DYSREGULATION OF FOLATE-DEPENDENT MITOCHONDRIAL *DE NOVO* THYMIDYLATE BIOSYNTHESIS AFFECTS MITOCHONDRIAL DNA INTEGRITY

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DYSREGULATION OF FOLATE-DEPENDENT MITOCHONDRIAL *DE NOVO* THYMIDYLATE BIOSYNTHESIS AFFECTS MITOCHONDRIAL DNA INTEGRITY

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Mitochondrial DNA (mtDNA) Depletion Syndrome (MDS) is a genetic disorder caused by mutations in nuclear-encoded mitochondrial proteins involved primarily in nucleotide or mtDNA synthesis. Folate-dependent one-carbon metabolism (FOCM) is a metabolic network compartmentalized in mitochondria, nucleus, and cytosol, where one-carbon units participate in the *de novo* synthesis of purines, thymidylate, and methionine metabolism. Either increase or decrease in mitochondrial dTTP pools reduces mtDNA content. However, FOCM has never been investigated as a pathway leading to loss of mtDNA integrity in MDS patients.

Folate deficiency reduces *de novo* dTMP synthesis and increases uracil levels in nuclear DNA (nuDNA). Uracil misincorporation is not mutagenic but affects DNA integrity by inducing chromosome fragmentation and eventually apoptosis. Our knowledge of factors that contribute to uracil accumulation in mtDNA is limited but is expected to mirror uracil misincorporation in nuDNA. Enzymatic disruption of nuclear *de novo* dTMP synthesis due to lack of SHMT in mice and lack of mitochondrial SHMT2 in CHO cells results in reduced dTMP synthesis capacity. Similarly, mice deficient in MPV17, a protein of unknown function associated with hepatocerebral MDS, have reduced mitochondrial dTMP pools. This study investigates the role of genetic (MPV17 and SHMT2) and non-genetic factors (folate, glycine, and serine) on mtDNA integrity, in the context of mitochondrial *de novo* dTMP synthesis.

These results reveal that mtDNA integrity is compromised in HeLa cells deficient in either MPV17 or SHMT2. MPV17-deficient cells exhibited reduced mitochondrial folate levels, suggesting it interacts

with mitochondrial FOCM. Both, cells cultured in folate-depleted medium and MPV17-deficient cells, exhibit elevated uracil accumulation in mtDNA by at least 3-fold. Mitochondrial dTMP synthesis capacity and its incorporation into mtDNA were not affected in MPV17-deficient cells, but the elevated uracil levels imply that availability of dTMP for mtDNA synthesis is compromised. I propose that MPV17 is a mitochondrial dTMP transporter. SHMT2-deficient cells exhibited 164% more mtDNA content than control cells; glycine and serine in the culture medium did not have an effect, indicating that SHMT2 catalytic activity is fundamental for mitochondrial function. These data suggest that FOCM is involved in maintaining mtDNA integrity, which is highly susceptible to uracil misincorporation.

BIOGRAPHICAL SKETCH

Judith Raquel Alonzo De La Nuez was born to Altagracia del Carmen De La Nuez Burgos and José Ramón Alonzo on February 24th, 1989 in Santiago, the Dominican Republic. At the age of 14, the author moved to Boston, Massachusetts, where she graduated from high school in 2005. The author enrolled at the University of Massachusetts-Boston (UMB), where she met her now husband, Francis Isabel. At UMB, Judith worked in an organic chemistry laboratory in the synthesis of biologically active heterocyclic and organofluorine compounds. She also completed a summer internship with the Dana-Farber Harvard Cancer Center's Continuing Umbrella of Research Experiences program at a biology lab at the Beth Israel Deaconess Medical Center. This summer internship led to a staff position as a research assistant at Harvard Institutes of Medicine, where she worked in a biochemistry/immunology lab until 2010.

After graduating with honors from UMB with an undergraduate degree in Biochemistry, Judith worked as a research associate at a biotechnology company in Natick, Massachusetts. Her love for science and research in the biomedical area motivated her to further her education by pursuing a Ph.D. The author joined the graduate field of Biochemistry, Molecular, and Cell Biology at Cornell University, where she conducted graduate research under the mentorship of her advisor Prof. Patrick J. Stover, Ph.D. At the Stover lab, Judith worked on folate metabolism, adopting a greater appreciation for understanding gene and diet interaction. Judith aims to continue making contributions to the biological sciences through research as well contributing to the community by mentoring younger students.

I would like to dedicate this work to my mother and my husband.

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LIST OF ABBREVIATIONS

1C	One-carbon
AHS	Alpers-Huttenlocher Syndrome
CSF	Cerebrospinal Fluid
DGUOK	deoxyguanosine kinase
DHFR	Dihydrofolate reductase
dNTP	2'-deoxyribonucleoside-5'-triphosphates
dT	thymidine
dTMP	Thymidylate
dU	Deoxyuridine
dUMP	Deoxyuridylate
FNT	Formate/Nitrite Transporter
FOCM	Folate-mediated one-carbon metabolism
FPGS	Folylpoly-γ-glutamate synthetase
GCMS	Gas chromatography-mass spectrometry
HPLC	High-performance liquid chromatography
KD	Knockdown
MDS	Mitochondrial DNA Depletion Syndrome
MFT	Mitochondrial Folate Transporter
MPV17	mitochondrial inner membrane protein MPV17).
mtDNA	Mitochondrial DNA
MUG	mismatch specific uracil-DNA glycosylase
nuDNA	Nuclear DNA
PEO1	mitochondrial DNA helicase twinkle
POLG	mitochondrial DNA polymerase gamma
RRM2B	p53 inducible ribonucleotide reductase subunit M2 B),
SAMC	S-adenosylmethionine mitochondrial carrier
SHMT	Serine hydroxymethyltransferase
SMUG1	single-stranded monofunctional uracil-DNA glycosylase
SUCLA2	succinyl-CoA ligase ADP binding beta subunit
SUCLG1	succinate-CoA ligase GDP binding alpha subunit
TDG	thymine-DNA glycosylase
THF	Tetrahydrofolate
TK	Thymidine Kinase
TYMS	Thymidylate synthase
UNG	Uracil-DNA glycosylases

CHAPTER 1- Introduction

Folate-dependent One-Carbon Metabolism and Mitochondrial DNA Integrity

Many genetically heterogeneous diseases associated with impaired mitochondrial function are associated with the loss of mitochondrial DNA (mtDNA) integrity. Loss of mtDNA integrity is defined as deletions or duplications in the mitochondrial genome, changes in mtDNA copy number, or mutations present either in the mitochondrial genome or nuclear-encoded genes involved in mtDNA replication, synthesis, or repair. In addition to genetic factors, mtDNA integrity is also affected by environmental and nutritional factors. Identification of genetic and non-genetic factors that affect mtDNA integrity, and their interactions, may lead to a better understanding of mitochondrial physiology and more effective therapies for diseases caused by mitochondrial dysfunction. This review explores the relationship between folate-dependent one carbon (FOCM) metabolism and mtDNA integrity in the context of mitochondria-related pathologies in mammals.

Background on mtDNA

Nuclear DNA (nuDNA) replication is cell cycle-dependent whereas replication of the mitochondrial genome occurs continuously throughout the cell cycle. In humans, there are 10^3-10^4 circular, double-stranded mitochondrial DNA (mtDNA) copies per cell (1). These may be identical mtDNA copies (homoplasmy) or a mixture of inherited and mutant mtDNA (heteroplasmy). Low levels of heteroplasmy are present in most individuals and are not associated with clinical phenotypes. If accumulation and expansion of mutant mtDNA exceed a certain threshold (threshold may vary depending on the given tissue) through multiple rounds of mtDNA replication, a clinical phenotype may develop over time (2-4).

Loss-of-function mutations in genes involved in mtDNA synthesis have been associated with mtDNA depletion syndrome (MDS). MDS are autosomal recessive disorders that result in tissue-specific reductions in mtDNA copy number. These include *POLG*, which encodes for mitochondrial DNA polymerase gamma, and *PEO1,* which encodes for mitochondrial DNA helicase twinkle. Other genes associated with MDS are: succinyl-CoA ligase ADP binding beta subunit (*SUCLA2*), succinate-CoA ligase GDP binding alpha subunit (*SUCLG1*), deoxyguanosine kinase (*DGUOK*), thymidine kinase 2 (*TK2*), thymidine phosphorylase (*TYMP*), p53 inducible ribonucleotide reductase subunit M2 B (*RRM2B*), and mitochondrial inner membrane protein Mpv17 (MPV17). Mpv17 is the only protein from the list with unknown function; all other proteins are involved in mtDNA integrity by participating in nucleotide synthesis, mtDNA synthesis and repair (5).

Maintaining an adequate and balanced nucleotide supply is vital to preserve DNA integrity for both the nuclear and mitochondrial genome. Regulation of 2'-deoxyribonucleoside-5'-triphosphates (dNTP) concentrations within the mitochondrial matrix is essential because dNTPs are determinants of DNA replication fidelity (6,7). In non-replicating cells, mtDNA synthesis depends primarily on mitochondrial salvage pathways enzymes, known as deoxyribonucleoside kinases, for mitochondrial dNTPs synthesis. For dividing cells, in addition to salvage nucleotide pathways, mtDNA synthesis also relies on *de novo* nucleotide synthesis to sustain the continuous demand for nuDNA and mtDNA synthesis. The *de novo* and salvage pathways for nucleotide synthesis are active in the cytosol, the nucleus for dTMP synthesis during S-phase, (8) and mitochondria for dTMP synthesis (9).

Deoxynucleoside/deoxynucleotide transporters in the mitochondrial membrane allow for the transport of nucleotides from the cytosol into the mitochondria. Concentrations of individual

purine and prymidine nucleotides are asymmetrical and tend to vary across tissues (7,10,11). A reduction of dNTP pools, or concentration imbalances across any of the four individual DNA nucleotides, may affect mtDNA replication rate and thus its integrity. For example, excess of dTTP depletes dCTP pools, which can become a limiting factor in mtDNA synthesis and thus delay mtDNA replication. However, excess of dATP, dGTP, or dCTP do not reduce mtDNA replication rate (12).

Both excess and depletion of dTTP pools result in mtDNA depletion, suggesting that dTTP synthesis may be highly regulated to maintain mtDNA integrity. There are intrinsic characteristics to dTTP that distinguish it from other DNA bases. Some unique characteristics of dTMP are: 1) it is the only DNA nucleotide for which *de novo* synthesis is known to take place in cellular compartments other than the cytosol (9,13-15), 2) it is synthesized *de novo* on demand at sites of DNA replication and repair within the nucleus (15), and 3) it is not essential for DNA replication as perturbed dTMP synthesis results in the accumulation of its precursor, deoxyuridine monophosphate (dUMP), which can be incorporated into DNA. Misincorporation of dUTP into DNA and its excision repair can compromise DNA integrity (Figure 2) (16).

FOCM and dTMP synthesis

In mammals, the *de novo* synthesis of dTMP occurs within the nucleus as well as the mitochondria to fulfill the demand for dTTP for nuDNA and mtDNA replication and repair, respectively. In both compartments, *de novo* dTMP synthesis is folate-dependent. The exchange rate between mitochondrial dTMP pools and dTMP pools from other cellular compartments is unknown. The role of Folate-dependent One-Carbon Metabolism (FOCM) in maintaining mtDNA integrity has not been studied extensively.

Folate-dependent one-carbon metabolism (FOCM):

FOCM is an interconnected metabolic network in which folate cofactors coordinate the transfer of one-carbon (1C) units for methylation reactions, *de novo* purine synthesis in the cytosol, and *de novo* nuclear, mitochondrial, and cytosolic dTMP synthesis (Figure 1.1). This network of reactions is highly compartmentalized in the cell. Serine is a primary source of 1C units in humans (17). Glycine, sarcosine, and dimethylglycine are also 1C donors for mitochondrial FOCM (18-20). Mitochondrial FOCM generates formate from serine and glycine, the primary 1C donor for cytosolic and nuclear FOCM.

Mitochondria-derived formate is transported into the cytosol, likely through a carrier-mediated process due to its inhibitory effect on cytochrome *c* oxidase (21). In bacteria and other lower organisms, formate is transported across cellular membranes by FocA, a member of the formate/nitrite transporter (FNT) channel family (22-24). Formate is incorporated into cytosolic FOCM, where it provides one-carbon units for the *de novo* synthesis of purines and 5,10-methylenetetrahydrofolate (5,10-methyleneTHF). 5,10-methyleneTHF can be converted to 5-methyltetrahydrofolate (5-methylTHF) in an irreversible reaction to support the remethylation of homocysteine to methionine.

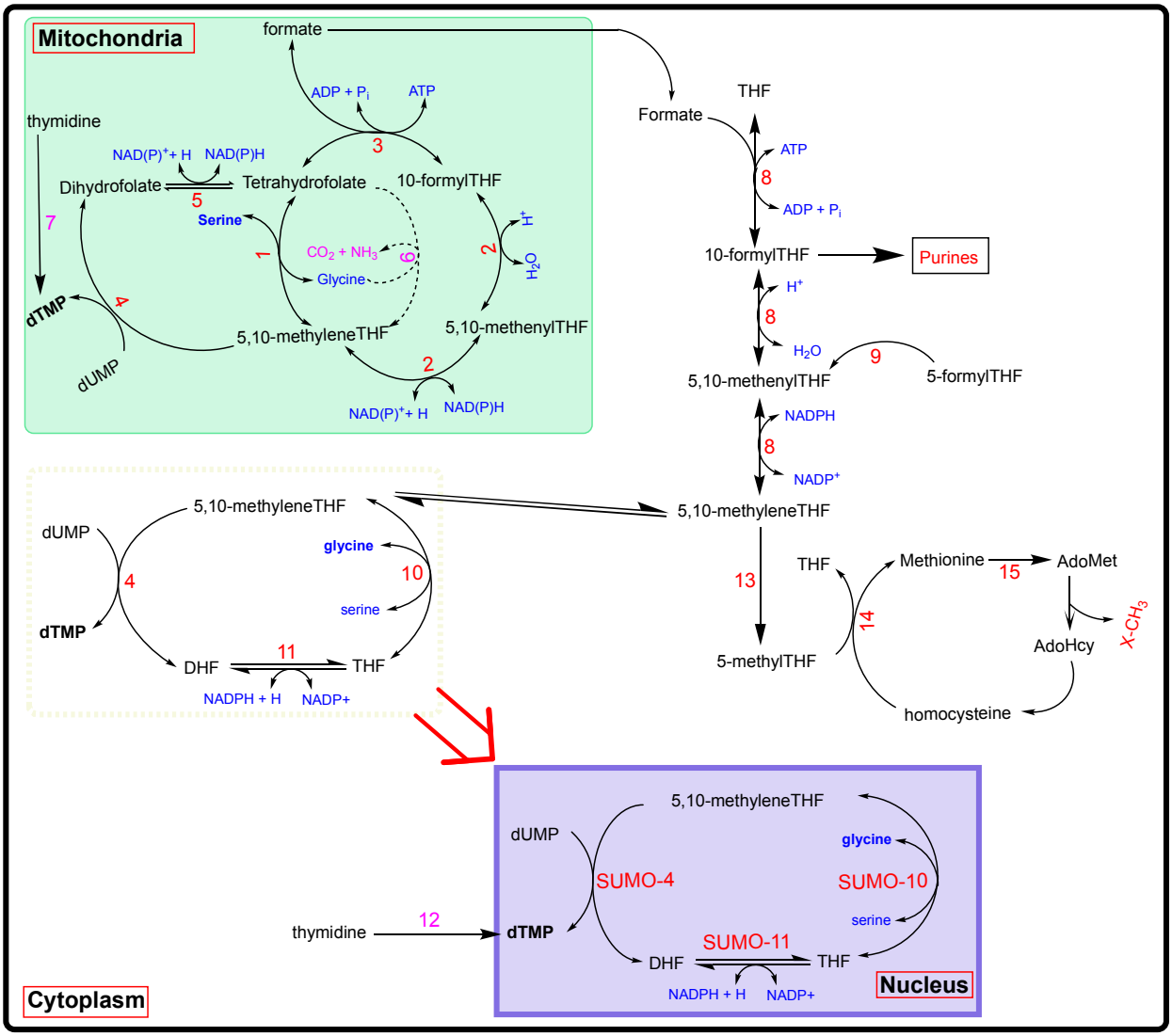

Figure 1.1: Folate-dependent One Carbon Metabolism (FOCM). FOCM is compartmentalized in the mitochondria, nucleus, and cytoplasm. One-carbon units are derived from from serine, glycine, and formate. Folate cofactors carry chemically-activated one carbons for the *de novo* synthesis of dTMP, purines, and methionine metabolism. 1= serine hydroxymethyltransferase 2 (SHMT2); 2= methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) or methylenetetrahydrofolate dehydrogenase 2 like (MTHFD2L); 3= methylenetetrahydrofolate dehydrogenase 1 like MTHFD1L; 4= thymidylate synthase (TYMS) or SUMOylated-TYMS in nucleus; 5= dihydrofolate reductase 2 (DHFR2); 6=glycine cleavage system; 7= thymidine kinase 2; 8=methylenetetrahydrofolate dehydrogenase 1 (MTHFD1); 9=methenyltetrahydrofolate synthetase (MTHFS); 10=serine hydroxymethyltransferase (SHMT), SUMOylated-SHMT1 or SUMOylated-SHMT2α; 11=dihydrofolate reductase (DHFR), SUMOylated-DHFR; 12=thymidine kinase 1 (TK1); 13=methylene tetrahydrofolate reductase (MTHFR); 14= methionine synthase (MTR); 15= methionine adenosyl transferase (MAT).

