3.3, 3.4).

Arsenic exposure was associated with biomarkers of impaired folate and glucose metabolism. Plasma formate and homocysteine concentrations were elevated in arsenic-exposed pregnant dams (Figure 3.5), consistent with previous studies demonstrating that arsenic results in degradation of the MTHFD1 protein (56). MTHFD1 is responsible for the condensation of mitochondrially-produced formate with THF in the cytosol and nucleus. Accelerated turnover of MTHFD1 protein decreases the rates of incorporation of formate into the cellular folate-activated

one-carbon pool, leading to decreased rates of homocysteine remethylation and increased rates of formate and homocysteine export into blood. Elevated plasma homocysteine is a biomarker of impaired folate metabolism (5) and was previously observed in a patient with *MTHFD1* deficiency (78).

Metabolomics profiling of study dams revealed a signature of diabetes, with lower anhydroglucitol, higher 3-hydroxybutytate (BHBA), and higher palmitate levels in arsenic-exposed dams (Figure 3.6). Arsenic exposure has been shown to be associated with an increased risk of impaired glucose tolerance at 24-28 weeks gestation in pregnant women and therefore may be a risk factor for gestational diabetes (41). Diabetes is a risk factor for having an NTD affected pregnancy (79), with uncontrolled hyperglycemia being the main teratogenic factor associated with diabetes-related congenital malformations (80). Maternal hyperglycemia itself has been shown to cause NTDs (81) and conceivably might have contributed to the NTD incidence observed in this study. Hyperglycemia has been previously demonstrated to induce PARylation, a marker of DNA strand breaks that interacts with γH2AX-medaited signaling (82, 83). The diabetic markers in this study showed an interaction between arsenic exposure and dietary folic acid or *Shmt1* genotype, indicating a link between folate-mediated one-carbon metabolism and risk for arsenic-induced diabetes.

In summary, these findings affirm a role for *Shmt1* in NTD pathogenesis. Previously we have shown that As_2O_3 targets MTHFD1 and SHMT1 by increasing SUMOylation and proteolytic degradation of these enzymes leading to impaired *de novo* dTMP synthesis (56). Whereas *Shmt1*$^{+/-}$ and *Shmt1*$^{-/-}$ mice are sensitive to folic acid-responsive NTDs, arsenic-induced NTDs are not folic acid-responsive. The provision of folate cofactors cannot overcome the effect of arsenic on two essential enzymes for *de novo* dTMP synthesis. In contrast, As_2O_3-

induced impairments in fetal growth as measured by crown-rump length and DNA damage in wild-type and *Shmt1*$^{-/-}$ MEFs was folic acid-responsive. These results indicate that DNA damage correlates with growth restriction but not NTD pathogenesis. One limitation of this study is that MEFs may not be a good model of the neuroepithelium, which is highly sensitive to impairments in one-carbon metabolism. Non-targeted metabolomics analyses indicate that As_2O_3-induced metabolic changes reflect those seen in gestational diabetes, suggesting a potential relationship between diabetes and impaired *de novo* dTMP synthesis. Recently, folic acid supplementation in diabetic patients was shown to lower markers of DNA damage in buccal cells (84), though the causal mechanisms whereby folic acid prevents DNA damage in diabetes have not been elucidated.

Supplementary Material:

Supplemental Table 1. Least squares fit model and ANOVA analysis for embryonic crown-rump length.

A. Level As x diet	**Least Sq Mean**	**Std Error**	**p value**
no,FC	8.0622010	0.05119105	<0.0001
no,FD	7.9380107	0.06418398	
25,FC	7.9042939	0.08070540	
25,FD	6.8478281	0.06807675	
B. Level As x gen	**Least Sq Mean**	**Std Error**	**p value**
no,-/-	7.8469124	0.05741446	0.62
no,+/+	8.1532993	0.05815181	
25,-/-	7.2554072	0.06636866	
25,+/+	7.4967149	0.08249104	
C. Level diet x gen	**Least Sq Mean**	**Std Error**	**p value**
FC,-/-	8.0282728	0.06237259	<0.0001
FC,+/+	7.9382221	0.06962231	
FD,-/-	7.0740468	0.06145672	
FD,+/+	7.7117921	0.07079654	

Supplemental Table 2. Dose response analysis of the effect of As_2O_3 on gH2AX and Kolmogorov-Smirnov (KS) test p-values.

	P value (Kolmogorov-Smirnov test with Bonferroni): Shmt1$^{+/+}$ 25 nM Fol dose As_2O_3 vs Shmt1$^{+/+}$ 25 nM Fol 0 As_2O_3							
As_2O_3 μM	P value all cells	significance level	P value G1	significance level	P value S	significance level	P value G2	significance level
0	1	NS	1	NS	1	NS	1	NS
1	1	NS	1	NS	2.22E-18	*	6.29E-11	*
2	1	NS	1	NS	1.94E-36	**	4.88E-54	**
3	1	NS	1	NS	1.45E-171	**	1.77E-281	***
4	1	NS	1	NS	2.74E-238	***	8.74E-297	***
5	4.15E-17	*	1	NS	0.00E+00	***	1.14E-206	***
6	3.84E-162	**	0	***	0.00E+00	***	0.00E+00	***
dose response model	$y = 847.71e^{0.2321x}$		$y = 484.62e^{0.2901x}$		$y = 1147.6e^{0.2427x}$		$y = 1739.8e^{0.174x}$	
R^2	0.8708		0.7977		0.9019		0.9592	

	P value (Kolmogorov-Smirnov test with Bonferroni): Shmt1$^{-/-}$ 25 nM Fol dose As_2O_3 vs Shmt1$^{-/-}$ 25 nM Fol 0 As_2O_3							
As_2O_3 μM	P value all cells	significance level	P value G1	significance level	P value S	significance level	P value G2	significance level
0	1	NS	1	NS	1	NS	1	NS
1	3.05E-49	**	3.13E-82	**	8.53E-18	*	8.53E-18	*
2	6.71E-21	**	3.17E-147	**	6.34E-126	**	6.34E-161	**
3	1.12E-36	**	9.93E-99	**	3.39E-160	**	3.39E-160	**
4	4.47E-210	***	4.46E-177	**	6.50E-267	***	6.50E-267	***
5	6.14E-85	**	0.00E+00	***	8.68E-187	**	8.68E-187	**
6	1	NS	0.00E+00	***	3.52E-272	***	3.52E-272	***
etoposide	9.65E-246	***	0.00E+00	***	0	***	0	***
dose response model	$y = 1018.6e^{0.247x}$		$y = 498.32e^{0.301x}$		$y = 1363.6e^{0.3318x}$		$y = 1882.6e^{0.185x}$	
R^2	0.9858		0.987		0.9537		0.9877	

Supplemental Table 3. Targeted metabolite panel.