Cytosolic and Nuclear de novo dTMP synthesis:

The enzymes involved in nuclear *de novo* synthesis of dTMP (Figure 1.1) are serine hydroxymethyltransferase (SHMT), thymidylate synthetase (TYMS), dihydrofolate reductase (DHFR), and methylenetetrahydrofolate dehydrogenase 1 (MTHFD1). These enzymes are present in the cytosol during G1 phase of the cell cycle, and SUMOylated and translocated from the cytosol into the nucleus during S and G_2/M phases of the cell cycle or in response to DNA damage where they form a miltienzyme complex at sites of DNA synthesis and repair (13,25). Two *SHMT* genes are encoded in the mammalian genome. The *SHMT1* gene encodes a cytosolic/nuclear isozyme, and *SHMT2* encodes a SHMT2α isoform present in the cytosol and nucleus, and a SHMT2 isoform present in mitochondria (14,26). SHMT1 and SHMT2α act as scaffold proteins for the assembly of the dTMP biosynthesis enzymes at the nuclear lamina where they form a complex (13,15). The absence of SHMT disrupts the complex and thus impairs nuclear *de novo* dTMP synthesis (15). A dominant negative SHMT1 that lacks catalytic activity but retains lamin binding capacity also protects and enhances nuclear *de novo* synthesis of dTMP by allowing multienzyme complex formation (15), similar to wild-type SHMT1. This suggests that SHMT's scaffold properties, in addition to its catalytic role, support nuclear dTMP synthesis (15).

Mitochondrial de novo dTMP synthesis:

Isoforms of cytosolic/nuclear SHMT1/2α and DHFR exist in the mitochondrial matrix and inner membrane (SHMT2 and DHFR2 respectively), as well as TYMS (9). The mechanism for mitochondrial import of TYMS is unknown. Mitochondria also contain MTHFD1 isoenzymes, namely MTHFD2 and MTHFD1L. *MTHFD2* encodes methylenetetrahydrofolate dehydrogenase and cyclohydrolase activities, and *MTHFD1L* encodes formyltetrahydrofolate synthetase activity (27). In-tact mitochondria are capable of converting dUMP to dTMP (9) (Figure 1.1).

Mitochondria have nucleoside transporters as well as enzymes involved in the salvage synthesis of nucleotides to sustain the demand for mtDNA synthesis. Mitochondrial pyrimidine nucleotide transporter PNC1 preferentially transports thymine, in addition to uracil, and cytosine (deoxy)nucleoside di- and triphosphates by an antiport mechanism, for mtDNA synthesis (28). Thymidine kinase 2 (TK2) is the mitochondrial enzyme responsible for the phosphorylation of deoxythymidine (dT) to generate dTMP for mtDNA synthesis. There is evidence that in addition to dT, mitochondria can also transport dTMP from the cytosol by an unspecified but rapid and highly selective transport mechanism that discriminates dTMP from other phosphorylated forms of thymidylate (di- and triphosphate) (29). Once inside the mitochondria, dTMP can be phosphorylated to dTDP and dTTP by a mitochondrial thymidine monophosphate kinase (TMPK2) (30). The transport of dTMP from the cytosol to mitochondria is not affected by an excess of other nucleosides or nucleotides, except for excess dUMP, which inhibits dTMP transport by 30% (29). This highly specific dTMP transporter has yet to be identified. The exact level of contribution of each dTMP source (transport, salvage, or *de novo* synthesis) to the mitochondrial dTMP pool is unknown.

Mitochondrial FOCM

FOCM in mitochondrial includes pathways for generating formate, dTMP, and f-Met-tRNA. Formate is the primary 1C donor for cytosolic and nuclear FOCM. In mitochondria the SHMT2-catalyzed reaction is reversible, but it favors the use of serine as an 1C donor to THF for the synthesis of glycine and 5,10-methyleneTHF, a cofactor that supports dTMP and formate synthesis (31,32). How mitochondrial and nuclear FOCM respond to changes in glycine and serine availability and their regulation is not well understood. SHMT2 is ubiquitously expressed;

however, tissues including liver, kidney, and brain also have an alternative 1C unit source, the glycine cleavage system (GCS), which catabolizes glycine to 5,10-methyleneTHF (33-35). Sarcosine and dimethylglycine can also be used as 1C sources (19,20).

FOCM is connected across cellular compartments by the flux of 1C donors. Nuclear, cytosolic and mitochondrial FOCM are connected by the exchange of formate, glycine and serine. However, there is very little exchange of folate derivatives. Folates are absorbed by the cell via folate transmembrane carriers or endocytosis via folate receptors (36-38). The mitochondrial folate transporter (MFT) allows for transport of cytosolic reduced-monoglutamated folate forms (THF, 5-formyl-THF) into the mitochondria, where they are then polyglutamylated for their mitochondrial retention (38-40). Mitochondrial folates account for about 50% of total cellular folates, the nuclear compartment contains 10%, and the remain folate cofactors are present in the cytosol (38,41). Folylpoly-γ-glutamate synthetase (FPGS) is required to synthesize polyglutamate peptides present on folate derivatives to activate their cofactor function by increasing their affinity for folate-dependent enzymes, and enable their accumulation within the cell and subcellular compartments by limiting movement across organelles (42,43). Multiple studies have shown that depletion of the cytosolic folate pools does not alter mitochondrial folate pools, and vice versa (42,44-46). Mammalian cells that lack MFT are glycine auxotrophs and both mitochondrial and cytosolic FOCM are disturbed by a lack of formate (38), emphasizing the importance of mitochondrial FOCM.

Folate and mtDNA integrity

Folate deficiency is associated with loss of mtDNA integrity in multiple studies (47-51), although the mechanisms by which folate status and FOCM affect mtDNA integrity are unknown. Mitochondrial disorders associated with loss of mtDNA integrity have heterogeneous symptoms

and a high degree of tissue specificity. The most common deletion in the mitochondrial genome is known as the human 4977bp common deletion (also known as the common deletion and 5kb deletion). It occurs between two 13bp repeats at positions 13447-13459bp and 8470-8482bp of the major arc (between the origins of replication of heavy and light strands) of the mitochondrial genome and leads to three mitochondrial diseases: Pearson's syndrome, Kearns-Sayre syndrome, and chronic progressive external ophthalmoplegia (52). There is a homologous common deletion found in rats, the 4834bp deletion. An association between folate levels and the common deletion in rats and mice has been reported in liver, pancreas, heart, brain, and kidney, but not in colon or lymphocyte mtDNA (47-49,51,53).

Rats consuming a high-folate diet (50mg/kg folic acid, daily) have significantly fewer 4834bp common deletions in liver mitochondria than rats fed either a folate-replete (2mg/kg folic acid, daily) or low-folate diet (no added folic acid) as measured by real-time PCR (53). As part of the same study, rats treated with cyclophosphamide, a potent chemotherapy drug that interferes with DNA replication by introducing alkyl radicals into DNA strands, were also subjected to the same experimental diets to quantify mtDNA deletions. Rats treated with cyclophosphamide have significantly more mtDNA deletions than untreated rats. Rats fed a high-folate diet and treated with 50 or 110mg/kg cyclophosphamide had fewer 4977bp common deletions compared to rats on low-folate diet with these drugs (53). Although there is not a potential mechanism to explain how folic acid supplementation protected mtDNA from the common deletion, similar results have been observed in other studies (47,48,51).

In a separate study in liver mitochondrial samples from old rats, both the 4834bp common deletion and random deletions (spontaneous-smaller deletions) were measured in the mitochondrial genome (47). Random deletions in mtDNA from liver accumulate with age

irrespective of folate status, while common deletions do not show an increase with age but are folate responsive (47); folate supplementation rescued the 4834bp common deletion. The mtDNA region covered by the 4977bp common deletion encodes for enzymes of complex I, complex IV, and complex V, as well as tRNA genes that encode tRNAs that carry five amino acids, including glycine and serine (52), which are the main 1C donors for mitochondrial FOCM.

In nuDNA, the number of double strand breaks increases in conditions where *de novo* dTMP biosynthesis is suppressed (54). In humans, the 5'-end of the 4977bp common deletion is A:T rich (64%) (Table 1.1). This region is also enriched with nucleotide repeats. Under folate-deficient conditions, this common deletion region may be more susceptible to uracil misincorporation, resulting in breaks leading to loss of mtDNA integrity.

4977bp common deletion Region	Location	A:T content
5'-end	8401bp-8470bp	64%
4977bp common deletion	8470bp-13447bp	56.5%
3'-end	13459bp-13560bp	53.5%

Table 1.1: A:T content in human 4977bp common deletion.
Human mitochondrial genome NC_012920.1 was used for reference**.**

Uracil misincorporation in DNA:

The two primary sources of uracil in DNA are cytosine deamination and uracil misincorporation during DNA replication due to disturbed dTTP pools. Cytosine deamination refers to the spontaneous deamination of cytosine to uracil, while uracil misincorporation results from dUTP incorporation into DNA in lieu of dTTP during DNA synthesis or repair through the action of DNA polymerases. The rate of uracil accumulation in DNA from spontaneous cytosine deamination ranges from 70-200 events per cell per day in nuclear DNA, while uracil misincorporation into the nuclear genome due to disturbed dTMP pools is estimated to 10^4 uracil bases per human cell per day (55). Most DNA polymerases, including human mitochondrial DNA polymerase gamma (mitochondrial DNA polymerase for synthesis and repair), cannot distinguish between dUTP and dTTP (56). Cytosine deamination is mutagenic whereas misincorporation of deoxyuridine triphosphate (dUTP) in DNA due to deficient dTTP pools is not. Uracil misincorportation results in an A:U base pair that can be corrected during future rounds of DNA replication as both dUTP and dTTP nucleotides pair with dATP. On another hand, uracil in DNA arising from cytosine deamination would result in C:G base pairing converting to an U:G base pairing, thus future DNA strands would contain a transversion mutation. The presence of uracil in DNA increases risk for chromosome breakage and is associated with diseases such as cancer, neuronal damage, vascular disease, neural tube defects, and others (16,57-59). Similar outcomes are hypothesized for mtDNA.

Two enzymes directly protect DNA from uracil accumulation, dUTP pyrophosphatase (dUTPase) and Uracil-DNA glycosylase (UNG). dUTPase hydrolyses dUTP to dUMP, providing substrate for dTMP synthesis. dUTPase is essential to limit dUTP pools and thereby protects DNA integrity (60). In the event that dUTP is incorporated into DNA, UNG removes uracil bases from

DNA, which can then be replaced with the correct T base using the base excision repair machinery. Humans have two UNG isoforms, mitochondrial UNG1 and nuclear UNG2, which are generated from different promoters and alternative splicing of the same *UNG* gene. The catalytic domains of UNG1 and UNG2 are identical except for the N-terminus, which targets UNG1 to the mitochondria (61). When the dUTP/dTTP ratio is elevated, DNA polymerases will misincorporate dUTP during both DNA replication and repair (Figure 1.2) (56). This may lead to multiple rounds of excision of the misincorporated dUTP, which can result in DNA breaks. In some cases, this leads to what is known as "thymine-less cell death," as the repair system becomes overloaded and chromosome fragmentation occurs, leading to cell death (16).

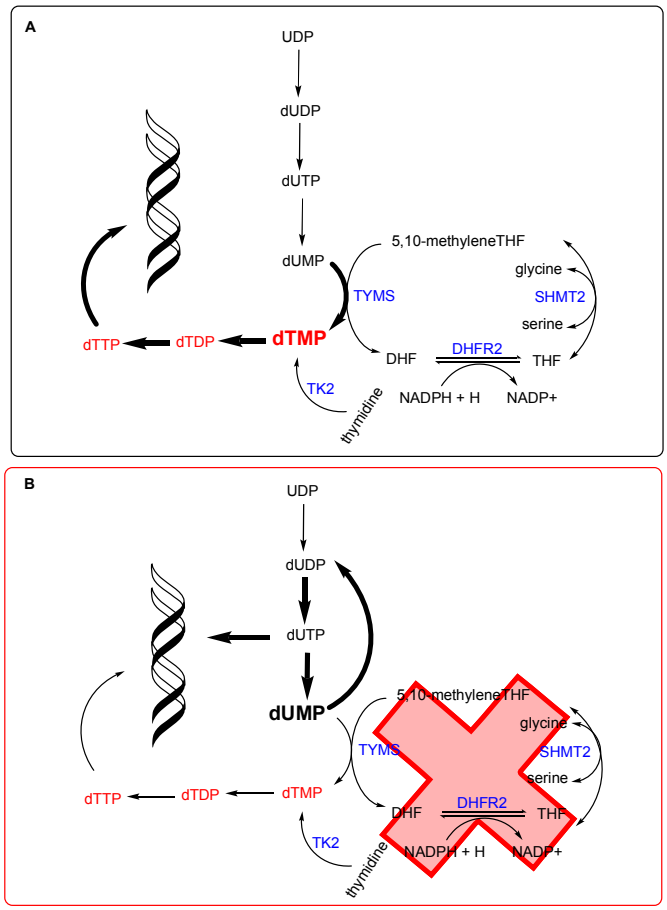

Figure 1.2: Mitochondrial *de novo* synthesis of dTMP. Biosynthesis of dTMP in folate replete conditions **(A)** versus *de novo* dTMP synthesis in folate-deficient conditions **(B)**. Disruptions to folate-dependent *de novo* dTMP synthesis by lack of 1C units, reduced folate pools, or enzymatically, may decrease dTMP pools, allowing for accumulation of dUMP. dUTP can be misincorporated into mtDNA during DNA synthesis.

Saccharomyces cerevisiae contain a single UNG isoform with nuclear- and mitochondrial-localization signals. Both, lack of- and increased expression of UNG result in a mitochondrial mutator phenotype (62). Immortalized human breast epithelial MCF 12A transfected with UNG and treated with a UNG inhibitor can repair DNA damage caused by agents known to deaminate mtDNA and have normal cell growth with no induced mutations in the mitochondrial genome (63). The authors attributed this lack of phenotype to other DNA glycosylases being present, such as thymine-DNA glycosylase (TDG), mismatch specific uracil-DNA glycosylase (MUG), and single-stranded monofunctional uracil-DNA glycosylase (SMUG1). In another study, mice consuming a folate-deficient diet lacking UNG had a 2-fold increase in mtDNA content in brain tissues when compared to wildtype mice and *UNG-/-* mice consuming a control-folate replete diet (51). The increase in mtDNA was accompanied by an increase in the abundance of D-1 mtDNA deletion (>2-fold) when compared to wildtype *UNG* in the folate-deficient diet (51). The levels of the D-1 deletion did not differ by genotype for mice fed the control diet. In these studies, the inability to excise uracil from mtDNA by UNG resulted in a loss of mtDNA integrity. The mtDNA stability in these models was affected by the presence of dietary folate, suggesting that other FOCM-related factors, such as nucleotide synthesis, may contribute to mtDNA integrity.