	FC			FC+AsIII			FD			FD+AsIII			noAsIII	AsIII	two-way ANOVA, p value				
Metabolite	**Shmt1 +/+**	**Shmt1 -/-**	**All**	**Shmt1 +/+**	**Shmt1 -/-**	**All**	**Shmt1 +/+**	**Shmt1 -/-**	**All**	**Shmt1 +/+**	**Shmt1 -/-**	**All**	**All**	**All**	**geno-type**	**diet**	**AsIII**	**geno-type x diet**	**geno-type x AsIII**	**diet x AsIII**
Formate, μM	165.92 +/- 9.59	239.79 +/- 18.04	202.86 +/- 13.06	234.17 +/- 16.21	304.71 +/- 25.08	269.44 +/- 16.63	224.68 +/- 25.09	241.06 +/- 18.32	232.87 +/- 15.23	305.86 +/- 27.39	289.57 +/- 34.66	297.71 +/- 21.58	217.86 +/- 10.19	283.58 +/- 13.64	0.0077	NS	<0.0001	0.00231	NS	NS
Homocysteine, μM	9.45 +/- 0.77	8.31 +/- 0.30	8.88 +/- 0.42	11.7 +/- 0.89	8.92 +/- 0.60	10.31 +/- 0.61	19.89 +/- 3.18	21.22 +/- 5.07	20.52 +/- 2.85	42.99 +/- 6.99	25.19 +/- 3.39	34.09 +/- 4.30	14.6 +/- 1.65	22.2 +/- 2.87	0.0138	<0.0001	0.0003	NS	0.0482	0.0313
Cystathionine, μM	822.9 +/- 80.13	799.4 +/- 41.74	811.15 +/- 44.05	799.2 +/- 38.93	995.5 +/- 142.5	897.35 +/- 75.34	1357.9 +/- 144.49	1278.33 +/- 153.65	1320.21 +/- 102.72	2092.7 +/- 286.79	14177 +/- 167.63	1755.2 +/- 179.24	1059.15 +/- 67.23	1326.28 +/- 118.0	NS	<0.0001	0.0162	0.0313	NS	NS
Cysteine, μM	206.8 +/- 3.75	200.8 +/- 7.52	203.8 +/- 4.15	190.8 +/- 9.04	183.3 +/- 10.17	187.05 +/- 6.68	172.7 +/- 8.34	188.89 +/- 9.29	180.37 +/- 6.33	166.3 +/- 11.42	175 +/- 6.73	170.65 +/- 6.52	192.38 +/- 4.10	178.85 +/- 4.79	NS	0.0016	0.0246	NS	NS	NS
Methionine, μM	66.12 +/- 5.89	53.59 +/- 5.12	59.86 +/- 4.06	50.31 +/- 3.31	58.85 +/- 3.86	54.58 +/- 2.66	52.56 +/- 4.91	59.6 +/- 4.24	55.89 +/- 3.29	51.41 +/- 4.73	63.24 +/- 6.02	57.32 +/- 3.97	57.93 +/- 2.58	55.95 +/- 2.37	NS	NS	NS	NS	NS	NS
α-amino-butyric, μM	9.57 +/- 0.54	9.12 +/- 0.35	9.35 +/- 0.32	12.02 +/- 1.56	8.68 +/- 0.58	10.35 +/- 0.90	9.69 +/- 1.05	8.61 +/- 0.53	9.18 +/- 0.60	11.82 +/- 1.04	10.04 +/- 0.62	10.93 +/- 0.63	9.26 +/- 0.33	10.64 +/- 0.54	0.0144	NS	0.0298	NS	NS	NS
Glycine, μM	87.2 +/- 4.47	97.1 +/- 5.00	92.15 +/- 3.46	97.6 +/- 5.35	128.6 +/- 18.78	113.1 +/- 10.15	84.67 +/- 8.86	135.67 +/- 13.93	110.17 +/- 10.12	105 +/- 12.69	123.5 +/- 12.07	114.25 +/- 8.79	100.68 +/- 5.13	113.68 +/- 6.63	0.0002	NS	NS	NS	NS	NS
Serine, μM	186.2 +/- 12.64	152.8 +/- 13.14	169.5 +/- 9.66	136.5 +/- 7.81	142.7 +/- 9.69	139.6 +/- 6.10	163.2 +/- 9.67	185 +/- 13.51	173.52 +/- 8.34	173 +/- 12.59	188.6 +/- 15.95	180.8 +/- 10.05	171.46 +/- 6.26	160.2 +/- 6.67	NS	0.0062	NS	NS	NS	0.0479
Gly/Ser	0.50 +/- 0.06	0.67 +/- 0.05	0.59 +/- 0.04	0.73 +/- 0.05	0.91 +/- 0.10	0.82 +/- 0.06	0.54 +/- 0.07	0.76 +/- 0.09	0.65 +/- 0.06	0.62 +/- 0.07	0.67 +/- 0.05	0.64 +/- 0.04	0.62 +/- 0.04	0.73 +/- 0.04	0.0015	NS	0.014	NS	NS	0.0358
Folate, nM	102.73 +/- 23.10	92.56 +/- 4.37	97.64 +/- 10.76	57.87 +/- 12.72	54.16 +/- 4.12	56.01 +/- 6.04	12.72 +/- 6.49	17.38 +/- 8.39	15.05 +/- 4.86	18.02 +/- 0.41	9.28 +/- 3.73	13.65 +/- 2.58	56.35 +/- 13.66	34.83 +/- 7.11	NS	<0.0001	0.0097	NS	NS	0.0143

Supplemental Table 4. Multi-factor ANOVA results for selected metabolites a) Hexadecanoic acid, b) 1,5-Anhydroglucitol, c) 3-Hydroxybutyric acid and d) 2-Picolinic acid taking into account the factors genotype (WT or *Shmt1*$^{-/-}$), diet (folic acid supplementation or no supplementation) or AsIII treatment (25 ppm AsIII or no AsIII) and each possible combination, n=10 dams per group.

a)

Hexadecanoic Acid	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Genotype	1	51.7	51.7	7.56	0.00752	**
Diet	1	9.9	9.9	1.453	0.23187	
AsIII	1	46.8	46.8	6.844	1.08E-002	*
Genotype:Diet	1	0.1	0.1	0.018	0.89436	
Genotype:AsIII	1	2.2	2.2	0.321	0.57283	
Diet:AsIII	1	11.7	11.7	1.704	0.19593	
Residuals	73	499.6	6.84			

b)

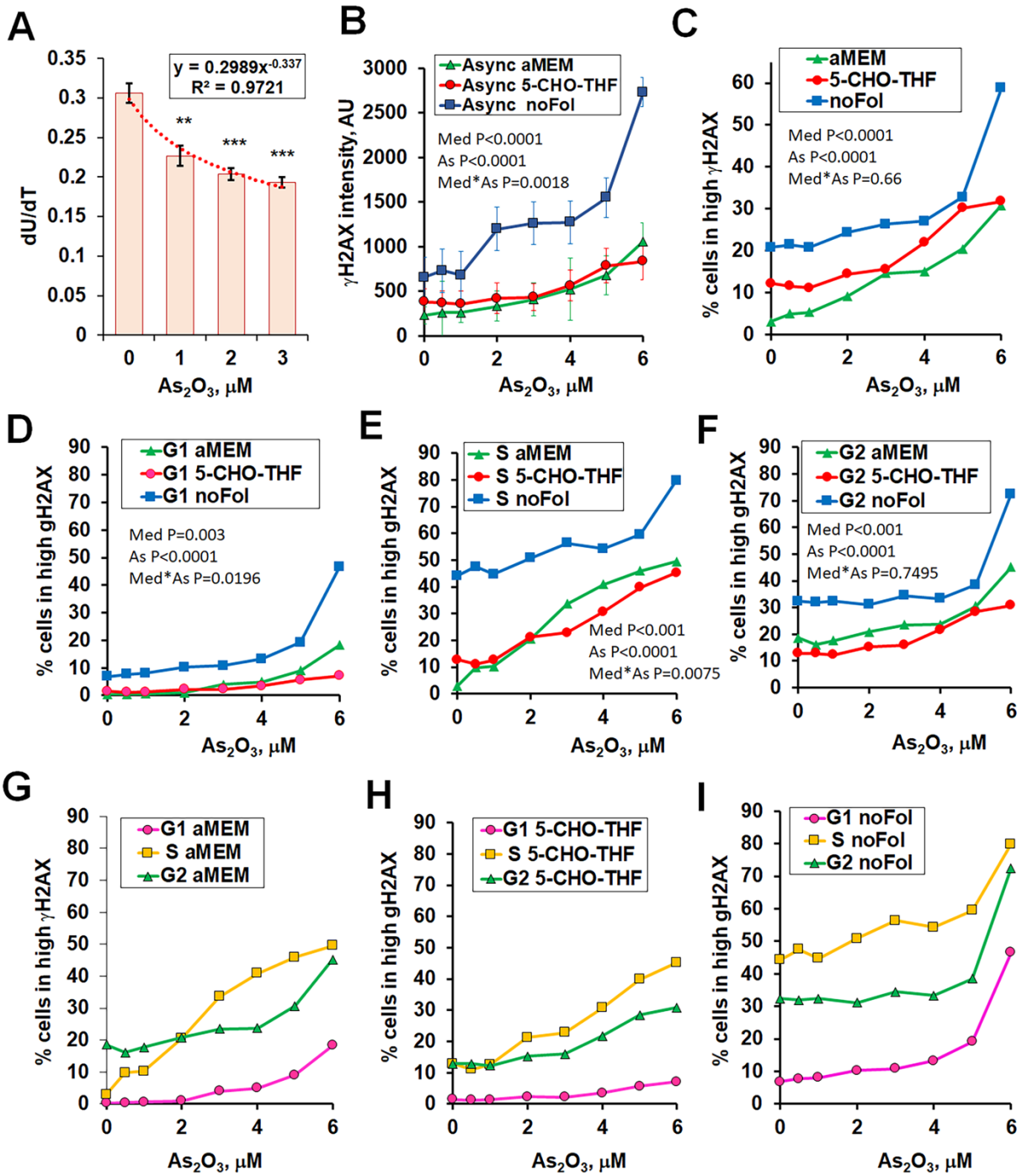
1,5-Anhydroglucitol	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Genotype	1	0.94	0.94	4.467	0.037979	*
Diet	1	0.014	0.014	0.065	0.798741	
AsIII	1	3.999	3.999	19.011	4.19E-005	***
Genotype:Diet	1	0.05	0.05	0.236	0.628655	
Genotype:AsIII	1	0.624	0.624	2.967	0.089206	
Diet:AsIII	1	3.201	3.201	15.218	0.000211	***
Residuals	73	15.355	0.21			

c)

3-Hydroxybutyric Acid	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Genotype	1	8.47	8.47	2.092	0.1524	
Diet	1	20.81	20.81	5.14	0.0263	*
AsIII	1	19.33	19.33	4.776	3.21E-002	*
Genotype:Diet	1	17.75	17.75	4.383	0.0398	*
Genotype:AsIII	1	1.82	1.82	0.449	0.5049	
Diet:AsIII	1	16.28	16.28	4.021	0.0487	*
Residuals	73	295.55	4.049			

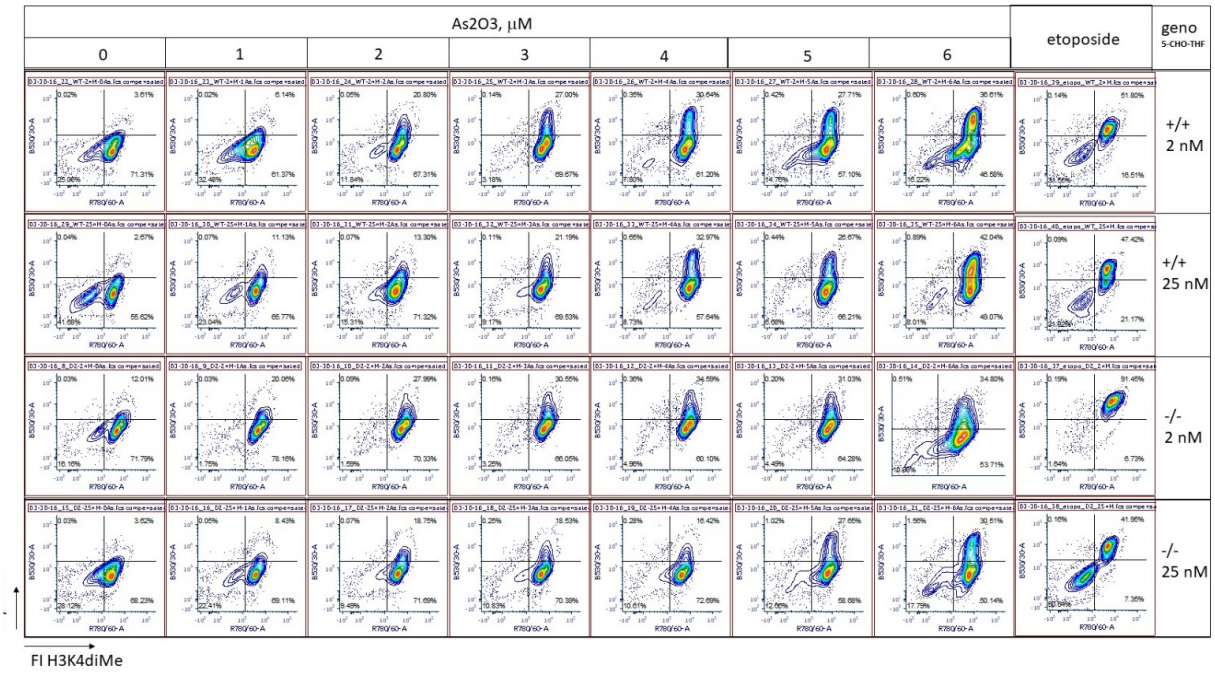
d)

2-Picolinic Acid	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Genotyp	1	8.456	8.456	141.643	< 2e-16	***
Diet	1	1.878	1.878	31.457	3.44E-007	***
AsIII	1	3.027	3.027	50.704	6.22E-010	***
Genotype:Diet	1	0.004	0.004	0.068	0.79571	
Genotype:AsIII	1	1.358	1.358	22.741	9.23E-006	***
Diet:AsIII	1	0.955	0.955	15.995	0.00015	***
Residuals	73	4.358	0.06			



Supplemental Figure 1. Arsenic exposure inhibits thymidylate biosynthesis and induces H2AX histone phosphorylation (gH2AX) in dose-dependent manner in *Shmt1*$^{-/-}$ MEFs. A. The contribution of the de novo and salvage pathways to dTMP synthesis was performed by exposing *Shmt1*$^{-/-}$ MEFs to [14C]-deoxyuridine and [3H]-thymidine. The ratio of (14C-dU)/(3H-dT) DPM in nuclear DNA is a measure of relative capacity of de novo synthesis and salvage pathway dTMP synthesis. [14C]-deoxyuridine is incorporated into DNA through the conversion to dTMP by TYMS in a folate-dependent manner, whereas [3H]-thymidine is incorporated into DNA through the salvage pathway after its phosphorylation by TK1. *Shmt1*$^{-/-}$ MEFs were plated in triplicate in six-well

plates and allowed to grow for three doublings in DMEM supplemented with 200 µM methionine, 20 nM Leucovorin, 500 nM [3H]-thymidine (Perkin-Elmer), and 10 µM [14C]-deoxyuridine (Moravek). Genomic DNA was isolated using Roche kit per manufacturer' instructions, and dU/dT ratio measured by scintillation counting (Beckman). **B-H**. *Shmt1*$^{-/-}$ MEFs were isolated, cultured in aMEM, Fol, or noFol media (modMEM lacking folic acid and ribo- and deoxyribonucleosides with (Fol) or without (noFol) supplementation with 25 nM (*6S*)-5-formyltetrahydrofolate) for 10 days, treated with indicated concentrations of As_2O_3 for 24 h prior to cell collection and analyzed by flow cytometry, where DNA stain allowed for stratification of cells by phases of cell cycle. **B**. Effect of media folate on mean per-cell gH2AX fluorescence intensity +/- CV in response to As_2O_3 for cells in all stages of cell cycle (asynchronous cells). **C**. Effect of media folate on % cells in high gH2AX in response to As_2O_3 for cells in all stages of cell cycle (asynchronous cells). **D.** Effect of media folate on % cells in high gH2AX in response to As_2O_3 for cells in G1 phase of cell cycle. **E.** Effect of media folate on % cells in high gH2AX in response to As_2O_3 for cells in S phase. **F.** Effect of media folate on % cells in high gH2AX in response to As_2O_3 for cells in G2. **G**. Effect of cell cycle stage on % events of high gH2AX in response to As_2O_3 for cells grown in aMEM media. **H.** Effect of cell cycle stage on % events of high gH2AX in response to As_2O_3 for cells grown in 5-CHO-THF-supplemented media. **I**. Effect of cell cycle stage on % events of high gH2AX in response to As_2O_3 for cells grown in folate-depleted media.



Supplemental figure 2. Density plot for gH2AX and H3K4me2 landscape in response to arsenic and etoposide. *Shmt1*$^{+/+}$ and *Shmt1*$^{-/-}$ MEFs were isolated, cultured modMEM lacking folic acid and ribo- and

deoxyribonucleosides and supplemented with 2 or 25 nM (*6S*)-5-formyltetrahydrofolate) for 10 days, treated with indicated concentrations of As_2O_3 for 24 h or with 50 mM etoposide for 30 min prior to cell collection and analyzed by flow cytometry. Single cells are plotted (FSC express software) as a function of their gH2AX and H2K4me2 signal (Y-axis: gH2AX fluorescence intensity, X axis: H2K4me2 fluorescence intensity). Upper right quadrant of each plot represents the % of cells that display simultaneously high gH2AX and high H2K4me2 signal.

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CHAPTER 4: A novel reduced folate, 5-Formyl, 10-FormylTHF, is just as effective as folic acid in reducing NTDs in *Shmt1*$^{-/-}$ mice exposed to arsenic trioxide

Manuscript in preparation with additional material that will be added for publication

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Abstract:

Neural tube defects are birth defects that occur early in development when the neural tube fails to close. Supplementation with folic acid before and during pregnancy can prevent up to 70% of NTDs. It has been suggested that folic acid may be able to rescue NTDs caused by arsenic toxicity. However, our recent mouse study found that folic acid was not able to rescue arsenic-induced NTDs. We hypothesize that a novel stable reduced folate, which doesn't rely on dihydrofolate reductase for its bioactivation, may be able to rescue arsenic-induced NTDs. The purpose of this study was to determine the efficacy of a novel stable reduced folate, 5-formyl, 10-formylTHF (DIFF), as compared to folic acid in its ability to prevent arsenic-induced NTDs and adverse pregnancy outcomes. *Shmt1*$^{-/-}$ mice were used as a model for low penetrance folic acid responsive NTDs. The female mice were weaned onto one of three diets: DIFF, folic acid, or folate deficient diet. After 8 weeks on diet the mice were placed on 25 ppm As_2O_3 in their drinking water for one week. The females were then hand bred and pregnant females were

dissected at E12.5 to analyze embryos for NTDs. Arsenic was shown to dramatically lower levels of dihydrofolate reductase in mouse embryonic fibroblasts (MEFs). However, DIFF was just as efficient at reducing arsenic induced NTDs as folic acid. Likewise, embryo-crown rump length, number of viable embryos, and number of resorptions were rescued from both folic acid and DIFF diets compared to the folate deficient diet. Dam's plasma from the DIFF diet group had folate levels that were not statistically different from the folic acid diet group. This novel reduced folate is not superior to folic acid in rescuing arsenic induced NTDs. However, it is just as efficient as folic acid in terms of protecting litter health from arsenic exposure. The added benefit of DIFF as a supplement is that it is more rapidly metabolized and less likely to accumulate in the blood like folic acid, mitigating the current folic acid concerns.