Because of the limited research on uracil in mtDNA, most of our knowledge of UTP incorporation into DNA is based on studies of nuDNA. A decrease in dTTP pool levels as a result of folate deficiency results in accumulation of dUTP, shifting the dTTP/dUTP ratio and allowing for uracil misincorporation by DNA polymerases (56). Increased levels of uracil in DNA is a biomarker of folate deficiency (16,64). Folate-deficient conditions disrupt dTMP synthesis and negatively affect nucleotide pools and DNA integrity (Figure 1.2). One study did quantify the effect of impaired mitochondrial FOCM on uracil levels in mtDNA. Disruption of SHMT2 activity

increased uracil levels in mtDNA in g*lyA* Chinese hamster ovary (CHO) cells, which are glycine autotrophs lacking mitochondrial SHMT2 activity (9). When compared to wild-type CHO cells, *glyA* CHO contained 40% more uracil, presumably due to impaired mitochondrial dTMP biosynthesis pathways (9). Other studies have shown that disruptions of mitochondrial dTTP synthesis or dTTP pool levels result in mitochondrial disorders. Mutations in thymidine kinase 2 and thymidine phosphorylase, both involved in mitochondrial dTTP synthesis, are associated with myopathic depletion syndrome and mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) respectively (12,65-68).

Mitochondrial DNA content and Mitochondrial Mass

The number of mitochondria per cell (mitochondrial mass) varies among tissues, and each mitochondrion has a varying number of mtDNA copies (mtDNA content). Changes in mtDNA content are not associated with, or the result of, mtDNA heteroplasmy. MDS, which is defined as a 30% reduction in residual mtDNA content, is caused by mutations in nuclear-encoded genes involved in nucleotide and mtDNA synthesis (69,70). In some presentations of MDS, the reduction in mtDNA content that is required to see a clinical phenotype may be less than 30% (71,72). An MDS patient may show a reduction of mtDNA content in one tissue, with normal or increased levels of mtDNA content in other tissues. The liver mtDNA content in a patient with hepatic MDS was reduced to 12% of the mean of control samples, while mtDNA content of skin fibroblasts from the same patient was 35% higher than observed in controls (73). Tissue-specific differences in mtDNA content is characteristic of many mitochondrial related disorders.

There is no established mechanism to account for the tissue variation in reduced or increased mtDNA content. In addition to MDS, mtDNA content is altered in certain cancers. A

meta-analysis conducted for 11,847 cases and 15,438 controls revealed a negative association between mtDNA copy number and the risk of hepatic carcinoma. The same study found a positive association between mtDNA content and lymphoma, breast cancer, and melanoma (74). Other factors that may alter mtDNA content besides genetic and nutritional factors are age, oxidative stress, and exposure to environmental agents damaging to DNA.

Loss of mtDNA integrity in the form of mutated mtDNA and/or reduced mtDNA content may result in impaired electron transport chain activity, given that mtDNA encodes for many of the subunits required for oxidative phosphorylation. However, defects in the respiratory chain do not always correlate with changes in mtDNA content. The ratio of mtDNA content to mitochondrial mass may be a better proxy for MDS than mtDNA content alone (Table 2, adapted from Navarro-Sastre, Tort et al. 2012 (75)), which may account for some of the tissue-specificity variation seen on MDS patients. A 20% residual mtDNA fits the definition of MDS, but a hepatocerebral MDS patient still exhibited normal respiratory chain activity (Table 2). In contrast, an encephalomyopathy MDS patient with moderate to normal mtDNA depletion was reported to have deficient respiratory chain function and a 50% residual mtDNA content, consistent with a lower mtDNA content to mitochondrial mass ratio.

Form of MDS	Residual % mtDNA	mtDNA content/mitochondrial mass	Deficient Respiratory chain	Genetic cause
Hepatocerebral	20%	58%	Normal	DGUOK, patient 4
Encephalomyopathy	50%	27%	deficient	SUCLA2, patient 7
Alpers-Huttenlocher syndrome	11%	8%	deficient	POLG, patient 10

Table 1.2: Ratio of mitochondrial DNA content and mitochondrial mass in MDS patients. The number of mtDNA copies per mitochondrion may be a better indicator of respiratory chain status. Data and patient numbers correspond to those listed on Navarro-Sastre, Tort et al. 2012 (75).

Folate and MDS:

Two patients with the hepatocerebral form of MDS, caused by mutations in different genes (*DGUOK* and MPV17), have been reported to exhibit signs of perturbed FOCM (73). FOCM provides one-carbon units for re-methylation of homocysteine to methionine in the cytosol, as well as for *de novo* purine and dTMP synthesis. These patients presented elevated levels of plasma methionine and *S*-adenosylmethionine, 21-fold and 8.6-fold above the upper limit of reference ranges, respectively (73). Elevated methionine is one marker of disturbed folate metabolism (76,77). All other genetic causes known to elevate blood methionine and *S*-adenosylmethionine were evaluated, but mutations in *DGUOK* and MPV17 were the only apparent genetic abnormalities. Mechanisms linking MDS to methionine synthesis are lacking.

Alpers-Huttenlocher syndrome (AHS) is a cerebrohepatopathy form of MDS caused by mutations present in *POLG*. It is characterized by seizures, hepatic failure, and developmental regression. At least one AHS patient has been described with high levels of folate receptor blocking autoantibodies in the cerebrospinal fluid (CSF), causing CSF folate levels to be lower than normal. The patient had drastically reduced levels of 5-methylTHF in CSF, potentially due to folate receptors autoantibodies blocking the transport of 5-methylTHF across the blood-brain barrier (78). After two weeks of folinic acid treatment, there was a reduction in the number and magnitude of seizures, communication skills improved, and difficulties swallowing were less prominent (78). The exact mechanism whereby folate therapy attenuates the symptoms of AHS are unknown. In both of these reports, FOCM is perturbed in response to, or as a cause of, loss of mtDNA integrity, and folate in the form of 5-methylTHF appears to be associated with changes mtDNA content (50,73). It is not clear if there is any connection of relevance between mtDNA integrity and 5-methylTHF levels, given that 5-methylTHF does not accumulate in mitochondria and the enzyme

that catabolizes it, methionine synthase, is localized in the cytosol.

Conclusion

Mitochondrial-related disorders are diverse both in symptoms and cause, and are affected by age, genetics and environmental factors including nutrition. Folate appears to play an important role in mtDNA; understanding the connection between folate, FOCM, and mtDNA integrity may inform better diagnostic and treatment.

Disruptions to *de novo* dTMP in CHO cells that lack SHMT2 have elevated uracil levels in mtDNA, suggesting that SHMT2 may play an important role in maintaining mtDNA integrity. How disruption of *de novo* dTMP synthesis by dysregulation of FOCM or folate enzymes affect mtDNA integrity in humans has not been previously studied. I would like to explore how changes in SHMT2 expression and changes in glycine and serine concentration affect mtDNA in immortal human cervical cancer cells, HeLa cells, since most of the human FOCM work done by the Stover group has been done in these cell lines. Similarly, MPV17 loss-of-function has been associated with elevated levels of plasma methionine and *S*-adenosylmethionine in humans (73) as well as reduction in mitochondrial dTMP pools in mice (79), both signs of disturbed FOCM. MPV17 biological function(s) and the mechanisms by which its loss-of-function leads to MDS are not known. I would like to explore a potential connection between MPV17 and FOCM.

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CHAPTER 2 - Mpv17 Prevents Uracil Accumulation in Mitochondrial DNA

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Keywords: folate, one-carbon metabolism, mitochondrial transport, nucleoside/nucleotide biosynthesis, mitochondrial disease, mitochondrial thymidylate synthesis, MPV17, Mitochondrial DNA Depletion Syndrome (MDS), deoxythymidine monophosphate (dTMP).

ABSTRACT

Mpv17 is a protein of unknown function that is associated with mitochondrial DNA (mtDNA) depletion syndrome (MDS). MPV17 loss-of-function has been reported to result in tissue-specific nucleotide pool imbalances, which can occur in states of perturbed Folate-mediated one-carbon metabolism (FOCM), but Mpv17 and FOCM have never been linked. FOCM is a metabolic network that provides one-carbon units for the *de novo* synthesis of purine and thymidylate nucleotides (e.g. dTMP) for nuclear DNA (nuDNA) and mtDNA replication. In this study, we investigated the impact of reduced Mpv17 expression on markers of impaired FOCM in HeLa cells. Depressed MPV17 expression reduced mitochondrial folate levels by 43%, and increased uracil levels in mtDNA by 3-fold, a marker of impaired dTMP synthesis. Mitochondrial *de novo* and salvage dTMP biosynthesis capacities were unchanged by reduced Mpv17 expression, but the elevated levels of uracil in mtDNA suggested that other sources of mitochondrial dTMP are compromised in Mpv17 deficient cells. These results indicate that Mpv17 facilitates a third

source of dTMP, potentially by serving as dTMP transporter from the cytosol to mitochondria to sustain mtDNA synthesis. Mpv17 and related hepatocerebral MDS is linked to impaired FOCM in mitochondria by providing access to cytosolic dTMP pools, and by severely reducing mitochondrial folate pools.

INTRODUCTION

Mitochondrial inner membrane protein Mpv17 is a ubiquitously expressed protein of unknown function (1-3). Over 30 distinct mutations throughout the MPV17 gene have been associated with mitochondrial DNA depletion syndrome (MDS), which is characterized by a tissue-specific reduction in mitochondrial DNA (mtDNA) copy number (4-7). Despite a lack of knowledge of its biochemical function(s), Mpv17 has been shown to protect against mitochondrial dysfunction and apoptosis, and regulate reactive oxygen species (8,9). It has been proposed to function as a nonselective mitochondrial channel protein which opens under conditions characteristic of damaged mitochondria to preserve mitochondrial homeostasis by decreasing the membrane potential and thus preventing the formation of reactive oxygen species (10).

Loss-of-function mutations in genes involved in regulation or synthesis of nucleotides can result in MDS. Mice that lack Mpv17 function exhibit a 35% and 30% reduction in dTTP and dGTP pools respectively in liver mitochondria, but not in other tissues tested (11). Maintenance and regulation of cellular dTTP synthesis and pool size for mtDNA replication is critical, as both depleted and expanded dTTP pool sizes have been associated with mtDNA depletion (12).

Folate-mediated one-carbon metabolism (FOCM) is a network of interconnected metabolic pathways that use tetrahydrofolate (THF) cofactors to carry and chemically activate single carbon units for remethylation of homocysteine to methionine and the *de novo* synthesis of purine

nucleotides and thymidylate (dTMP) (13) (Figure 2.1). FOCM functions in the mitochondria, nucleus, and cytosol. The synthesis of dTMP occurs in the mitochondria (14) and the cytosol/nucleus (15) through both salvage pathway synthesis catalyzed by thymidine kinase (TK1, TK2) as well as through folate-dependent *de novo* synthesis. Mitochondrial folate-dependent dTMP synthesis involves the enzymes serine hydroxymethyltransferase 2 (SHMT2), dihydrofolate reductase 2 (DHFR2, formally known as DHFRL1), and thymidylate synthase (TYMS). SHMT2 transfers one-carbon (1C) units from serine to THF to synthesize glycine and 5,10-methyleneTHF, the 1C donor for the conversion of deoxyuridylate (dUMP) to thymidylate (dTMP) in a reaction catalyzed by TYMS. In this reaction the folate cofactor serves as both a 1C donor and a source of 2 electrons, generating dihydrofolate (DHF) as a product. THF is regenerated from DHF by DHFR2 to complete the dTMP cycle (14).

FOCM in mitochondria is the primary source of formate, which when translocated to the cytosol serves as the primary source of 1Cs for homocysteine remethylation, and *de novo* purine and thymidylate biosynthesis. Nuclear dTMP biosynthesis via FOCM requires isoforms of each of the mitochondrial dTMP synthesis enzymes (SHMT1/SHMT2α, TYMS, and DHFR). These enzymes are SUMOylated and translocated from the cytosol to the nucleus during S-phase where they form a multienzyme complex for nuclear dTMP synthesis at sites of nuclear DNA (nuDNA) replication and repair (14-16). Folate-dependent *de novo* dTMP synthesis is compromised in states of perturbed FOCM and folate deficiency, leading to increased uracil misincorporation into DNA (17,18).

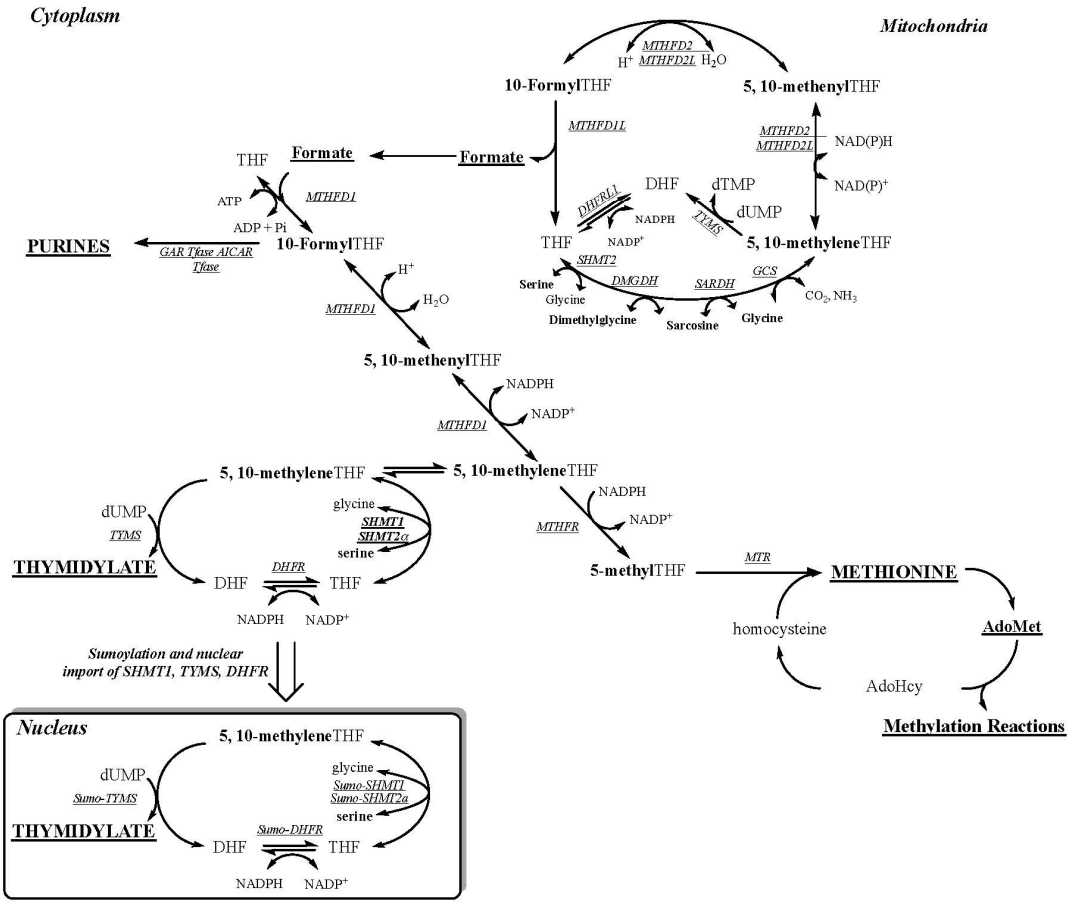


Figure 2.1: Folate-Mediated One-Carbon Metabolism (FOCM). One-carbon metabolism is required for the synthesis of purines, thymidylate (dTMP) and methionine. The hydroxymethyl group of serine is a major source of 1C units, which are generated in the mitochondria in the form of formate via SHMT2, or in the cytoplasm through the activity of SHMT1 or SHMT2α. Mitochondrial-derived formate can enter the cytoplasm and function as a one-carbon unit for folate metabolism. The synthesis of dTMP occurs in the nucleus and mitochondria. At S phase, the enzymes of the thymidylate (dTMP) synthesis pathway undergo SUMO-dependent translocation to the nucleus. The remethylation of homocysteine to methionine by MTR requires vitamin B12. The one carbon is labeled in "bold". The "inset" shows the thymidylate synthesis cycle which involves the enzymes, SHMT1, SHMT2α, TYMS and DHFR. THF, tetrahydrofolate; DHF, dihydrofolate, MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; SHMT, serine hydroxymethyltransferase; DHFR, dihydrofolate reductase; TYMS, thymidylate synthase; MTHFD1, methyleneTHF dehydrogenase; AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; GAR Ftase, glycinamide ribonucleotide transformylase; AICAR Ftase, (GAR Tfase) and aminoimidazolecarboxamide ribonucleotide transformylase; GCS, glycine cleavage system.