Introduction:

Arsenic is a naturally occurring element and environmental toxin that can be found in air, water, soil, and food [1]. While the WHO safety cutoff for arsenic in the drinking water is 0.01 ppm, millions are at risk for chronic arsenic exposure with drinking water levels exceeding 1.6 ppm [2-4]. Chronic arsenic exposure can cause a multitude of pathologies such as cancer, peripheral neuropathy, and birth defects [2, 4]. Various animal models have demonstrated arsenic is a teratogen and can cause neural tube defects (NTDs) [5-7]. NTDs are congenital birth defects that occur early in development and are often paralyzing or fatal. Arsenic is metabolized by a series of chemical and/or enzymatic redox reactions that depend on the availability of S-adenosyl methionine (AdoMet) [8, 9]. AdoMet is produced by one-carbon folate metabolism, a pathway that is also well known for its association to risk of NTDs.

Previous studies have analyzed the interactions between folate metabolism and arsenic exposure [10-12]. Recently, our lab discovered that low doses of arsenic trioxide target and degrade two important enzymes in folate metabolism [13]. It was hypothesized that arsenic's mechanism of toxicity was through impairment of the one-carbon folate metabolism pathway. A mouse study was performed to determine if folic acid supplementation was sufficient to rescue arsenic-induced NTDs in a genetically susceptible mouse model of impaired folate metabolism. Although dams fed folic acid while on arsenic-contaminated water exhibited a lower percentage of NTDs, the rescue effect was not significant when compared with diet alone rescue effects (Kamynina *et al.* submitted manuscript).

Folic acid is the synthetic stable form of folates that are found naturally in food sources. Folic acid is more stable and bioavailable than food folates and can be found in both dietary supplements as well as fortified grain products. Although folic acid fortification has been established in the US since 1998, there has been more recent concern about unmetabolized folic acid [14, 15]. The specific concern is not about excess folates in general, but specifically unmetabolized folic acid in the blood. Folic acid requires metabolism by the enzyme dihydrofolate reductase (DHFR) to become tetrahydrofolate (THF), a useable form in the folate pool. Humans have lower levels of this enzyme in their liver as compared to other mammals. Thus, genetic differences as well as high or repeated doses of folic acid may increase levels of unmetabolized folic acid in the blood. Various studies have found unmetabolized folic acid in serum after consuming supplements or fortified foods demonstrating a dose dependency and accumulative effect of repeated consumption [16-18].

In this study we found that arsenic trioxide targets DHFR for degradation. Since this enzyme is essential to reduce folic acid into the available folate form, we hypothesized that folic

acid is not able to rescue arsenic-induced NTDs because the reduction in DHFR impairs folic acid reduction and renders it insufficient. Thus, a reduced stable form of folate, 5-formyl, 10-formylTHF (DIFF), would be bioavailable without the need of DHFR for reduction and may be able to rescue arsenic-induced NTDs.

Materials and Methods:

Mouse embryonic fibroblasts and Immunoblotting

Wildtype (*Shmt1*$^{+/+}$) mouse embryonic fibroblasts (MEFs) were cultured in modified minimal essential media (MEM). The modified MEM media comprised alpha-MEM (Hyclone) lacking glycine, serine, methionine, pyridoxine, folate, and all nucleosides/nucleotides. The media was supplemented with 10% (vol/vol) dialyzed FBS, 200 μM methionine, 2.5 g/L sodium bicarbonate, and 1 mg/L pyridoxine. To the media either 0.2 or 2 μM of folic acid or 2 or 20 nM of leucovorin (most stable reduced folate) was added. The MEFs were treated with 3 μM As_2O_3 for 48 hours. The cells were lysed with (0.15 M NaCl, 5 mM EDTA pH 8, 1% Trition X100, 10 mM Tris-Cl, 5 mM DTT, and a dilution of 1:100 Sigma PI Cocktail 8340) and sonicated. Immunoblotting was performed as in [13].

Study Mice

The Institutional Animal Care and Use Committee (IACUC) at Cornell University, Ithaca, NY, approved all the procedures and experiments in this study using animals according to the guidelines in the Animal Welfare Act and all state and federal laws. All mice were maintained on a 12h light/dark cycle in a temperature-controlled room. The *Shmt1*$^{-/-}$ mice were produced as previously described in [19]. *Shmt*$^{+/-}$ mice were maintained from wildtype C57/B6 females

mated with heterozygous males. *Shmt1*$^{-/-}$ study breeders were collected from heterozygous crosses. Females from the *Shmt1*$^{-/-}$ study breeders were weaned onto one of three diets: AIN-93G folate deficient (no folate diet), AIN-93G with 2 mg/kg folic acid (folic acid diet), or AIN-93G with 2 mg/kg 5-formyl, 10-formyltetrahydrofolate (reduced folate diet named DIFF for diformylfolate). The females remained on diet for at least 8 weeks before being placed on 25 ppm As_2O_3 administered *ad libitum* in the drinking water. After one week exposure to the arsenic water, females were hand-mated with *Shmt1*$^{-/-}$ males. Vaginal plugs were identified in the morning and when found were considered to be embryonic day E0.5. Pregnant females were euthanized by cervical dislocation and blood was collected from cardiac puncture at E12.5. The uteri were removed and resorptions sites were recorded. The embryos were analyzed for NTDs, crown-rump length, and viability (based on observation of beating heart and color of the embryo).

Plasma Metabolites

Blood from cardiac puncture was collected in EDTA tubes and then centrifuged to separate plasma from red blood cells. The samples were immediately snap frozen in liquid nitrogen and then stored in -80°C. Stable isotope dilution capillary gas chromatography-mass spectrometry was used to measure total plasma homocysteine, cystathionine, total cysteine, methionine, serine, glycine, and α-aminobutyric acid as previously described in [20], n=15 per diet group. Plasma folate was quantified with the *Lactobacillus casei* assay described in [21], n=4-6 per diet group.

Statistics

The comparisons of proportions of NTDs were calculated using the "N-1" Chi-squared test. All others were analyzed with Tukey's Honest Significant Difference test.

Results:

DHFR protein levels are reduced with arsenic exposure

Western blot of DHFR in *Shmt1*$^{-/-}$ MEFs exposed to 3 μM As_2O_3 for 48 hours was performed. The MEFs were cultured in media with different concentrations and/or different folates (folic acid or leucovorin, the most stable reduced folate). All samples that were exposed to arsenic showed a greater than 85% reduction in DHFR protein expression (**Figure 4.1**).

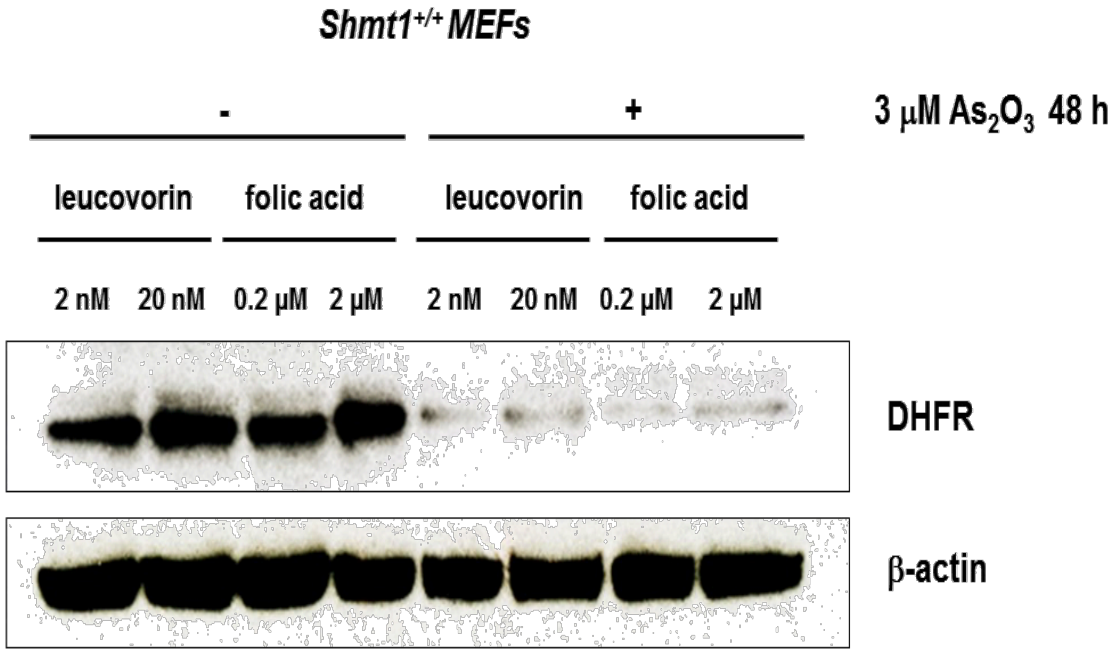

Figure 4.1: Arsenic trioxides causes protein loss of DHFR

Mouse embryonic fibroblasts of *Shmt1*$^{+/+}$ genotype were cultured either in 0.2 μM or 2 μM folic acid or in 2 nM or 20 nM leucovorin, the most stable reduced folate. The cells were treated with arsenic trioxide (As_2O_3) for 48 hr. A western blot of DHFR from the MEFs was performed with β-actin as a loading control.

Dam plasma metabolite levels are similar for DIFF and folic acid diets.

Plasma metabolites for 15 samples in each diet group were sent for analysis. Of the 7 metabolites measured, three did not show a significant difference between diet groups, cysteine, alpha-aminobutyric acid, and glycine (**Table 4.1**). Homocysteine, cystathionine, methionine, and serine were all significantly higher after in the no folate group than both the folic acid and the DIFF groups. There were no significant differences between the folic acid and the DIFF groups. A subset of dam plasma was also measured using the *Lactobacillus casei* assay. Dams consuming the DIFF diet had an average plasma folate level of 120.32 ± 4.75 fmol/μL (n=6, Table 1). The folic acid diet group was slightly higher with an average of 144.54 ± 7.06 fmol/μL (n=4). These two diets are not significantly different. However, they are both significantly higher than the folate deficient diet (76.39 ± 8.00 fmol/μL) with p-values less than 0.005 (n=6).

Table 4.1: Plasma metabolites of *Shmt1*$^{-/-}$ dams as a function of diet

Metabolite	DIFF	Folic Acid	No Folate	p value
Homocysteine (μM)	10.43 ± 0.58^{a}	9.37 ± 0.55^{a}	40 ± 9.73^{b}	<0.005
Cystathionine (nM)	945.9 ± 69.7^{a}	942.1 ± 68.4^{a}	2736.5 ± 651.6^{b}	<0.05
Cysteine (μM)	174.6 ± 4.3	176.8 ±5.3	164.9 ± 7.2	NS
Methionine (μM)	62.04 ± 2.18^{a}	60.95 ± 2.35^{a}	76.06 ± 4.24^{b}	<0.05
α-Ambinobutyric acid (μM)	9.8 ± 0.79	8.19 ± 0.7	8.98 ± 0.44	NS
Gycine (μM)	157.6 ± 15.9	149.6 ± 11.8	258.2 ± 48.3	NS
Serine (μM)	163.9 ± 9.7^{a}	167.1 ± 5.5^{a}	257.1 ± 11.8^{b}	<0.001
Folate (fmol/μL)	120.32 ± 4.75^{a}	144.54 ± 7.06^{a}	76.39 ± 8.00^{b}	<0.005

All plasma metabolites excluding folate were measured by stable isotope dilution capillary gas chromatography-mass spectrometry. All diet groups had n= 15 plasma samples from different dams. Folate was measured using the *L. casei* assay with n=6 samples in each diet group except folic acid group had n=4. The values are listed as the mean ± standard error of the mean. Different letters from the same line are significantly different as described by the row p-value.

DIFF is just as efficient as folic acid in rescuing NTDs

The folic acid and the DIFF diet groups were not significantly different from each other with 8.33% and 7.14% of viable embryos with NTDs respectively (all exencephaly, **Figure 4.2A**). However, the folate deficient diet group was substantially higher with 52.94% viable embryos with NTDS (all exencephaly) ($p< 0.005$). The percentage of the litter affected per viable embryos was analyzed and the no folate group had a substantially higher percentage of the litter with NTDs compared to both the folic acid and DIFF groups (35.49 ± 10.17 %, $p<0.0001$, Figure 4.2B). There was no difference in NTD litter severity between the folic acid and DIFF groups (2.12 ± 1.18 %, 1.53 ± 0.74 %, respectively).

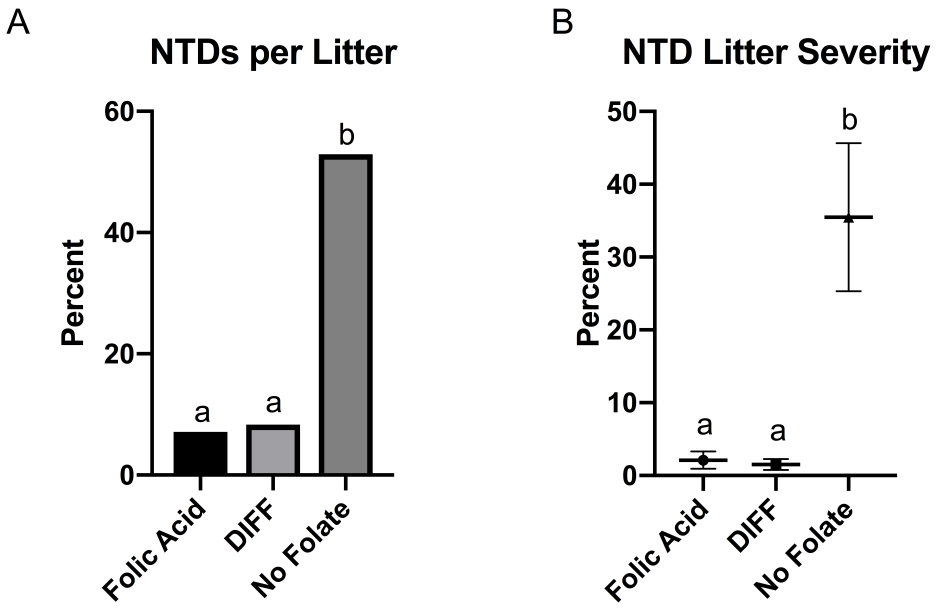

Figure 4.2: DIFF and folic acid reduce NTD risk

Embryos were analyzed for NTDs. Exencephaly was the only NTD type observed. **A.**The percent represents affected litters over the total of litters analyzed. The comparisons of proportions of NTDs were calculated using the "N-1" Chi-squared test. Different letters show significance with ab<0.005. **B.** The percent of the litter affected with NTDs. Tukey's HSD test was used. Graph displays mean and SEM. Different letters show significance with ab<0.0001.

Embryo growth is increased in groups where dams are fed DIFF or folic acid.

Embryo crown-rump length was measured and averaged for each litter dissected at E12.5. Consistent with previous studies, dams fed the folic acid diet had embryos with significantly increased growth as compared to the no folate diet (8.21 ± 0.16 mm vs 6.48 ± 0.34 mm, $p<0.001$, **Figure 4.3A**) The DIFF diet group also had significantly higher growth (7.77 ± 0.14 mm, $p<0.001$). Again, there was no significant growth difference between the DIFF and the folic acid groups.

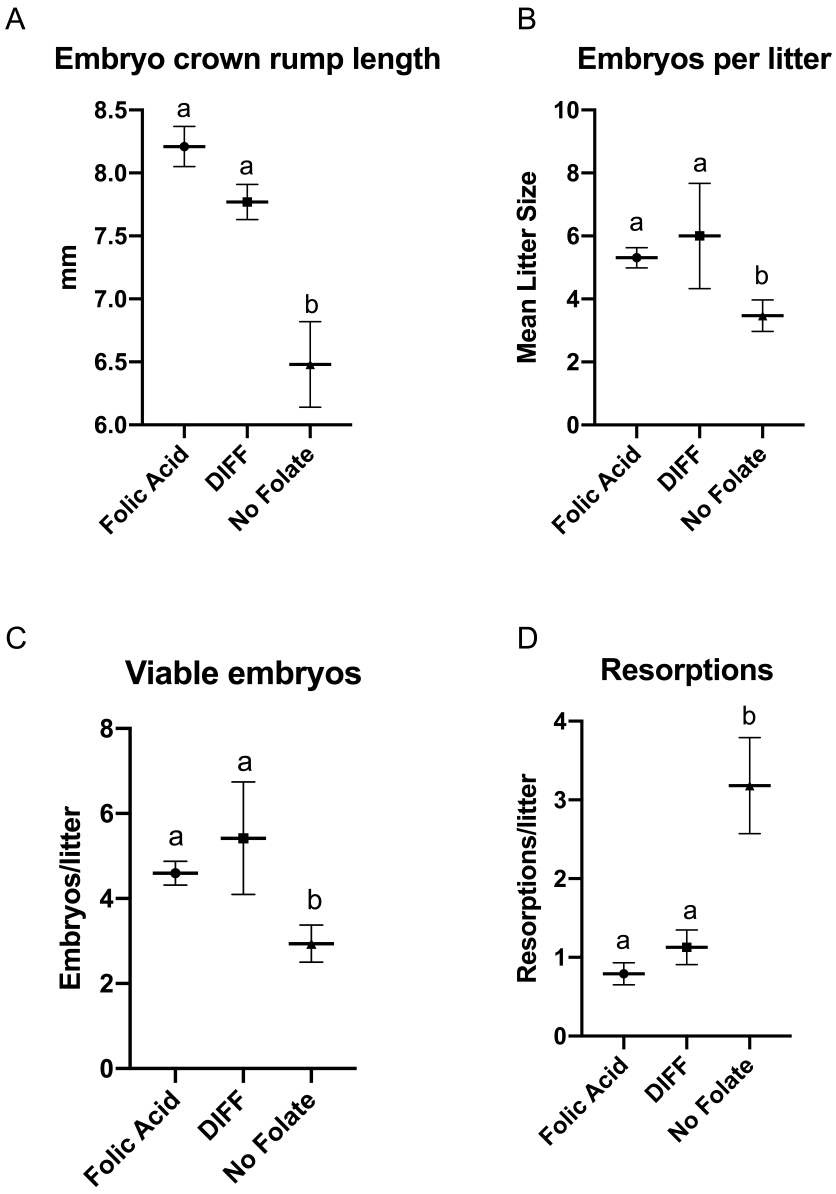

Figure 4.3: Folate deficient diet causes adverse reproductive outcomes that are rescued by both DIFF and folic acid diets