Under folate deficient conditions, lack of folate-activated 1C units for dTMP synthesis results in dUMP accumulation leading to dUTP synthesis, which can be misincorporated into DNA by DNA polymerases, which do not distinguish between dUTP and dTTP during DNA synthesis (19). Uracil-DNA glycosylases (UNG) cleave the misincorporated U base leaving an abasic site; multiple rounds of repair can result in DNA strand breaks, genomic instability, and cell death (20). These mechanisms have been studied more extensively in nuDNA than in mtDNA. In this study, the effect of Mpv17 expression on markers of impaired FOCM, including nucleotide synthesis and uracil misincorporation, was investigated.

RESULTS

Impact of Mpv17 expression on mitochondrial folate-dependent nucleotide synthesis

HeLa cells with reduced Mpv17 expression generated by shRNA (MPV17 knockdown, KD) exhibited an 80-85% reduction in Mpv17 protein levels when compared cell lines treated with the with scrambled shRNA (Figure 2.2). The deoxyuridine (dU) suppression assay measures the cellular capacity to synthesize dTMP via the *de novo* ([^{14}C]-dU) and salvage ([^{3}H]-thymidine, dT) pathways for DNA synthesis. The decreased Mpv17 expression did not affect the relative contribution of the *de novo* to salvage thymidylate pathway to mtDNA synthesis as indicated by the dU suppression assay (Figure 2.3); the activity of both the salvage and *de novo* dTMP synthesis were elevated similarly in mitochondria of MPV17 knockdown cell lines compared to mitochondria of control lines (Figures 2.4a-c). Mitochondrial TK2 and TYMS protein levels were unaffected by reduced Mpv17 expression when compared to control MPV17-expressing cell lines (Figure 2.4h). Separation of DNA bases by high-performance liquid chromatography (HPLC) showed that both dTMP precursors, [^{14}C]-dU and [^{3}H]-dT, were incorporated into mtDNA

primarily as dTTP and not dUTP (Figure 2.5). In contrast, incorporation of [^{3}H]-dT, via the salvage pathway, and [^{14}C]-dU, via the folate-dependent *de novo* dTMP pathway, into nuDNA were not affected by reduced Mpv17 expression (Figures 2.4d-e, 2.6). *De novo* purine synthesis capacity in the cytosol was also quantified in MPV17-deficient cells via the formate suppression assay. Reduced Mpv17 expression did not affect the relative ratio of *de novo* to salvage purine synthesis capacity in HeLa cells, as demonstrated by the ratio of [^{14}C]-formate (via folate-dependent *de novo* synthesis) and [^{3}H]-hypoxanthine (via salvage synthesis) incorporated into DNA (Figure 2.7). Both salvage and *de novo* purine synthesis were reduced in MPV17 KD cells when compared to control lines (Figures 2.4f-g), but the magnitude of the difference is small and unlikely biologically important.

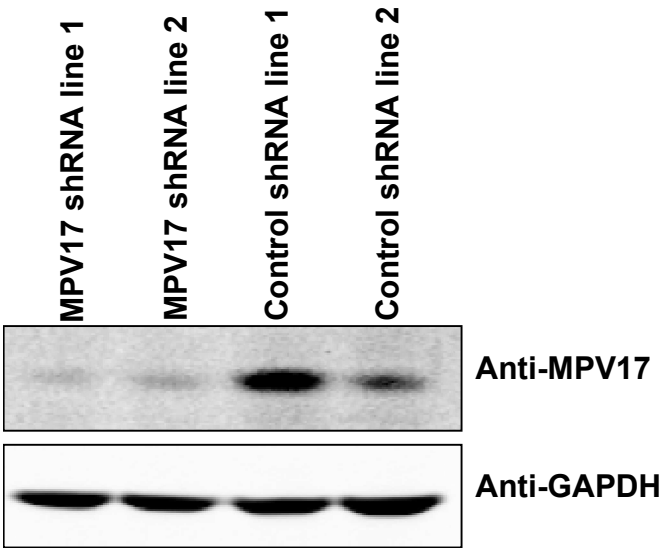


Figure 2.2: MPV17 stable knockdown (KD) cell lines. MPV17 KD cell lines were generated by clonal selection using shRNA in a HeLa cells background. Mpv17 protein expression was reduced by 80-85% in KD lines. Western blot was quantified using ImageJ.

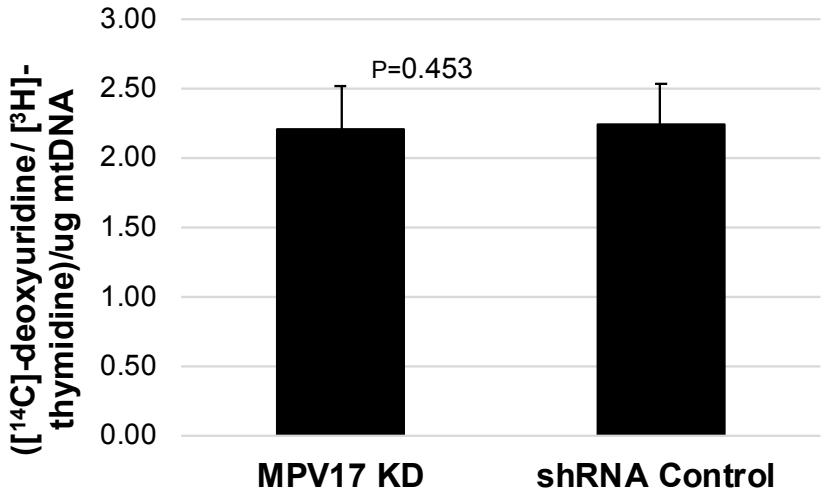
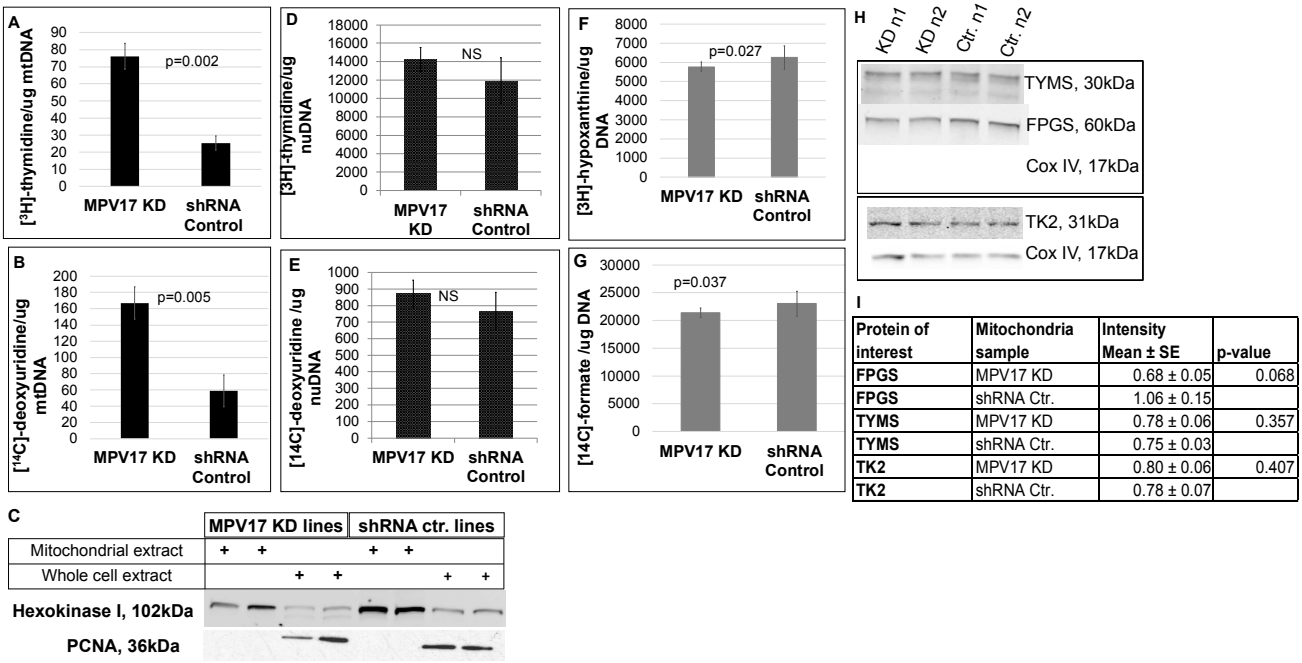


Figure 2.3: Relative contribution of the *de novo* to salvage mitochondrial dTMP biosynthesis capacity is not affected by Mpv17 expression. [^{14}C]-deoxyuridine is incorporated into dTMP via FOCM; [^{3}H]-thymidine is incorporated into dTMP by TK2 via the dTMP salvage pathway. ^{14}C and ^{3}H channels were counted in dual DPM mode on a scintillation counter. The *de novo* to salvage ratio was calculated and normalized to micrograms of mtDNA (N=3). Data shown as means ± standard deviation. Statistical significance was determined by 2-tailed Student's t-test (NS= not significant $p>0.05$). KD= MPV17 shRNA knockdown; Ctr.= scrambled shRNA Control.



Protein of interest	Mitochondria sample	Intensity Mean ± SE	p-value
FPGS	MPV17 KD	0.68 ± 0.05	0.068
FPGS	shRNA Ctr.	1.06 ± 0.15	
TYMS	MPV17 KD	0.78 ± 0.06	0.357
TYMS	shRNA Ctr.	0.75 ± 0.03	
TK2	MPV17 KD	0.80 ± 0.06	0.407
TK2	shRNA Ctr.	0.78 ± 0.07	

Figure 2.4: MPV17 knockdown (KD) increased mitochondrial dTMP synthesis. Panel A: [^{3}H]-thymidine incorporation into newly synthesized mtDNA via salvage pathway; KD n=3, Ctr. n=2. **Panel B:** [^{14}C]-deoxyuridine incorporation into mtDNA via folate-dependent *de novo* dTMP pathway; KD n=3, Ctr. n=2. **Panel C:** MtDNA was extracted from isolated mitochondria. Representative western blot of protein extracts of mitochondrial fractions and respective whole cell extracts is shown. Mitochondrial fractions were free of nuclear contamination (Proliferating Cell Nuclear Antigen-PCNA, nuclear marker, and Hexokinase I, mitochondrial marker). **Panels D-E:** [^{3}H]-thymidine incorporation into newly synthesized nuDNA via salvage pathway **(D)** and [^{14}C]-deoxyuridine incorporation into nuDNA via folate-dependent *de novo* dTMP pathway **(E)**; n=4 each group, NS. **Panels G-F:** Newly synthesized nuDNA containing purines made via salvage pathway **(F)** and folate-dependent *de novo* pathway **(G),** as quantified by [^{3}H]-hypoxanthine and [^{14}C]-formate incorporation, respectively; n=6 each group. **Panels A-B, D-G:** ^{3}H and ^{14}C channels were counted in dual DPM mode on a scintillation counter. **Panels H-I:** Western blots of mitochondrial protein extracts from different biological replicates probed for Thymidine Kinase 2 (TK2), thymidylate synthase (TYMS), and folylpolyglutamate synthase (FPGS). Densitometry was performed using ImageJ. The intensities of non-saturated bands were quantified and normalized to Cox IV, which served as mitochondrial marker and mitochondrial protein loading control. Data shown as means ± standard deviation (panels A-H) or means ± standard error (SE) (panel I). Statistical significance was determined by 2-tailed Student's t-test (NS= not significant, $p>0.05$). KD= MPV17 shRNA knockdown; Ctr.= scrambled shRNA Control.

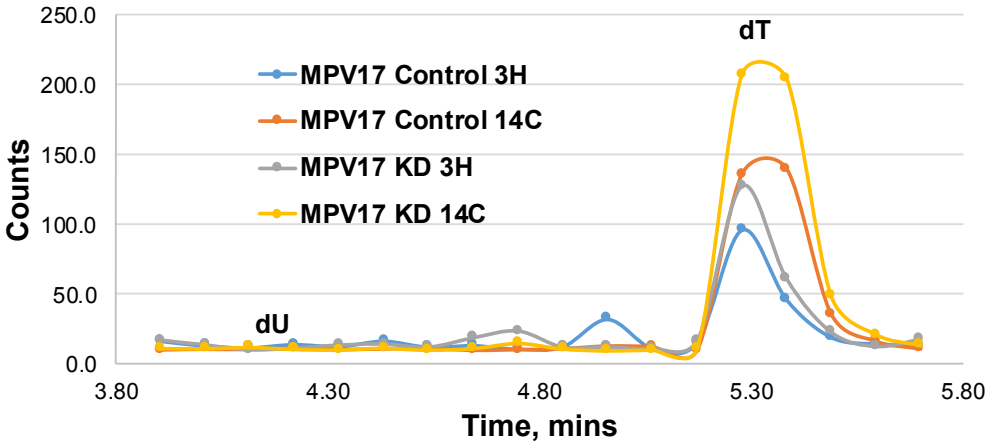


Figure 2.5: DNA bases from mtDNA individually separated by HPLC. [^{3}H]-thymidine and [^{14}C]-deoxyuridine were incorporated into mtDNA exclusively as T bases; KD n=3, Ctr. n=3. [^{3}H] and [^{14}C] labels were quantified using a liquid scintillation counter. Data shown as means ± standard deviation. Statistical significance was determined by 2-tailed Student's t-test (NS= not significant $p>0.05$). KD= MPV17 shRNA knockdown; Ctr.= scrambled shRNA control.

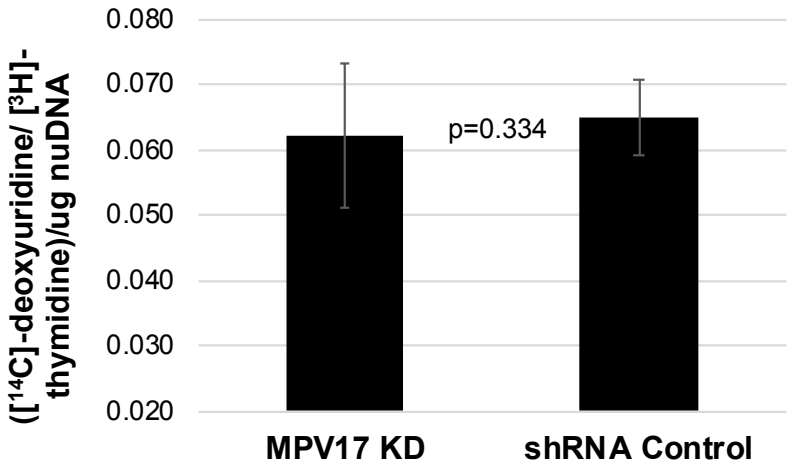


Figure 2.6: Relative contribution of the *de novo* to salvage nuclear dTMP biosynthesis capacity is not affected by Mpv17 expression in nuDNA. [^{14}C]-deoxyuridine is incorporated into dTMP via FOCM; [^{3}H]-thymidine is incorporated into dTMP by TK1 via the salvage pathway. ^{14}C and ^{3}H channels were counted in dual DPM mode on a scintillation counter. The *de novo* to salvage ratio was calculated and normalized to micrograms of nuDNA. Data shown as means $\pm$ standard deviation. Statistical significance was determined by 2-tailed Student's t-test (NS= not significant $p>0.05$). KD= MPV17 shRNA knockdown; Ctr.= scrambled shRNA control. N=4, each group.