All used Tukey's HSD test and graphs display mean and SEM. Different letters per graph demonstrate significance **A.** Embryo crown-rump length**.** Only viable E12.5 embryos were analyzed. ab<0.001 **B.** Average number of embryos per litter ab<0.005 **C.** Average viable embryos per litter. Discolored embryos with no observable heartbeat were considered dead ab<0.005 **D.** Average number of resorptions per litter ab<0.0001

Dams fed the folic acid diet or the DIFF diet had significantly less adverse reproduction outcomes than the no folate diet group

The average litter size was significantly larger ($p<0.005$) in both the folic acid and DIFF groups compared to the no folate group (6 ± 1.67, 5.31 ± 0.32, vs. 3.47 ± 0.5, respectively) (Figure 4.3B). Similar to the litter size, the no folate diet group had significantly less ($p<0.005$) viable embryos per litter than both the folic acid and DIFF groups (Figure 4.3C). Both the average number of total embryos as well as viable embryos per litter were not significantly differ between the DIFF and folic acid diet groups. They were both significantly higher than the no folate group ($p< 0.01$). The average number of resorptions per litter was also significantly higher for the folate deficient group (3.18 ± 0.61) compared to both the DIFF and folic acid groups (0.79 ± 0.14 and 1.13 ± 0.22 respectively, $p< 0.001$, Figure 4.3D).

The Dam's pregnancy rate or weight gain during pregnancy was not altered by diet

Dams were bred with males until the presence of a copulatory plug and then removed to check for pregnancy. If the dam did not become pregnant, she was bred again. The pregnancy rate is the average percentage of times the dam needed to be bred for a successful pregnancy. All three diet groups had similar percentages of successful breeding at about 70% corresponding to about 1-2 times bred (**Figure 4.4A**). The dams were also weighed at the time of plug observation and then again at the day of dissection. The dam's weight change during pregnancy was not significantly different between the diet groups (Figure 4.4B). However, the dam's weight gain per embryo in the litter did show significance in that the no folate group had more weight gain per embryo than both the DIFF and folic acid groups ($p<0.0005$ Figure 4.4C). This result is

likely due to the no folate group having significantly less embryos per litter than the other two diet groups.

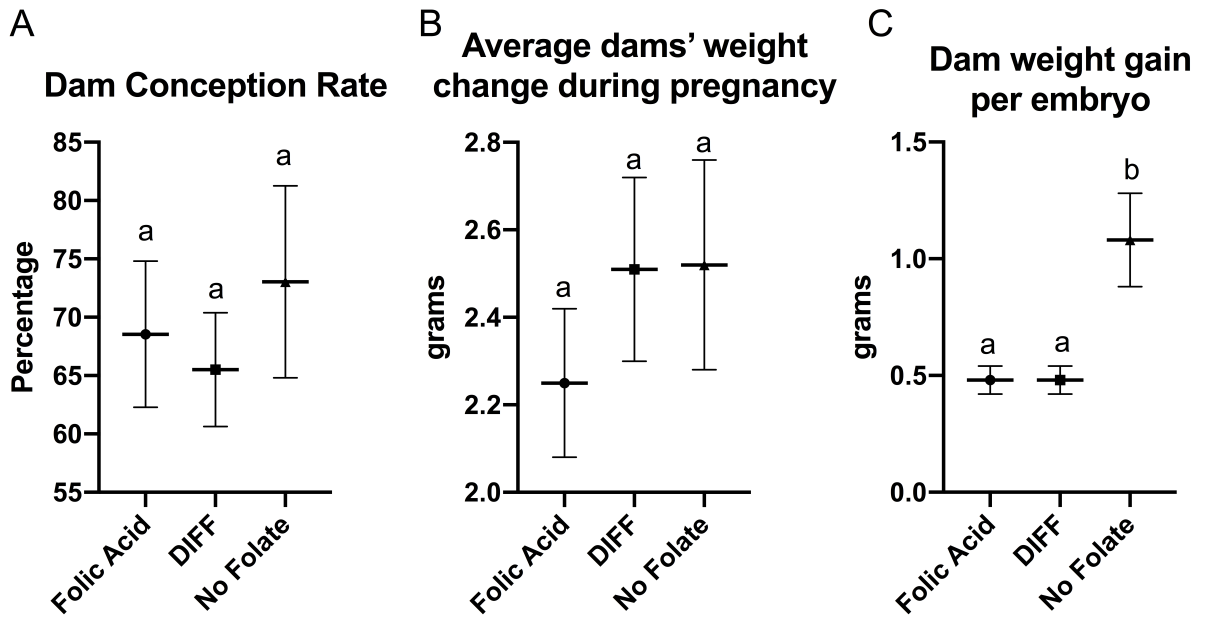

Figure 4.4 Dam conception rate and weight change are not affected by diet

All used Tukey's HSD test and graphs display mean and SEM. Different letters per graph demonstrate significance. **A.** Pregnancy success percentage per dam **B.** Average dam's weight change during pregnancy **C.** Dam weight gain per embryo during pregnancy ab<0.0005)

Discussion:

This study has demonstrated that DIFF has the same beneficial reproductive effects as folic acid supplementation. There was no difference between the DIFF diet and the folic acid diet in plasma folate levels and both were sufficient in rescuing the metabolic changes seen in the plasma of dams on the folate deficient diet. In addition, the DIFF diet was able to rescue NTDs as adequate as folic acid when compared to the folate deficient diet group. Likewise, DIFF and folic acid did not just decrease NTDs per litter, but they also decreased the percentage of the litter exhibiting NTDs. This reduction in severity is another important measure of NTD

penetrance. Furthermore, the DIFF diet also increased embryo and viable embryo counts per litter similar to folic acid as well as rescuing the impaired embryo-crown rump length growth in the folate deficient group. Similarly, DIFF reduced resorptions per litter in the same manner as the folic acid group. Thus, we suggest that this stable novel DIFF may be an adequate replacement to folic acid in terms of preventing adverse reproductive outcomes, including NTDs.

Although the DIFF diet was as effective as folic acid in decreasing the penetrance of NTDs, because it was not significantly better than folic acid, it cannot be considered to rescue arsenic-induced NTDs. However, the benefit of DIFF as a supplement is that it does not require enzymatic activity to become an active form in the folate pool. Thus, its metabolism would be more rapid than that of folic acid and decrease the likelihood of accumulation in the blood, mitigating the current concern of folic acid supplementation [15].

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Phillip Chung assisted with plug checking and dissections. This work was supported by a R01 HD059120 to PJS from the US Public Health Service and F30 HD093288 to ERL. **Conflicts of Interest**: The authors have no conflicts of interest to disclose. **Author contributions:** ERL, MP, and SPS were primarily responsible for data collection. Data analysis and interpretation was conducted by ERL, MP, MSF, and PJS. The manuscript was written by ERL, MSF, and PJS. All authors read and approved the final manuscript.

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CHAPTER 5: Mice deficient in *Shmt2α* do not exhibit neural tube defects even when challenged with a folate deficient diet

Manuscript in preparation with additional material to be added for publication

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Abstract:

Neural tube defects (NTDs) have a high association with maternal folate status. Although there are more than 300 genetic mouse models of NTDs, the *Shmt1*-deficient mouse is the only folate-responsive NTD model resulting from impairment of a folate-dependent enzyme. The goal of this study was to determine if loss of Shmt2α, an isoform of Shmt1, is sufficient to cause NTDs and if so, whether those NTDs are folate-responsive. CRISPR/Cas-mediated genome editing was used to create *Shmt2α*$^{-/-}$ mice. Female mice were weaned onto either a folic acid deficient (-FA) or a folic acid sufficient (+FA) diet for 8 weeks. The mice were time mated and dissected at E12.5. Embryos were examined for NTDs and litter data, plasma, and embryo crown-rump length was collected. *Shmt2α*$^{-/-}$ mice did not exhibit NTDs in either the +FA or the –FA diet groups. There were no significant differences in any of the litter or embryo data. Maternal plasma metabolites

confirmed folate deficiency and no other metabolic alterations. The loss of *Shmt2α* is not sufficient to induce NTDs even when on a folic acid deficient diet. Because only 25% of *de novo* thymidylate biosynthesis relies on SHMT2α in mice nuclei, it is likely insufficiently impaired or compensated by 75% contributed by SHMT1.