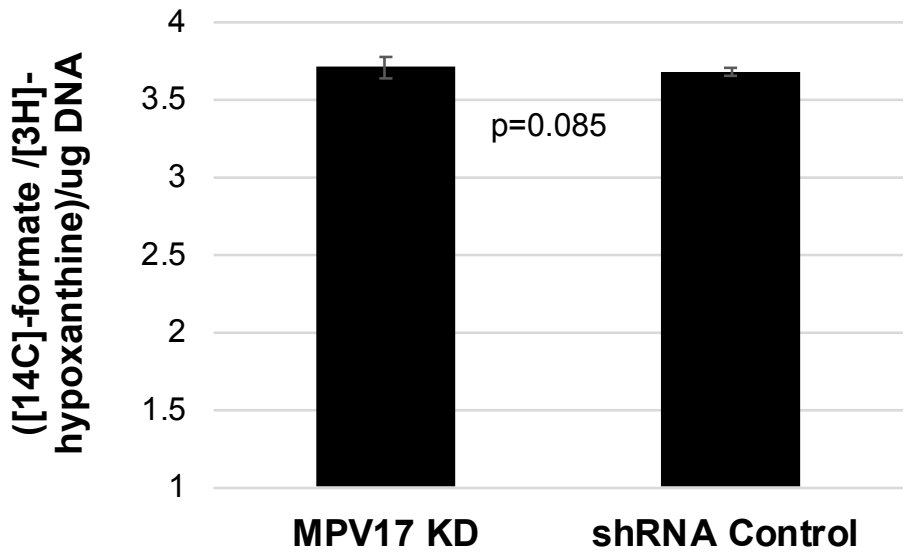


Figure 2.7: Relative contribution of the *de novo* to salvage purine biosynthesis capacity is not affected by Mpv17 expression. [14C]-formate provides carbons 2 and 8 to the purine ring via FOCM; [3H]-hypoxanthine contributes to purine synthesis via a salvage pathway. ^{14}C and ^{3}H channels were counted in dual DPM mode on a scintillation counter. The *de novo* to salvage ratio was calculated and normalized to micrograms of DNA (N=3). Data shown as means $\pm$ standard deviation. Statistical significance was determined by 2-tailed Student's t-test (NS= not significant $p>0.05$). KD= MPV17 shRNA knockdown; Ctr.= scrambled shRNA Control.

Impact of Mpv17 on cellular folate levels

Intracellular folates are compartmentalized in the mitochondria, nucleus, and cytosol as discrete pools (13,18,21-23). Folate cofactors enter the cell in a monoglutamate form and are converted to a polyglutamate form by the enzyme folylpolyglutamate synthetase (FPGS). The polyglutamate peptide serves to retain the cofactors within the cell and subcellular compartments, and increase the affinity of the cofactor for enzymes (24,25). Mitochondrial FPGS levels were reduced (30%) in MPV17 KD cell lines, with a trend toward significance ($p=0.068$) (Figures 2.4h-I). Total cellular folate levels were similar for control and MPV17 KD lines (Table 2.1). However, mitochondrial folate levels in MPV17 KD cells were 43% lower than mitochondrial folate levels in control cell lines (Table 2.1, $p \leq 0.0001$).

	Folates in Whole Cell[NS]	Folates in Mitochondria Fraction[‡]
	fmol/ug protein ± SEM	fmol/ug protein ± SEM
MPV17 KD	107.64 ± 17.98	74.08 ± 6.11
shRNA Control	100.95 ± 14.18	129.63 ± 5.29

Table 2.1: Cells deficient in MPV17 have 43% less mitochondrial folate.
Total intracellular folate in whole cell lysates and isolated mitochondria were quantified in a *Lactobacillus casei* microbiological assay. Whole cell lysates: shRNA control n=3, MPV17 KD n=6, NS. Mitochondria: n=4 each group, ‡$p \leq 0.0001$. Data shown as means ± standard error of the mean (SEM). Statistical significance was determined by 2-tailed Student's t-test (NS= not significant, $p > 0.05$). MPV17 KD= MPV17 shRNA knockdown.

The effect of Mpv17 on folate accumulation and turnover was investigated in MPV17 KD and control lines cultured with labeled [^{3}H]-(6S) 5-formylTHF. Cells with reduced Mpv17 expression accumulated 29% less labeled folate than control lines (Figure 2.8a). Similarly, the rate of [^{3}H]-(6S) 5-formylTHF uptake was lower for MPV17 KD cells than for control lines over time (Figure 2.8b). Whole-cell folate turnover rates were similar in MPV17 deficient cells and control cell lines (Figure 2.8c).

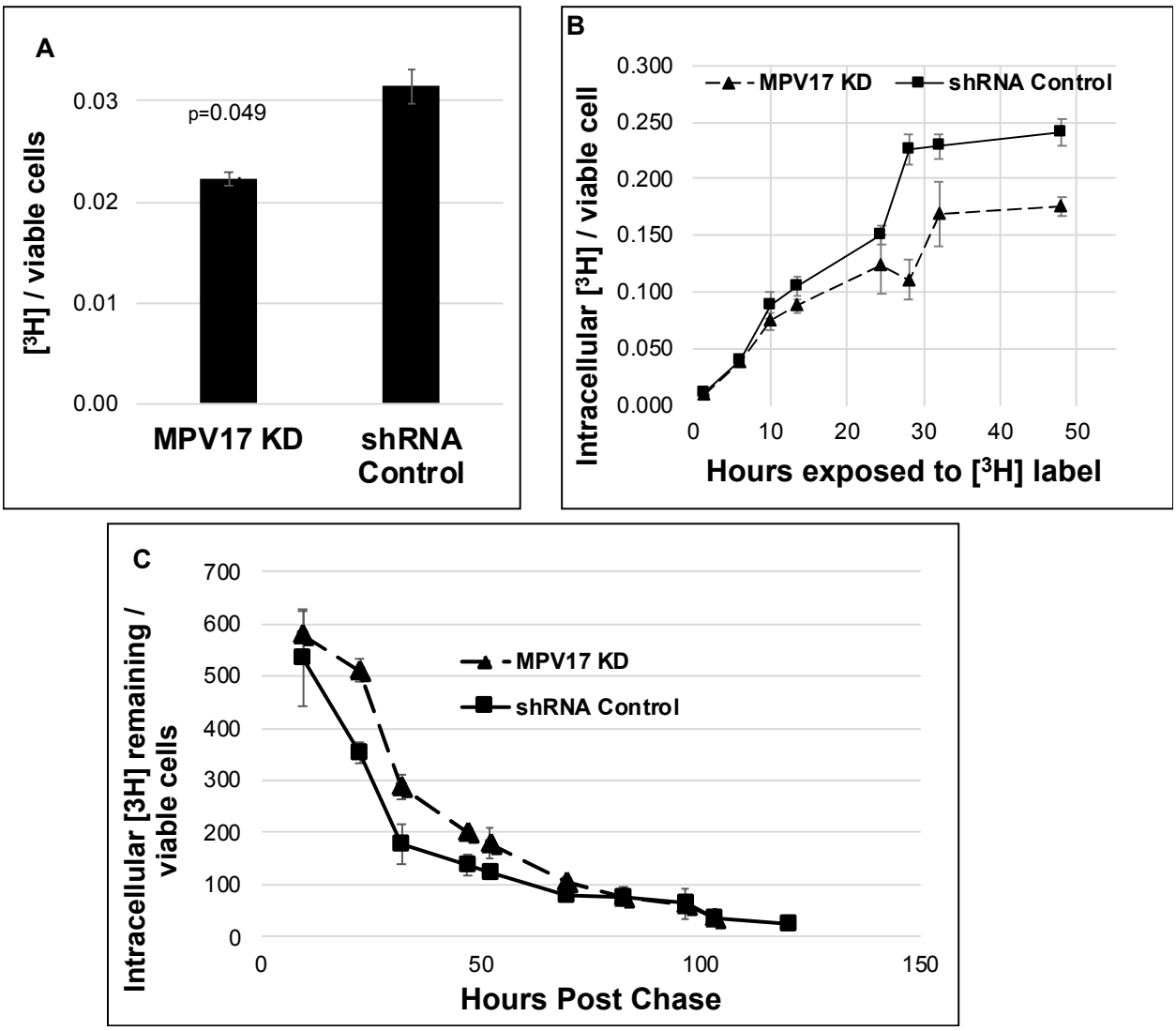


Figure 2.8: Mpv17 and folate accumulation. Panel A: MPV17 KD cells incubated with (6S)-[^{3}H]5-formyl-THF for 12 hours accumulated 29% less of the label than control lines; n=3 each group. **Panel B:** Accumulation of [^{3}H]-labeled folates in viable cells over 50 hours. Folate uptake in cells deficient in MPV17 is less than control lines; n=3 each group. **Panel C:** The rate of [^{3}H]-labeled folate depletion in cells pulsed with (6S)-[^{3}H]5-formyl-THF for 13 hours was similar between MPV17 KD cells and control lines; n=3 each group. Data shown as means ± standard deviation. Statistical significance was determined by 2-tailed Student's t-test (NS= not significant, $p>0.05$). KD= MPV17 shRNA knockdown; Ctr.= scrambled shRNA Control.

Effect of reduced Mpv17 levels on mitochondrial DNA integrity from HeLa Cells

Uracil misincorporation in DNA is a marker of low folate status (20,26). In folate deficiency the dUTP to dTTP ratio is increased, leading to uracil misincorporation in DNA, double-strand breaks, and DNA instability (27). Uracil levels in mtDNA and nuDNA from MPV17 KD and control cells were quantified by gas chromatography-mass spectrometry (GCMS). HeLa cells cultured in folate-deplete medium for at least four doublings exhibited an 84%± 14% increase in uracil levels in mtDNA when compared to mtDNA from cells cultured in folate-replete medium (Table 2.2). Similarly, uracil levels in mtDNA were three-fold higher in cells with reduced Mpv17 expression than uracil levels from control lines (Figure 2.9a). However, reduced Mpv17 expression did not affect uracil levels in nuDNA (Figure 2.9b). Decreased Mpv17 expression did not change mtDNA copy number or mitochondrial mass in HeLa Cells as compared to control cell lines (Figures 2.10a-b).

Uracil in mtDNA from folate-deplete HeLa Cells			
	Replicate # 1	Replicate # 2	Replicate # 3
% Increase	99%	55%	97%
Mean ± SE	84%	±14%	

Table 2.2: Uracil levels in mtDNA from HeLa cells.
HeLa cells grown in folate-deplete media for four doublings or more exhibited least 55% more uracil in their mitochondrial genome than HeLa cells grown in folate-replete media. Table shows uracil levels quantified by GC-MS for three different biological replicates; Each biological replicate was the average of 3 independent measurements except replicate 3 (n=1). Data shown as percentage increase for each replicate and the mean ± standard error (SE).

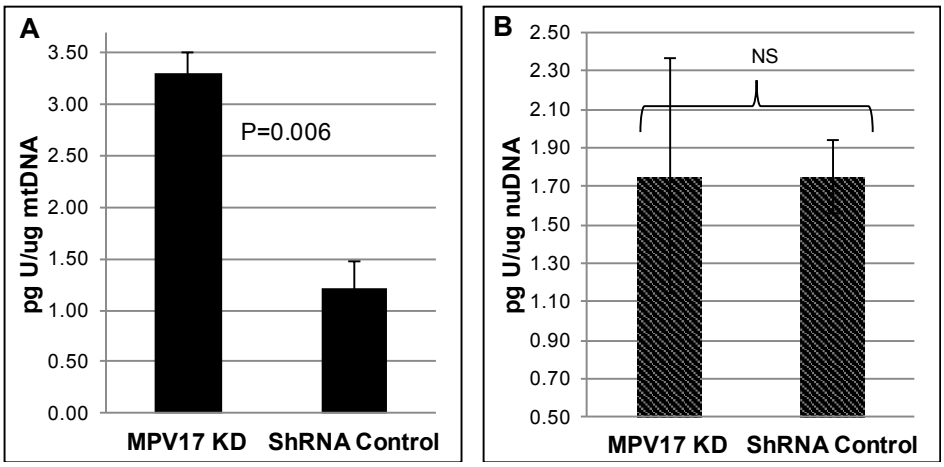


Figure 2.9: MPV17 deficiency increases uracil levels in mtDNA. Panel A: Uracil levels were 3-fold higher in mtDNA extracted from isolated mitochondrial of MPV17 KD cells than control lines, n=2 each group. **Panel B:** There was no difference in uracil levels from nuDNA with changes in Mpv17 expression. n=4 each group. Data shown as means ± standard deviation. Statistical significance was determined by 2-tailed Student's t-test (NS= not significant, $p>0.05$). KD= MPV17 shRNA knockdown; Ctr.= scrambled shRNA Control.

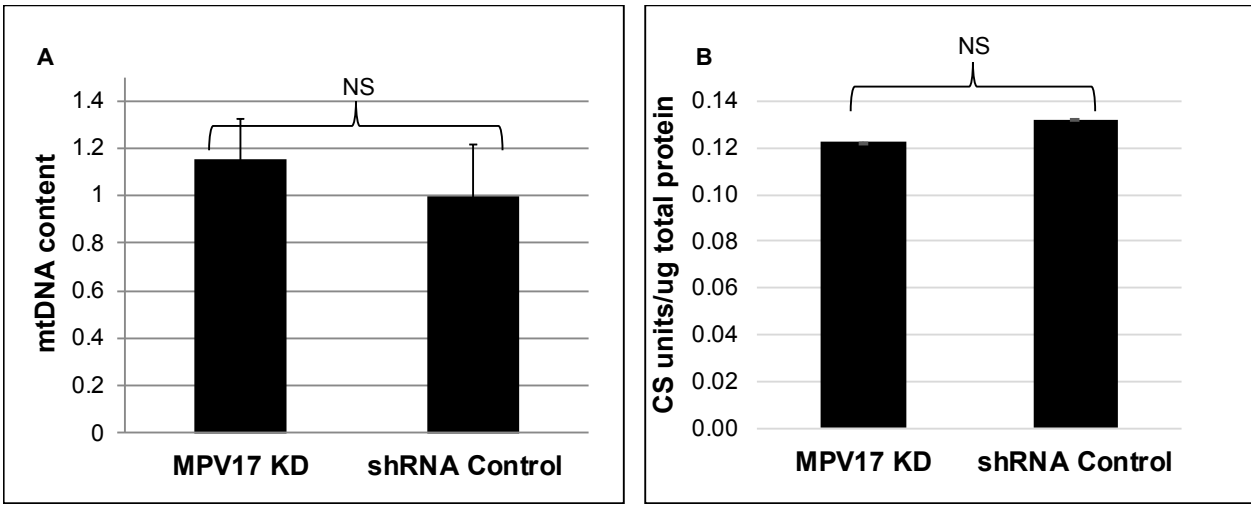


Figure 2.10: Mitochondrial content and mitochondrial mass are not affected by Mpv17 expression in HeLa Cells. Panel A: Mitochondrial copy number was not affected in Hela cells with reduced Mpv17 expression; mtDNA content in MPV17 KD lines is expressed relative to mtDNA content in control lines. KD n=5, ctr. n=4; each measurement done in triplicates. **Panel B:** Mitochondrial mass was not affected by lack of Mpv17 expression; mitochondrial mass was determined by citrate synthase activity assay and normalized to total protein. KD n=5, ctr. n=2, each measurement done in duplicates. Data shown as means ± standard error of the mean (A) or ± standard deviation (B). Statistical significance was determined by 2-tailed Student's t-test (NS= not significant, $p>0.05$). KD= MPV17 shRNA knockdown; Ctr.= scrambled shRNA control.

DISCUSSION

The interaction between Mpv17 and FOCM was investigated to provide insight into the role of Mpv17 in mitochondrial nucleotide synthesis and mtDNA depletion. These studies demonstrate that Mpv17 function is critical to maintain mitochondrial folate levels and prevent uracil accumulation in mtDNA, providing new insights into the role of MPV17 in MDS.

Mpv17 has been hypothesized to be a non-selective mitochondrial transporter (10,28). Liver mitochondria with reduced Mpv17 expression have been reported to have lower levels of dTTP and dGTP pools (11). Our data demonstrate that that MPV17 deficient cells synthesized and incorporated more dTMP from the *de novo* and salvage pathways into mtDNA (Figures 2.4a-e, 2.5) without an increase in the expression levels of dTMP enzymes (Figure 2.4h). However, the three-fold increase in uracil levels in the mitochondrial genome (Figure 2.9a) of MPV17 deficient cells indicates that dTMP pools are compromised independent of biosynthesis capacity. These data suggest that MPV17 is essential to prevent uracil accumulation in mtDNA, independent of dTMP synthesis capacity. The elevated levels of uracil in mtDNA are independent of uracil misincorporation in the nuclear genome or nuclear dTMP synthesis capacity, which were not affected (Figures 2.4d-e, 2.9b).

Nucleosides and nucleotides are transported into the mitochondria for mtDNA synthesis. The mitochondrial pyrimidine nucleotide transporter SLC25A33 (PNC1) preferentially transports uracil, in addition to thymine, and cytosine (deoxy)nucleoside di- and triphosphates by an antiport mechanism (29,30). Similarly, SLC25A36 (PNC2) transports cytosine and uracil (deoxy)nucleoside mono-, di-and triphosphates by uniport and antiport mechanism (29,30). There is evidence in mouse liver for an unidentified highly selective mitochondrial dTMP transporter, with lower affinity for other thymine forms (base, di-, and triphosphate) (31). Hence, there are

three potential sources of dTMP for mtDNA replication: 1) endogenous folate-dependent *de novo* biosynthesis from dUMP; 2) salvage pathway synthesis from thymidine, and 3), import of thymidylate nucleotides from the cytosol. Given the elevated levels of uracil in the mtDNA (Figure 2.9a) from cells with reduced Mpv17 expression without evidence for disturbed dTMP biosynthesis capacity from both the salvage and *de novo* synthesis pathway (Figure 2.4), as well as the reported cases of dysregulation of mitochondrial dTTP pools in MPV17-deficient cells (11), we hypothesize that Mpv17 is an important source of dTMP by serving as a dTMP transporter from the cytosol into the mitochondria for mtDNA synthesis (Figure 2.11). The apparent increase in both mitochondrial *de novo* and salvage dTMP biosynthesis (Figures 2.4a-b) suggests that these pathways are partially compensating for other mitochondrial sources of dTTP, namely nucleotide (dTMP, and potentially dUMP) transport from the cytoplasm by Mpv17.

Mitochondria contain about 50% of total cellular folate, the nucleus contains ~10%, and the remaining folates are present in the cytosol (32,33). The 43% reduction in mitochondrial folates (Table 2.1) correlates with the 29% decrease in [^{3}H] cellular folate accumulation (Figure 2.8a). This suggests that the mitochondrial folate pool is the main folate pool affected by Mpv17 expression and that the decrease in the accumulation of the labeled folate by MPV17 KD cells may be a reflection of the depletion of mitochondrial folate pools. There was no observed change in total folates levels from whole-cell lysates of MPV17 KD cells compared to control cell lines (Table 2.1) or in overall whole-cell folate turnover (Figure 2.8c), indicating that accumulation of cytoplasmic folate levels is not affected and is likely increased by reduced Mpv17 expression. The spatial compartmentalization of folate pools is possible by the addition of polyglutamate chains to folate substrates by folylpoly-γ-glutamate synthetase (FPGS). The polyglutamate chains limit the translocation of folates across organelles and increase selectivity for several folate-dependent

enzymes (24,25). Reduced FPGS protein levels in mitochondria in cell with reduced Mpv17 expression may contribute to lower folate levels in that compartment.

The mechanism for reduced mitochondrial folate pools in MPV17 deficiency is not clear. Cells deficient in MPV17 cultured with labeled [^{3}H]-leucovorin, have reduced uptake of the tritium label as shown by the quantitation of the label accumulation into the cell over time (Figure 2.8b). It is not clear from this data if the lower folate uptake levels represent lower uptake by the cell as a whole or lower uptake of folates exclusively by mitochondria. The expression of FPGS in MPV17-deficient cells may be the important determinate of reduced folate accumulation in mitochondria (Figures 2.4h-I). The pulse-chase data (Figure 2.8c) indicate that folate stability was not affected by the Mpv17 expression, suggesting that the turnover of folate is the same for both MPV17 KD and control lines.

Dietary folate has been shown to have an effect on mtDNA damage, specifically in protecting against mtDNA deletions by unknown mechanisms (34-36). MtDNA from HeLa cells grown in folate-deplete media for at least four doublings had 84% ± 14% more uracil than cells grown in folate-replete media (Table 2.2), suggesting that mtDNA is highly sensitive to uracil misincorporation under folate deficient conditions. A potential mechanism by which folate deficiency may be associated with mtDNA deletion is by generation of double-strand breaks as a result of unrepaired uracil misincorporation. Uracil in mtDNA because of lack of Mpv17 expression may be accompanied by mtDNA instability and may be the foundation for MPV17 MDS.

Mitochondrial mass and copy number are important indicators of mitochondria integrity. Depletion of mtDNA by Mpv17 is tissue dependent. Others have also shown that changes in mtDNA copy number are also affected by cellular state, regardless of the tissue type. In human

fibroblasts, Mpv17 deficiency results in mtDNA depletion only in quiescence cells, but not in proliferating cells (11). This may explain why there was no difference in mtDNA copy number nor mitochondrial mass on HeLa cells deficient in Mpv17 (Figure 2.10).

In summary, this study reports the impacts of reduced Mpv17 expression in HeLa cells on FOCM. These data show that reduced Mpv17 expression depletes mitochondrial folate pools, and that mitochondrial dTMP synthesis capacity is not sufficient to prevent uracil misincorporation, suggesting that the mitochondrial genome requires access to cytosolic dTMP pools. We postulate that there are three important sources of dTMP for mtDNA replication: *de novo* synthesis, salvage synthesis, and transport from the cytoplasm. We predict that Mpv17 may be involved in the transport of dTMP from the cytosol to the mitochondria to maintain dTMP pool (Figure 2.11) for mtDNA synthesis. In the absence of the cytosolic sources of dTMP, mtDNA replication becomes dependent on increased rates of *de novo* and salvage dTMP synthesis, as well as misincorporation of dUTP. Whereas the impact of reduced Mpv17 expression (and the resulting severe reduction in mitochondrial folate pools) did not impact mitochondrial *de novo* dTMP synthesis in HeLa cells, the relationship between Mpv17 expression, mitochondrial folate pools and mitochondrial folate metabolism should be investigated in animal models, and their relationship to MDS. This report provides evidence that MPV17-related hepatocerebral MDS may be linked to altered dTMP synthesis and impaired FOCM.

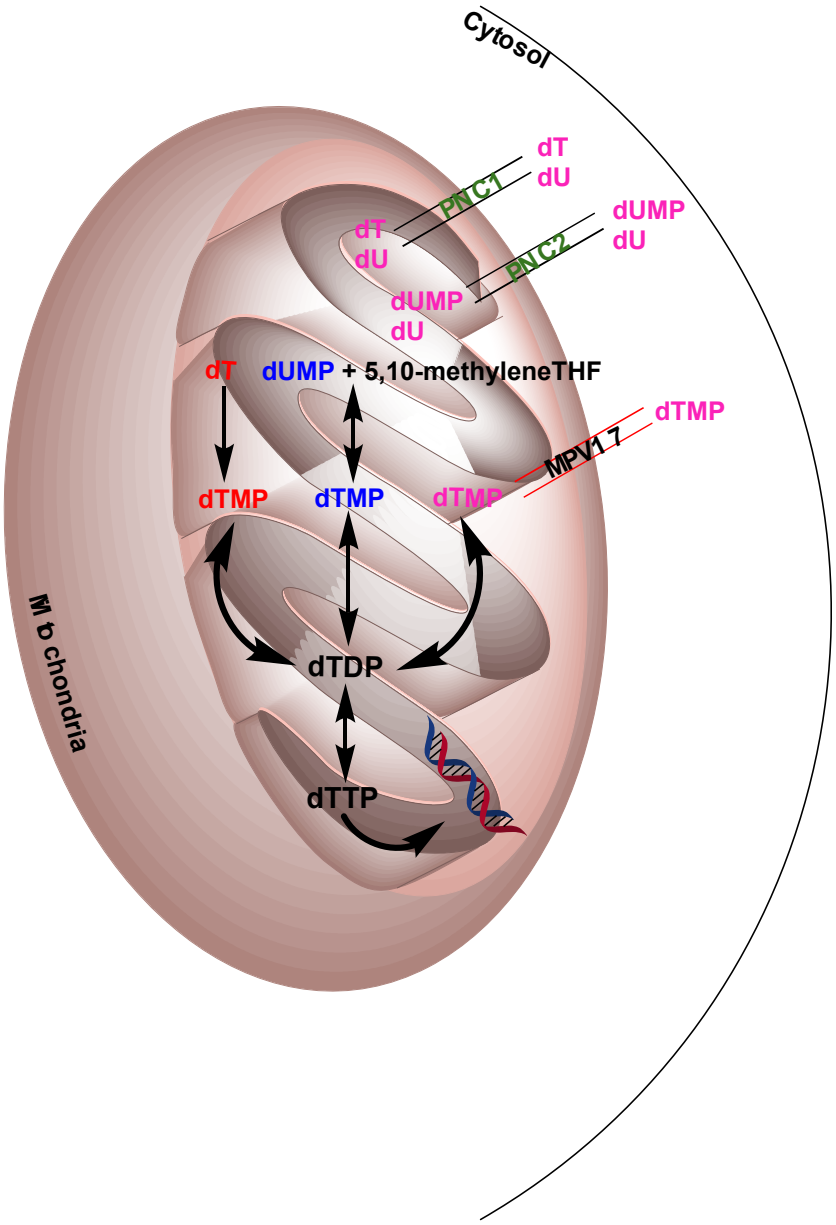


Figure 2.11: Proposed model: Three primary sources of the mitochondrial dTMP pool.
Source 1: ***Salvage dTMP synthesis using dT from cytosolic and mitochondrial pools***. Salvage synthesis of dTMP is independent of folate status; it relies on TK2 activity to convert dT to dTMP. Mitochondrial pyrimidine nucleotide transporter PNC1 facilitates the import of dT from cytosolic pools.
Source 2: ***de novo dTMP synthesis using dUMP from mitochondrial and cytosolic pools***. Cytosolic dUMP is imported into the mitochondria via PNC2 where it participates in folate-dependent *de novo* dTMP biosynthesis.
Source 3: ***Transport dTMP from cytosolic pools, to be incorporated into mitochondrial dTMP pool.*** dTMP synthesized in the cytosol via the salvage pathway or *de novo* pathway is imported into the mitochondria to help sustain mtDNA synthesis; the mitochondrial import of synthesized dTMP may be perturbed under Mpv17 deficient conditions.

METHODS

Stable cell lines: HeLa cells with reduced Mpv17 expression were generated using an MPV17 shRNA constructs (Origene Technologies, TR316619D ACCTGTCCTGGAAGGCACATCGGCTCTAA) with the Mirus Ingenio Electroporation Kit and NucleofectorTM Technology (Lonza). Clonal populations were selected using puromycin and transferred to 96-well plates. Initial puromycin concentrations were 0.25ug/ml, and were slowly increased up to 1ug/mL. Cells were isolated and expanded as individual cell lines while always maintaining puromycin selection. Control lines were generated under the same conditions but using scrambled shRNA construct (Origene Technologies, (5' GCACTACCAGAGCTAACTCAGATAGTACT 3'). MPV17 knockdown was validated by western blot normalized to protein concentration using a Lowry Protein Assay.

Culture conditions: Cells were cultured in HyClone Minimum Essential Medium Alpha (αMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 0.50 ug/ml puromycin to maintain selection. Modified αMEM (HyClone) lacking glycine, serine, methionine, folate, and nucleosides was used in experiments as indicated; modified αMEM was supplemented with 10% dialyzed fetal bovine serum, 200μM methionine, and 20nM (6S)-5-formyl-THF (folate-replete cells only).

Deoxyuridine (dU) suppression assay: This assay quantifies *de novo* dTMP synthesis capacity based on incorporation [^{14}C]-deoxyuridine, the precursor of folate-dependent *de novo* synthesis of dTMP, compared to [^{3}H]-thymidine incorporation from the salvage pathway, into DNA. Cells were plated 1:8 in modified αMEM containing 2μM [^{14}C]-2' deoxyuridine (American

Radiochemicals), and 25nM [^{3}H]-thymidine (Perkin Elmer). Cells were rinsed twice with 1X Phosphate-Buffered Saline (PBS) and detached from the tissue culture plates with 1X 0.25% Trypsin-EDTA (Corning); trypsin was neutralized with unlabeled media (for nuclear dU suppression) or with 10% FBS in 1X PBS (for mitochondrial dU suppression). For nuclear dTMP biosynthesis capacity, genomic DNA was extracted using DNAeasy Tissue and Blood Kit (Qiagen). For mitochondrial dTMP biosynthesis capacity, mitochondria were isolated, followed by mtDNA purification using Zyppy Plasmid Miniprep kit (Zymo Research). ^{3}H and ^{14}C channels were counted in dual DPM mode on an LS6500 scintillation counter (Beckman Instruments).

Mitochondrial extraction: Mitochondrial extraction was carried out on ice. Solutions and equipment were maintained 4°C or ice before and during the procedure. A total of 35 T175 flasks, 90-100% confluent, were collected per sample; cells were rinsed twice with 1X PBS and detached with 0.25% Trypsin-EDTA 1X (Corning); trypsin was neutralized with 10% FBS, 1% penicillin/streptomycin, in 1X PBS. Cell pellets were washed with 1X PBS and centrifuged (1000xg, 5mins) before proceeding with mitochondrial extraction. Extraction of mitochondria was performed with an OptiPrep (60% iodixanol, Sigma-Aldrich) discontinuous gradient with the following modifications. *OptiPrep discontinuous gradient*: Cell pellets were washed with homogenization medium (0.25 M sucrose, 1mM EDTA, 20mM HEPES-NaOH, pH 7.4, and freshly added protease inhibitor (PI)), followed by centrifugation. Pellet was resuspended in 5ml homogenization medium and homogenized in a Dounce homogenizer pestle A, followed by centrifugation (1000xg for 10 minutes in a fixed-angle rotor) to pellet nuclei. Pellet was suspended using a Dounce homogenizer pestle B and the mitochondria pelleted by centrifugation. Supernatants from both homogenization steps were combined and centrifuged at 17,000xg for 10

minutes to obtain a crude mitochondrial pellet. The crude mitochondrial pellet was resuspended with type B Dounce homogenizer. The crude fraction was adjusted to 36% (W/V) iodixanol with 50% iodixanol (diluted in 0.25M sucrose, 6mM EDTA, 120 mM Hepes-NaOH, pH 7.4, and fresh PI). The mitochondrial fraction+iodizanol solution was loaded on the bottom of ultracentrifuge tube and layered with equal parts of 25% and 20% iodixanol gradient solutions. One ml of the homogenization solution was added to the top layer. The sample was centrifuged at 100,000xg for 4 hours (Beckman SW41TI). The mitochondrial fraction was collected and diluted 3X with homogenization buffer. The diluted mitochondrial fraction was centrifuged at 30,000g for 30 minutes to pellet mitochondria.

Formate suppression assay: This assay quantifies the relative rate of *de novo* purine synthesis capacity as a ratio of (^{14}C)formate, the precursor of folate-dependent *de novo* synthesis, and (^{3}H)Hypoxanthine, a precursor for salvage purine biosynthesis. MPV17 stable knockdown and control cells were plated 1:6 in modified αMEM supplemented with 10% dialyzed fetal bovine serum, 200μM methionine, and 20nM (6S)-5-formyl-THF, 0.4nM [^{3}H]-hypoxanthine, and 4μM [^{14}C]-formate. At confluency, cells were rinsed twice with 1X Phosphate-Buffered Saline (PBS) and detached with 1X 0.25% Trypsin-EDTA (Corning). Nuclear DNA was isolated using DNAeasy Tissue and Blood Kit (Qiagen) according to manufacturer's protocols. ^{3}H and ^{14}C channels were counted in dual DPM mode on an LS6500 scintillation counter (Beckman Instruments).

Western blot Analyses and Densitometry: All primary antibodies were diluted in 5% Bovine Serum Albumin (BSA) and 0.02% sodium azide as indicated: Proliferating Cell Nuclear Antigen (PCNA,

nuclear marker, Cell Signaling, 1:1000, mouse); Hexokinase I and CoxIV (mitochondrial markers, Cell Signaling, 1:1000, rabbit); Folylpolyglutamate synthetase (FPGS, mouse, 1:1000, Zuckerman's laboratory, (37)); Mpv17 (Abcam, 1:80, rabbit or mouse), Anti-thymidine kinase 2, (Abcam, 1:100, rabbit); TYMS (Cell Signaling, 1:2000, rabbit). Secondary antibodies were diluted in 10% non-fat milk made in PBS. Densitometry was performed using ImageJ. Background was subtracted and intensities of non-saturated bands were quantified and normalized to loading control (Cox IV served as mitochondrial marker and mitochondrial loading control). Due to the size similarity, protein expressions of TK2 and TYMS were quantified on different western blots.