Introduction**:**

Worldwide each year, about 300,000 babies are born with neural tube defects (NTDs) [1]. These congenital defects are usually paralyzing or fatal. Maternal folate status has been a well-studied and highly associated risk factor for NTDs. Up to 70% of human NTDs can be prevented with maternal folic acid supplementation before and during pregnancy [2]. There are more than 300 genetic mouse models to study NTDs [3, 4], however, only a limited number have been tested for folic acid responsiveness. In addition, most of the mouse models with folate-dependent enzyme depletion lead to embryonic death or do not develop NTDs. The exception is the reduction of serine hydroxymethyltransferase (*Shmt1*$^{-/-}$ and *Shmt1*$^{+/-}$*)*. This mouse model exhibits folic acid responsive NTDs, with a penetrance of about 13% with folate deficiency [5]. It has been thought that this mouse is viable and fertile because of compensation by its isoform Shmt2α [6].

Shmt1 and Shmt2α enzymes with scaffolding and catalytic functions in the *de novo* thymidylate (dTMP) synthesis pathway of folate one-carbon metabolism (FOCM) [7]. Serine hydroxymethyltransferase activity is a component of the *de novo* dTMP biosynthesis pathway, with Shmt1 and Shmt2α providing 75% and 25% of activity, respectively in mice nuclei [6]. Impaired *de novo* dTMP synthesis, as seen in folate

deficiency, causes accumulation of uracil in the DNA leading to genomic instability [8]. The *Shmt1*$^{-/-}$ and *Shmt1*$^{+/-}$ models suggest that uracil misincorportation and the resultant genome instability is the cause of the folate responsive NTDs. The goal of this study was to create a mouse model with a loss of *Shmt2α* to study its relationship to risk of NTDs. The hypothesis was that NTD penetrance would be lower in *Shmt2α*$^{-/-}$ compared to the *Shmt1*$^{-/-}$ since it contributes less to *de novo* dTMP synthesis. It was also thought that the *Shmt2α*$^{-/-}$ mice would have folic acid responsive NTDs similar to the *Shmt1*$^{-/-}$ and *Shmt1*$^{+/-}$ mice.

Materials and Methods:

Targeted Mutagenesis

The mouse *Shmt2* gene encodes two transcripts through alternative splicing to produce the mitochondrial isozyme (SHMT2) and the nuclear/cytosolic isozyme (SHMT2α) [6]. The mouse *Shmt2* gene is located on mouse chromosome 10 (GenBank: NM_028230.4; Ensembl:ENSMUSG00000025403). The gene has twelve exons with the start codon ATG in exon 1. Exon 2 was selected as the target site to produce M22N (ATG to AAT) mutation in the splice site to selectively impair expression of SHMT2 without affecting SHMT2α expression. CRISPR/Cas-mediated genome engineering was used by Cyagen Biosciences Inc. to create a C57/BL/6J mouse with a M22N mutation in the Shmt2 locus.

Animal Husbandry

The Cornell University Institute Animal Care and Use Committee approved all animal studies following the guidelines in the Animal Use and Welfare Act as well as all state

and federal laws. *Shmt2α*$^{-/-}$ females from *Shmt2α*$^{-/-}$ X *Shmt2α*$^{-/-}$ breeders, were weaned onto either an AIN93G folic acid sufficient diet (+FA) or an AIN93G lacking folic acid (-FA) diet for at least 8 weeks. The females were then hand mated to *Shmt2α*$^{-/-}$ males and copulatory plugs were checked in the morning. If a vaginal plug was found it was counted at embryonic day 0.5 (E0.5). At E12.5 pregnant females were dissected and embryos were examined for NTDs. Data was collected on viability of each embryo, number of resorptions, and embryo crown-rump length. Maternal blood was collected by cardiac stick and immediately moved in EDTA tubes. The blood was centrifuged and plasma was separated from red blood cells. The samples were immediately frozen in liquid nitrogen.

Plasma Metabolite Analysis

A subset of plasma samples was analyzed for metabolite concentrations. Stable isotope dilution capillary gas chromatography-mass spectrometry was used to measure plasma homocysteine, cystathionine, methionine, glycine, serine, dimethylglycine (DMG), methylglycine (MG), and cysteine as describe in [9]. A total of 15 samples from the +FA group and 14 samples from the –FA group were analyzed. Plasma folate concentrations were measured with another subset of samples n=6 per diet group using the *Lactobacillus casei* microbiological assay as described in [10].

Results:

Shmt2α$^{-/-}$ mice do not exhibit NTDs in either the +FA or the –FA diet groups

A total of 38 litters fed the -+FA diet and 32 litters fed the –FA were analyzed (Table 5.1). No embryos from any litter in the study exhibited NTDs. The –FA diet group had smaller embryo crown-rump length (8.45 ± 0.19 mm) compared to the +FA diet group (8.61 ± 0.16 mm), however, the difference was not statistically significant. Other adverse reproductive outcomes such as embryo viability and resorptions were also not significantly different between the diet groups.

Table 5.1: Reproductive data from litters on +FA and -FA

Litter Average			
	+FA	**-FA**	**p-value**
NTDs	0	0	NS
Crown-rump (mm)*	8.61 ± 0.16	8.45 ± 0.19	NS
Resorbed	0.5 ± 0.15	0.5 ± 0.19	NS
Viable embryos	7.05 ± 0.3	6.66 ± 0.43	NS
Total Litters	38	32	

* Only E12.5 litters were assessed (+FA n=32 and –FA n=29). Folic acid supplemented diet group (+FA) and folic acid deplete diet group (-FA). NS= not significant ($p>0.05$). The values are listed as the mean ± standard error of the mean.

Plasma metabolites confirm folate deficiency in the –FA diet group

The plasma levels of total homocysteine and cystathionine were significantly elevated in the –FA group compared to the +FA group ($p<0.001$ and $p<0.005$, respectively, Table 5.2). In accordance, the *L. casei* assay showed the +FA group had plasma folate

concentrations significantly higher than the –FA group (135.3 ± 10.85 vs. 90.51 ± 5.37, $p<0.005$, Figure 5.1). All other plasma metabolites measured were not significantly different between the two diet groups.

Table 5.2: Dam plasma metabolites

Metabolite	+FA	-FA	p value
Homocysteine (μM)	9.63 ± 0.74	31.45 ± 2.7	<0.001
Cystathionine (nM)	1708.13 ± 156.8	2874.43 ± 281.75	<0.005
Cysteine (μM)	175 ±7	164.3 ± 4.3	NS
Methionine (μM)	54.78 ±5.29	48.41 ± 2.39	NS
α-Ambinobutyric acid (μM)	20.87 ± 1.63	19.57 ± 1.85	NS
Glycine (μM)	164.4 ± 18.8	158.9 ± 16	NS
Serine (μM)	160.6 ± 9.3	178.6 ± 6	NS

Folic acid (FA) group n=15 while no folate (-FA) group n=14. NS= not significant ($p>0.05$). The values are listed as the mean ± standard error of the mean.

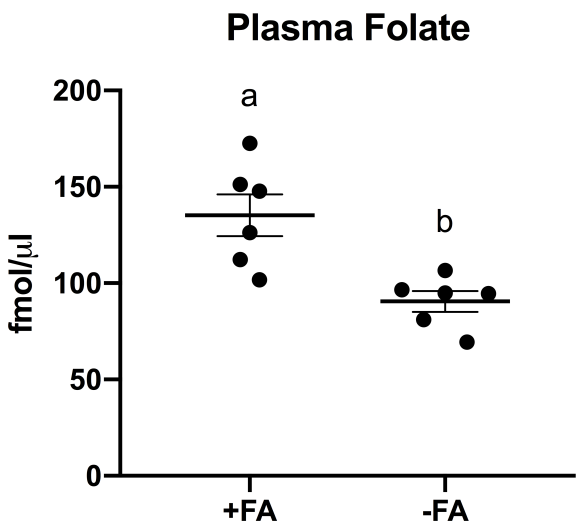

Figure 5.1: Dam plasma folate concentrations

Plasma folate levels were measured using the *L. casei* assay with n=6 per group. Significance between different letters p<0.005). Folic acid supplemented group (+FA) and folate deficient group (-FA)

Discussion:

The results from the study demonstrate that loss of *Shmt2α* alone is not sufficient to induce NTDs, even when these mice are fed a folate-deficient diet. This result is contrary to the loss of its isoform *Shmt1,* where the mice exhibit folic acid responsive NTDs. It was expected that NTD rates would likely be lower for *Shmt2α*$^{-/-}$ mice compared to *Shmt1*$^{-/-}$ mice since only 25% of *de novo* dTMP synthesis uses the Shmt2α enzyme [6]. It is possible with a much larger study. NTDs may be exhibited in the mice. However, due to expenses and large animal numbers, we did not expand the study.