Microbiological Lactobacillus Casei Assay: Folate in whole cell samples as well as in isolated mitochondria were quantified as described in (38,39). Total folates were normalized to protein concentration (quantified with Lowry Protein Assay) for each given sample.

Folate accumulation: Cells were plated in triplicates in 6-well plates in αMEM until 50% confluency. Growth medium was replaced with modified MEM containing 25nM 6S-[^{3}H]5-formylTHF and the cells cultured for 12 hours. Cells were harvested, rinsed twice with 1X PBS, and counted with an automated cell counter (Bio-Rad, TC-20). Cells were lysed with 0.2M ammonium hydroxide. Tritium in the cells was quantified in a LS6500 scintillation counter (Beckman Instruments) and normalized to the number of cells.

Folate uptake: Uptake of folate over time was determined. Cells were plated in triplicates in 6-well plates in αMEM. Once cells reached about 50% confluency, they were labeled with 25nM 6S-[^{3}H]5-formylTHF present in modified MEM for different time periods as shown in the graph.

Cells were detached with 0.25% Trypsin-EDTA (Corning) at the given time points, rinsed twice with 1X PBS, and counted with an automated cell counter (Bio-Rad, TC-20). Cells were lysed in 0.2M ammonium hydroxide. Tritium in the cells was quantified in a scintillation counter and normalized to the number of cells.

Measurements of folate turnover by Pulse-Chase: Cells were plated in 15cm plates and cultured in αMEM. When cells reached 70-80% confluency, media was replaced with radioactive 25nM 6S-[^{3}H]5-formylTHF modified MEM. After 13 hours in labeled media, cells were rinsed twice with 1X PBS, detached with 0.25% Trypsin-EDTA (Corning) and counted with an automated cell counter (Bio-Rad, TC-20). About 1/5 of the plate was harvested (time zero); the rest of the cells were passaged into 10x6-well plates to be harvested under the same conditions at different time points. Each measurement was performed in triplicate. Harvested cells were lysed with 0.2M ammonium hydroxide. Tritium remaining inside the cells was quantified in a scintillation counter and normalized to the number of cells.

Uracil content in DNA: Uracil present in both nuDNA and mtDNA was quantified by gas chromatography-mass spectrometry (GC-MS) and normalized to micrograms of DNA. For isolating nuDNA, whole cells were harvested by trypsinizing. NuDNA was extracted using Roche High Pure PCR Template Preparation Kit and eluted in DNase/RNase free water. NuDNA was briefly sonicated, and treated with RNase A for 30 minutes. For isolation of mtDNA, mitochondria were isolated as described above, followed by mtDNA extraction using Zyppy Plasmid Miniprep kit (Zymo Research) and eluted in DNase/RNase free water. MtDNA was briefly sonicated, and treated with RNase A for 30 minutes. DNA from nuclear and mitochondrial samples were

quantified as previously described (40) with the following modification; 50 pg of $^{15}N_6$ uracil was added as an internal standard to all samples and standards.

Mitochondrial content and mass: Mitochondrial DNA content, the number of mitochondrial genomes per cell, was quantified by real-time quantitative PCR (rt-qPCR; Roche LightCycler® 480) as previously described (41), using LightCycler® 480 SYBR Green I Master (Roche) and 3ug DNA per reaction. Mitochondrial mass, number of mitochondria per cell, was quantified with the Citrate Synthase Activity Assay Kit (Invitrogen) according to manufacturer's instructions. Citrate synthase activity is a marker for mitochondrial mass. Samples consisted of whole cell extracts normalized to total cellular protein. Protein was quantified by Lowry Protein Assay.

Isolation of nucleotides in mtDNA: This procedure confirms that dTMP precursors, dU and dT, were incorporated into mtDNA as dTTP. Cells were cultured and harvested in the presence of [^{14}C]-deoxyuridine and [^{3}H]-thymidine, as described in dU suppression. Mitochondria were isolated, followed by mtDNA purification using Zyppy Plasmid Miniprep kit (Zymo Research). DNA was digested to nucleosides as previously described (42). Nucleosides were separated by HPLC with a Synergi Fusion-RP column (Phenomenex) using a binary buffer system with a flow rate of 1 ml/min as previously described (18). Radioactivity in each fraction was quantified using a Beckman LS-6500 liquid scintillation counter in dual disintegrations/min mode.

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Conflict of interest:

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions:

JRA, MSF, and PJS designed the research and wrote the manuscript. CV generated the MPV17 shRNA knockdown stable cell lines used for this research. JRA conducted the research. MSF separated nucleotide bases by HPLC (Figure 2.5). All authors analyzed the results and approved the final version of the manuscript.

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CHAPTER 3 - Mitochondrial DNA content is affected by SHMT2 Expression but not by Exogenous Glycine and Serine Concentrations

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ABSTRACT

Serine hydroxymethyltransferase (SHMT) catalyzes the reversible conversion of tetrahydrofolate to 5,10-methyleneTHF for *de novo* dTMP synthesis, using serine and glycine as one-carbon (1C) donors. This reaction takes place in the mitochondria (SHMT2 isoform) as well as the nucleus (SHMT1 and SHMT2 isoforms). Lack of cytosolic/nuclear SHMT affects nuclear *de novo* dTMP biosynthesis capacity in mice. Similarly, Chinese Hamster Ovary (CHO) cells lacking SHMT2 activity have reduced mitochondrial dTMP synthesis capacity. This study explores how lack of SHMT2 and availability of 1C donors, glycine and serine, affect mitochondrial DNA (mtDNA) integrity in human cells in culture. Mitochondrial mass, mtDNA content, and uracil misincorporation were measured as indicators of mtDNA integrity. Uracil misincorporation in the mitochondrial genome was quantified to evaluate mitochondrial *de novo* dTMP biosynthesis capacity. Neither mtDNA content nor uracil levels in mtDNA of HeLa cells were affected by exogenous glycine or serine. Folate levels in culture medium did not affect mtDNA content. Reduced SHTM2 expression increased mtDNA content by 65% to 164% in HeLa cells, but has no effect on mtDNA content from MCF-7 cells. This suggests that in HeLa cells, enzymatic disruption of mitochondrial dTMP synthesis affects mtDNA content, and possibly mtDNA integrity.

INTRODUCTION

De novo thymidylate (dTMP) biosynthesis occurs in the cytosol, nucleus, and mitochondria. Serine hydroxymethyltransferase (SHMT), one of four enzymes required for the *de novo* dTMP biosynthesis, is a pyridoxal phosphate and folate-dependent enzyme that catalyzes the reversible conversion of serine and tetrahydrofolate (THF) generating 5,10-methyleneTHF and glycine (Figure 3.1). Methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) is also a source of 5,10-methyleneTHF using mitochondria-derived formate as a one-carbon (1C) donor for nuclear *de novo* dTMP synthesis (1). Thymidylate synthase (TYMS) transfers a 1C unit from 5,10-methyleneTHF to deoxyuridine monophosphate (dUMP) to generate dTMP and dihydrofolate (DHF). Dihydrofolate reductase (DHFR) then regenerates THF from DHF to allow for another round of dTMP biosynthesis. These reactions are part of folate-dependent one-carbon metabolism (FOCM) (Figure 3.1), an interconnected metabolic network in which folate cofactors donate 1C units for the *de novo* synthesis of dTMP, *de novo* synthesis of purines, and homocysteine remethylation to methionine to support methylation reactions.

SHMT is encoded by two genes, *SHMT1* and *SHMT2*, which share 63% amino acid sequence identity (2,3). *SHMT1* encodes for the cytosolic/nuclear isoform SHMT1, and *SHMT2* encodes two isoforms through alternative splicing of the hnRNA transcript, mitochondrial isoform SHMT2, and cytosolic/nuclear isoform SHMT2α. Nuclear/cytosolic isoforms of all enzymes known to be required for nuclear *de novo* dTMP synthesis (SHMT1/2, TYMS, MTHFD1, and DHFR) are translocated from the cytosol to the nucleus during S phase and during DNA damage for nuclear DNA (nuDNA) synthesis after being modified by the small ubiquitin-like modifier (SUMO) protein. After SUMO-dependent translocation, dTMP biosynthesis enzymes form a multienzyme complex associated with the nuclear lamina (4,5). Mitochondrial isoforms of *de novo* dTMP biosynthesis enzymes (SHMT2, TYMS, and DHFR2) reside in the mitochondrial matrix and mitochondrial inner membrane, for mitochondrial *de novo*

dTMP synthesis (6). SHMT2 has been identified as a protein associated with mtDNA nucleoids in humans (7), suggesting that its role in the mitochondrial dTMP synthesis may mirror that of its cytosolic/nuclear counterpart, facilitating *de novo* dTMP synthesis at sites of DNA replication.

SHMT1 acts as a scaffold for nuclear *de novo* dTMP multienzyme complex formation with the nuclear lamina. Disrupting the complex's formation by preventing SHMT binding to lamin or disruption of SUMOylation motifs, decreases *de novo* dTMP synthesis capacity in the nucleus (8-10). The contribution of the catalytic activity of nuclear SHMT is second to its lamin-binding activity in stimulating nuclear *de novo* dTMP synthesis, as cells expressing a catalytically inactive SHMT1 mutant protein also increased *de novo* dTMP synthesis capacity (8). Mice that lack SHMT1 show loss of nuDNA integrity, as demonstrated by increased levels of uracil due to disruption in the dTMP synthesis and increased double-strand breaks as measured by phosphorylated γH2AX (1); nuclear dTMP synthesis is not entirely abolished in *Shmt1*$^{-/-}$ mice, since SHMT2 still accounts for ~25% of total nuclear SHMT activity (10,11).

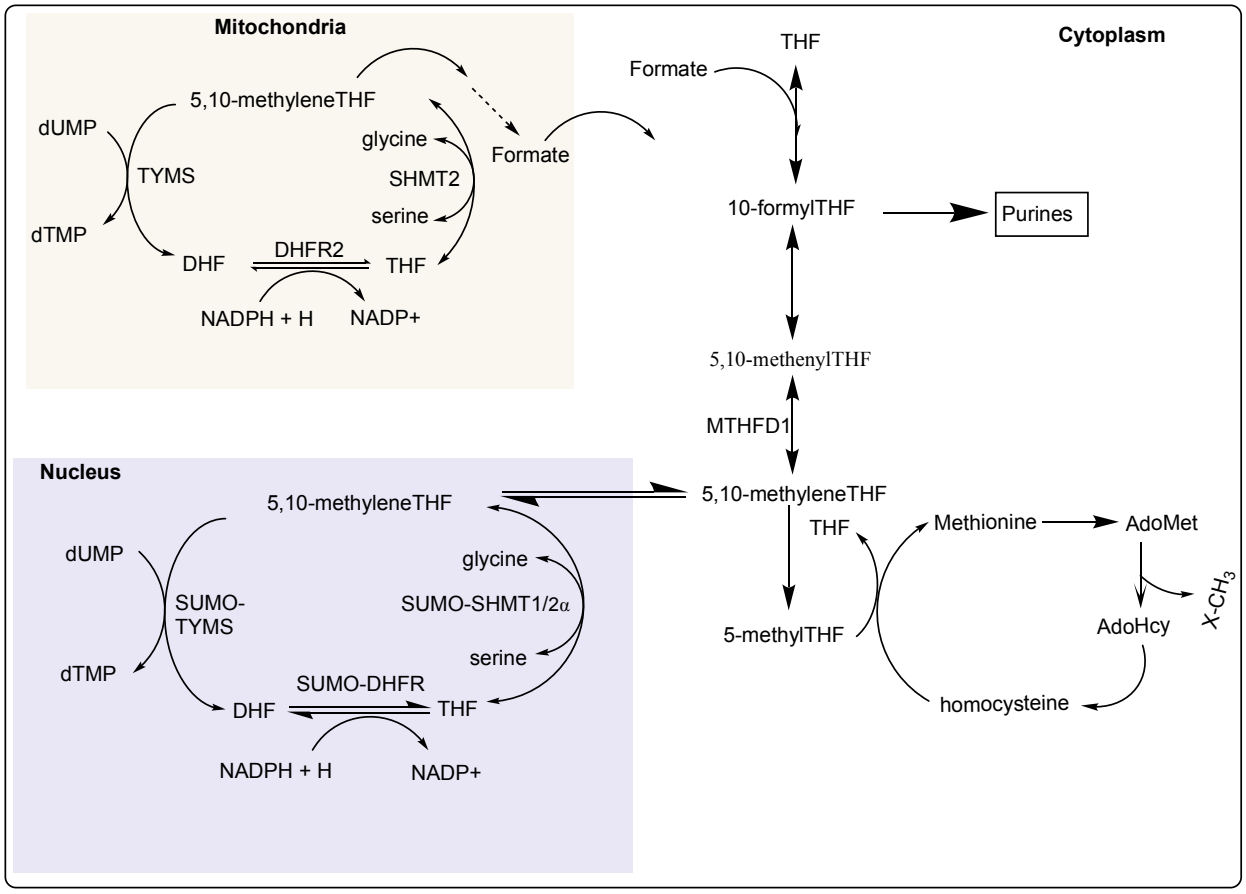

Figure 3.1: *De novo* dTMP biosynthesis in mitochondria and nucleus.
SHMT (SHMT1 and SHMT2) and Methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) synthesize 5,10-methyleneTHF for methylation of dUMP by TYMS. Synthesis of dTMP by TYMS releases DHF as a byproduct. DHF is then reduced by DHFR (or DHFR2 in mitochondria) to THF. Nuclear enzymes are SUMOylated. Formate is also generated as part of mitochondrial FOCM, which is transported to the cytoplasm as a 1C donor for purine and methionine synthesis.

Isolated mitochondria from mammalian cells incorporate labeled serine into mitochondrial dTMP via *de novo* dTMP synthesis (6). *GlyA* Chinese Hamster Ovary (CHO) cells lacking SHMT2 activity have a 94% reduction in mitochondrial dTMP synthesis capacity (6), suggesting that nuclear/cytosolic isoforms of SHMT cannot compensate for lack of mitochondrial SHMT in mitochondrial dTMP synthesis. In *Saccharomyces cerevisiae*, the direction of the reversible reactions by SHMT isoforms respond to changes in glycine and serine concentrations (12). In human cancer cells, it has also been shown that SHMT1 and MTHFD1 play fundamental roles in partitioning of folate derivatives to protect nuclear *de novo* dTMP synthesis at the expense of methionine and *S*-adenosylmethionine synthesis, and cytosolic/nuclear SHMT catalytic activity is influenced by exogenous glycine concentration (13). How the catalytic activity of mitochondrial SHMT2 may be affected by exogenous glycine and serine concentrations in human cells has not been explored.

The biological relevance of having three independent cellular compartments for *de novo* dTMP biosynthesis pathways is not understood. There is also limited knowledge of regulatory factors for mitochondrial *de novo* dTMP synthesis. Thymidine triphosphate is the only non-essential DNA nucleotide since its precursor; dUTP can be incorporated into DNA when dTTP pools are low, as DNA polymerases do not distinguish between dUTP and dTTP. The presence of dUTP in DNA as a result of diminished dTTP concentrations is not mutagenic, but does result in DNA instability, increasing the risk for DNA double-strand breaks. Abasic sites in the genome may lead to apoptosis if these are not adequately repaired (14-16). The mitochondrial genome is not exempt from these events; *glyA* CHO cells, are glycine auxotrophs and have 40% more uracil in mtDNA than wildtype CHO cells (6), suggesting that mtDNA integrity may be affected. The goal of this work is to determine the contributions of SHMT2 expression as well as exogenous serine and glycine availability on mtDNA integrity in human cells.

RESULTS

Exogenous serine and glycine do not affect HeLa mtDNA content or integrity.

MtDNA copy number was quantified in HeLa (human cervical cancer) cells in response to exogenous serine and glycine availability (0mM to 10mM, modified MEM) as well as standard culture conditions (αMEM). There was no difference in mtDNA content for HeLa cells cultured in standard medium conditions versus HeLa cells cultured in medium lacking both, glycine and serine (Figure 3.2a). Similarly, mtDNA content was not affected by exogenous glycine and serine added to the culture medium (Figure 3.2a). Mitochondrial mass, a measure of the number of mitochondria per cell, was evaluated in cells cultured with increasing glycine concentrations, however, exogenous glycine did not affect mitochondria mass (Figure 3.2c). Altering medium glycine and serine concentrations did not affect mtDNA content or mitochondrial mass. To monitor mitochondrial dTMP biosynthesis capacity, uracil levels in mtDNA were quantified under the same glycine and serine conditions. Uracil levels in mtDNA were refractory to serine and glycine availability (Figure 3.2b).