Plasma folate depletion was less than that usually seen with mice on folate deficient diets. There is usually about a 50-70% reduction in plasma folate, however in this study only a 30% reduction was achieved. It is possible these mice are more resistant to folate deficiency and this may be a reason the mice did not exhibit any NTDs. The

metabolic phenotype (excluding folate depletion associations), average adverse outcomes per litter, and embryo crown-rump length were all not statistically significant between the diet groups. The lack of metabolic changes and adverse outcomes per litter (excluding NTDs), are similar findings in the *Shmt1*$^{-/-}$ mouse model. However, it is contrary to the model in that the *Shmt1*$^{-/-}$ mouse model has embryos with significantly reduced embryo crown-rump length. The reduction in embryo-crown rump length is associated with risk of NTDs, as it has been demonstrated in multiple models [11-13].

In conclusion, the *Shmt2α* gene is not a risk factor for NTDs in mice, even when challenged with a folate-deficient diet. It would be of interest to see if loss of both *Shmt1*$^{-/-}$ and *Shmt2α*$^{-/-}$ would increase the penetrance of the *Shmt1*$^{-/-}$ induced NTDs and if those NTDs would remain folic acid responsive.

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Phillip Chung and Katarina Heyden assisted with plug checking and dissections. This work was supported by a R01 HD059120 to PJS from the US Public Health Service and F30 HD093288 to ERL. **Conflicts of Interest**: The authors have no conflicts of interest to disclose. **Author contributions:** ERL and SPS were primarily responsible for data collection. Data analysis and interpretation was conducted by ERL, MSF, and PJS. The manuscript was written by ERL, MSF, and PJS. All authors read and approved the final manuscript.

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CHAPTER 6: Future Directions

Chapter 1

In the literature review, it was revealed that many pathologies leading to NTDs have the same common pathway of DNA damage. In order to investigate this theory further, multiple mouse models should be used representing different etiologies of NTDs (genetic, nutritional, environmental) and embryos should be analyzed for DNA damage. γH2AX is a biomarker for DNA damage and identifies DNA double-strand breaks. Looking specifically at embryos just prior to neural tube closure with immunohistochemistry of the neuroepithelium would elucidate whether DNA damage is a precursor to failure of neural tube closure.

Chapter 2

NTDs caused by loss of p53 are not folic acid-responsive. This is true when analyzing the data by location of the lesion, caudal region (spina bifida) and cranial region (exencephaly). However the percentage of litters with at least one embryo with spina bifida did increase from 0% to 5.7% ($p=0.1$), when comparing the folic acid diet to the no folate diet, respectively. However,it may be an issue of statistical power and sample size. A larger population with the same percentages would show statistical significance. Investigating the role diet can play on NTD location would be a novel and exciting future direction to this study.

Loss of p53 causes an increase in expression of both MTHFD1 and DHFR enzymes. As expected the *p53*$^{-/-}$ MEFs had higher rates of *de novo* thymidylate biosynthesis. However, it was not anticipated that the *p53*$^{-/-}$ MEFs would have higher

measures of uracil in DNA. This phenotype is the same observed in the *Shmt1* model. It begs the question whether these NTDs are caused from increased uracil in DNA. The difference between these two models, is that the *Shmt1* mouse has folate responsive NTDs and the $p53^{-/-}$ mouse does not. Whether uracil is a significant player in NTD occurrence and if so, the specific mechanisms of action warrant further investigation. It would worthwhile to see if a mouse model with only increase in uracil in DNA, without folate nutrient changes, could recapitulate the NTD phenotype.

Another possible cause of the NTDs in the $p53^{-/-}$ mice is over-proliferation. The neural epithelium requires precise apoptosis and growth to form normally. Likewise, during development multiple cell types are dividing and migrating. Altered division, whether increased or decreased, could impair migration, which is essential to neural tube formation and closure. It is well known that p53 is a master regulator of cell cycle and loss of this transcription factor leads to excessive proliferation as seen in many cancers. It would be important to further investigate whether this proliferation trait caused by loss of p53 is the cause of the NTDs observed.

Chapter 3

Arsenic contamination can induce diabetes as been observed in epidemiological, *in vitro*, and *in vivo* data [1-6]. Our results in Chapter 2 demonstrate alterations in glucose metabolism of dams consuming contaminated arsenic trioxide water. Multiple studies have shown that diabetes mellitus is also a significant risk factor for NTDs [7-9]. To date there is only one mouse study that has demonstrated arsenic exposure leads to diabetes-induced NTDs [10]. This study did not show a complete rescue of NTDs after correcting

maternal hyperglycemia. Our study in Chapter 3 showed that folic acid supplementation could decrease arsenic-NTD incidence, but also not 100%. Thus, a combinatorial treatment of correcting maternal hyperglycemia in addition to folic acid supplementation may be a better practice for reducing the NTD penetrance. *In vivo* studies with multiple mouse models would be beneficial to evaluate this hypothesis. Likewise, it would also be valuable to explore whether maternal diabetes and arsenic exposure have an additive or synergistic effect on NTD occurrence. This information would be valuable to mothers who have diabetes and are living in areas with high arsenic contamination.

In Chapter 3 we found that arsenic exposure led to metabolic alterations in diabetes biomarkers in the dams. These changes were modified by both diet and genotype, suggesting a link between arsenic-induced diabetes and folate one carbon metabolism, specifically *de novo* thymidylate biosynthesis. Diabetes is known to cause oxidative stress and induce DNA damage [11, 12]. It has also been shown that folic acid can rescue DNA damage induced by diabetes [13]. It is possible that folic acid is working through the *de novo* thymidylate synthesis pathway to rescue diabetes-induced NTDs and this possible mechanism should be further evaluated.

Chapter 4

In Chapter 4 we observed that the novel reduced folate is just as efficient as folic acid in reducing NTDs, although there is not 100% reduction in arsenic-induced NTDs. Thus, the phenotypic outcomes of folic acid and reduced folate have been determined, but the mechanism of the reduced folate rescue effect has not been analyzed. It has not been determined if it acts through the same mechanism as the rescue effect of folic acid

in the *Shmt1*$^{-/-}$ mice. If the reduced folate acts in the same way as folic acid, it would be able to improve *de novo* thymidylate biosynthesis, decrease uracil in DNA, and rescue genomic instability. Likewise, it should be determined if both folic acid and reduced folate able to rescue DNA damage in the neuroepithelium.

Many limitations to our cellular assays are that MEFs may not be as similar to the neuroepithelium in folate metabolism. It would be beneficial to look at protein expression differences of folate-mediated one-carbon metabolism enzymes in the neuroepithelium compared to MEFs to see if they make a reasonable model with similar expression levels. Likewise, laser capture microdissection of the neuroepithelium and RNAseq would be worthwhile to determine differential expression in embryos with NTDs verses normal neural tube closure.

It is known that uracil accumulation in DNA is tissue specific [14]. Thus, some tissues may be more resistant to high levels of uracil compared to others. It's possible that the neuroepithelium is a cell type that is particularly sensitive to uracil in DNA as well as other types of DNA damage. This may be the reason with slight nutritional, environmental, and genetic alterations the other cells and tissues of the embryo develop normally, while the neuroepithelium is affected due to its sensitivity. It would be valuable to look into DNA repair proteins to see if this pathway may increase sensitivity in the neuroepithelium. Uracil in DNA is removed by a group of enzymes called Uracil DNA Glycosylases (UDG). Does loss of these enzymes cause NTDs in mouse models and would overexpression be able to rescue NTDs in mouse models with increased uracil, such as the *Shmt1*$^{-/-}$ and *p53*$^{-/-}$ models. Both of these models are also prone to cancer and

may be due to their increased uracil accumulation in DNA. This link between uracil, cancer, and NTDs should also be further explored.

Chapter 5

To further investigate the role of *de novo* dTMP synthesis and uracil in DNA as causative factors in NTDs, *Shmt2α*$^{-/-}$ MEFs should be analyzed for rates of *de novo* dTMP synthesis and uracil in DNA. Because NTDs were not observed in *Shmt2α*$^{-/-}$ mice, it would be expected that *de novo* dTMP synthesis would not be significantly altered and there would also not be an increase in uracil in DNA. If these results are found, it would further support the hypothesis that impaired *de novo* dTMP synthesis and uracil in DNA are causative factors in NTD penetrance.

In Chapter 5, the results showed that *Shmt2α*$^{-/-}$ mice do not exhibit NTDs including when additionally confronted with a folate deficient diet. These results were contrary to those of the *Shmt1*$^{-/-}$ and *Shmt1*$^{+/-}$ mice, which exhibit folic acid responsive NTDs. It would be useful to make a *Shmt2α*$^{-/-}$ *Shmt1*$^{-/-}$ mouse model to first see if it is viable and fertile. This result would establish the necessity of SHMT in *de novo* dTMP synthesis for viability. It would also be important to determine if loss of *Shmt2α*$^{-/-}$ would exacerbate the NTD penetrance due to increased impairment of *de novo* dTMP synthesis.

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