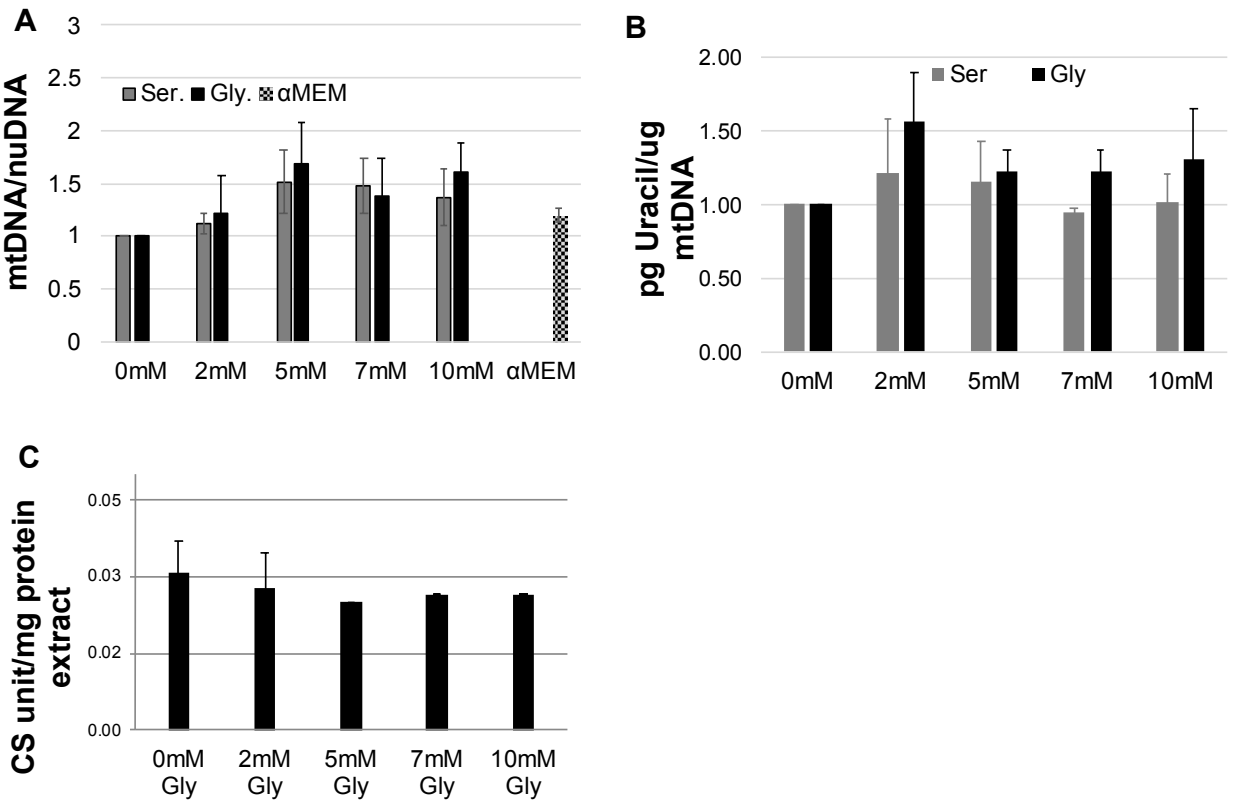

Figure 3.2: Exogenous serine and glycine concentrations do not affect mtDNA content in Hela cells. **A)** The mtDNA/nuDNA ratio is a measure of mtDNA copy number per cell, which was not sensitive to serine or glycine concentrations in media. αMEM contains 0.666mM serine and 0.666mM glycine. N=4, each in triplicates, NS. **B)** Uracil levels in mtDNA are unresponsive to changes in glycine and serine concentrations in the culture medium. Data is represented as picograms of uracil per micrograms of mtDNA, relative to 0mM glycine 0mM serine. N=3, NS. **C)** Mitochondrial mass corresponds to the number of mitochondria per cell. Citrate synthase (CS) activity, a marker of mitochondrial mass, was quantified in whole cell protein lysates. Serine concentration is 0.666mM. Mitochondrial mass not altered by different exogenous glycine concentrations. N=3, each in triplicates, NS. **Data shown as means standard error. Statistical significance was determined by 2-tailed Student's t-test (NS=not significant p>0.05).**

SHMT2 expression increases HeLa cell mtDNA content.

SHMT2 expression was reduced in two human cancer cell lines, HeLa (cervical cancer) and MCF-7 (breast cancer), using two independent siRNA *SHMT2* constructs (Figure 3.3a). Hela cells with reduced SHMT2 expression exhibited 65% to 164% more mtDNA content than Hela cells transfected with scrambled siRNA control (Figure 3.3b). MtDNA content in breast cancer MCF-7 cells was not affected by reduced *SHMT2* expression (Figure 3.3c). MtDNA content was also quantified in HeLa cells with wild-type SHMT2 expression cultured in folate-deficient media (Modified MEM) for at least four cell doublings. MtDNA content in HeLa cells was not affected by folate status in culture medium (Figure 3.4).

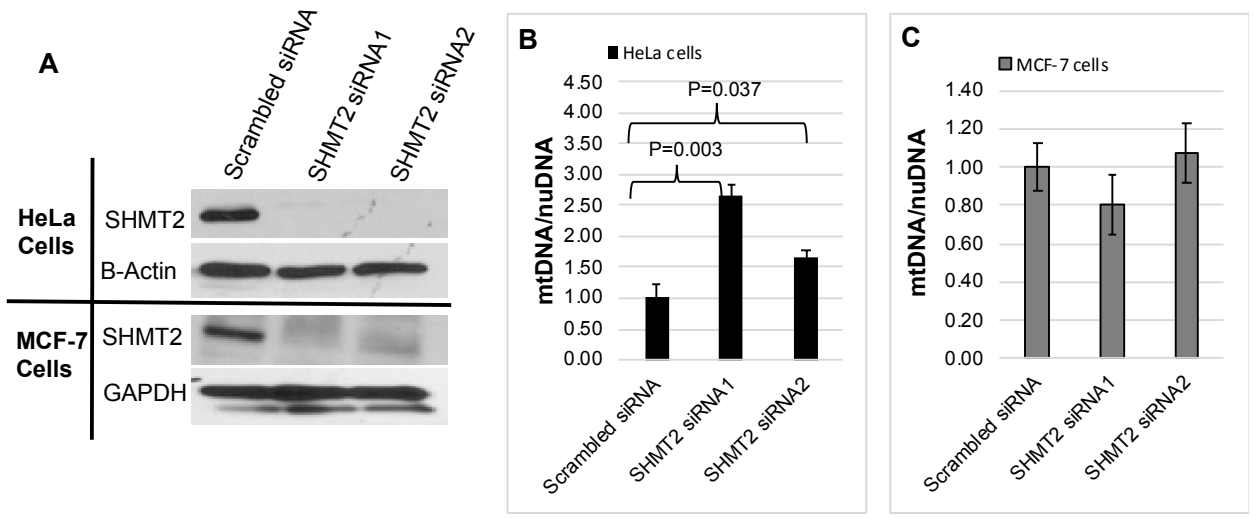

Figure 3.3: Reduced SHMT2 expression increases mtDNA content in HeLa cells. A) SHMT2 protein expression is reduced in cells transfected with two different SHTM2 siRNA constructs. Protein lysates correspond to matched DNA samples used for Figures **B** and **C**. **B)** MtDNA content is elevated 164% in cells transfected with siRNA 1 and 65% in cells transfected with siRNA 2 than in HeLa cells transfected with scrambled siRNA control. MtDNA/nuDNA represents copies of mtDNA per cell relative to scrambled siRNA control. n=3. **C)** MtDNA content in MCF-7 cells is not affected by SHMT2 expression. MtDNA/nuDNA represents copies of mtDNA per cell relative to scrambled siRNA control. n=3, NS. **Data shown as means standard error. Statistical significance was determined by 2-tailed Student's t-test (NS=not significant p>0.05).**

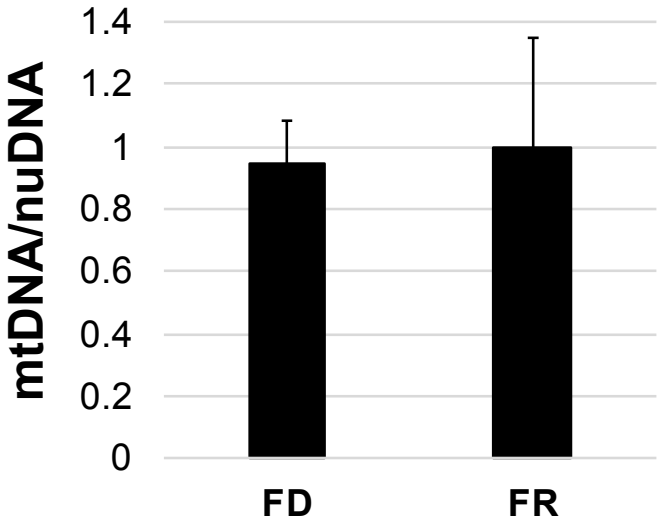

Figure 3.4: MtDNA content in Hela cells is not affected by folate status. MtDNA/nuDNA represents copies of mtDNA per cell relative to mtDNA content from Hela cells in folate-replete (FR) conditions. Cells were cultured in modified MEM lacking folate for at least four doublings. FD= folate deficient, n=4, in triplicates; FR=folate replete, n=2, in triplicates; NS. **Data shown as means standard error. Statistical significance was determined by 2-tailed Student's t-test (NS=not significant p>0.05).**

DISCUSSION

SHMT is a highly conserved protein (2) that links glycine/serine metabolism to folate metabolism by facilitating the use of 1C units from serine and glycine for FOCM (10,12,13,17). SHMT1 is a determinant of nuclear *de novo* dTMP synthesis capacity and genome stability by mediating the distribution of 5,10-methyleneTHF, which can carry 1C units for *de novo* dTMP biosynthesis or homocysteine remethylation (6,10,13). The role of cytosolic/nuclear SHMT in *de novo* dTMP biosynthesis has been shown to be not just enzymatic, but also structural, as SHMT1 and SHMT2α are required as scaffold proteins for *de novo* dTMP biosynthesis complex formation (6,9,10).

In mitochondria, the biological importance of SHMT2 is evident in that decreased SHMT2 protein levels result in limited *de novo* dTMP biosynthesis capacity and elevated uracil levels in mtDNA in *glyA* CHO cells (6). Mammalian cells lacking mitochondrial SHMT2 are glycine auxotrophs. It has been suggested that in some tissues, mitochondrial SHMT favors glycine synthesis from serine, while cytosolic SHMT favors serine synthesis, and thus SHMT activity may be affected by amino acids (glycine and serine) availability (12,18). Serine and glycine are major 1C donors for mitochondrial FOCM, via either SHMT2 or the glycine cleavage system (GCS) (19-21). The GCS is a system of four proteins that degrade glycine to provide the 1C units for 5,10-methyleneTHF formation (22). SHMT2 is expressed ubiquitously whereas the GCS shows a narrower range of expression (23). None of the cell lines used for this study, HeLa and MCF-7, express GCS enzymes. HeLa and MCF-7 cells lack GCS, which suggests that they may rely on SHMT2 for the provision of 1Cs for mitochondrial *de novo* dTMP synthesis. In this study, the effects of reduced SHMT2 expression and exogenous glycine and serine concentrations on mtDNA copy number, mitochondrial mass, and uracil levels in mtDNA were determined.

Mitochondrial DNA content was measured in HeLa cells cultured in standard medium conditions (αMEM) and compared to cells cultured in medium lacking both glycine and serine, as well as varying

concentrations of glycine and serine, ranging from 2mM to 10mM (Figure 3.2a). Similarly, impaired mitochondrial *de novo* dTMP synthesis was monitored by measuring uracil in mtDNA (Figure 3.2b), a proxy for dTMP biosynthesis capacity since uracil misincorporation in DNA results from an unfavorable dUTP/dTTP ratio (14). It was hypothesized that increasing glycine concentration in the culture medium could drive the SHMT2-catalyzed reaction away from 5,10-methyleneTHF synthesis, thus impairing mitochondrial *de novo* dTMP synthesis and resulting in mtDNA depletion and increased levels of uracil misincorporation in mtDNA. Similarly, it was anticipated that increasing cellular serine concentration would favor *de novo* mitochondrial dTMP synthesis. These data indicate that extracellular glycine and serine concentrations do not affect mtDNA content nor uracil misincorporation in HeLa cells (Figures 3.2a-b). This suggests that exogenous serine and glycine availability does not determine THF availability for mitochondrial *de novo* dTMP synthesis capacity in HeLa cells.

However, these results do not reflect the utilization of other 1C donors for mitochondrial FOCM, such as sarcosine and dimethylglycine (24,25), which may compensate for alterations of glycine/serine in the culture medium. Under some conditions and by unknown mechanisms, mitochondrial mass has been reported to adapt to changes in mtDNA content to protect mitochondrial function (26-28). Given that mtDNA content and mitochondrial uracil levels showed no dose-response effect to glycine and serine (Figures 3.2a-b), mitochondrial mass was measured in HeLa cells cultured in different glycine concentrations ranging from 0mM to 10mM. Nevertheless, mitochondrial mass in HeLa cells was also unresponsive to the increasing glycine concentrations in culture medium (Figure 3.2c).

SHMT2 protein expression is known to be elevated in many cancers (29,30). Lack of SHMT2 protein expression affected mtDNA integrity by increasing mtDNA content in HeLa cells by at least 65% (Figures 3.3a-b) but not in mCF-7 (Figures 3.3a & c). This suggests that mtDNA content in mammalian cells respond in a tissue-specific manner to changes in SHMT2 expression.

Similarly, mtDNA content in HeLa cells did not change when cells were cultured in folate-deficient media and wildtype SHMT2 expression (Figure 3.4). These results indicate that mtDNA content in HeLa cells is not affected by folate status in the media. Several other nuclear encoded genes are known to affect mtDNA content differently by undetermined reasons in a tissue-specific manner, such as thymidine kinase 2 and mitochondrial inner membrane protein MPV17 (31,32). Overall, the results of this study indicate that SHMT2 expression can be a determinate of mtDNA copy number in cancer cells, but that the exogenous serine/glycine ratio in culture medium does not affect mtDNA integrity or dTMP synthesis for mtDNA replication.

METHODS

Culture conditions: HeLa cells were cultured in HyClone Minimum Essential Medium Alpha (αMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin. Modified MEM (HyClone) refers to medium lacking glycine, serine, methionine, folate, and nucleosides. Modified MEM was supplemented with 10% dialyzed fetal bovine serum, 200μM methionine, 20nM (6S) 5-formyl-THF (replete folate cells only), and serine or glycine as indicated. For all conditions, cells were cultured for at least four doublings in proper medium.

Mitochondrial content: The number of mtDNA copies per cell was quantified by real-time quantitative PCR (qPCR; Roche LightCycler 480) as previously described (Venegas, Wang et al. 2011), using LightCycler 480 SYBR Green I Master (Roche) and 3ug DNA per reaction. Data is shown relative to 0mM glycine 0mM serine.

Uracil content in DNA: Uracil in mitochondrial DNA was quantified by GC-MS and normalized to micrograms of DNA used. Mitochondria were isolated using Qproteome Mitochondria Isolation Kit (Qiagen), according to manufacturer's instructions and the following modification. Mitochondria pellet was resuspended in disruption buffer again to undergo another round of homogenization; the final mitochondrial pellet was then resuspended in mitochondria storage buffer until needed. The mtDNA was isolated from using a QIAprep Spin Miniprep kit (Qiagen) and eluted in DNase/RNase free water. MtDNA was incubated with 10 units of Uracil-DNA-glycosylase, UNG, (EpiCentre) for 1 hour at 37C. UNG was then heat-inactivated for 3 minutes at 95C, placed on ice, centrifuged at maximum speed for 1 minutes and allowed to dry to completion. Samples were allowed to dry uncapped inside a desiccator for at least 3 days to continue with quantitation as previously described (11) with following modification: addition of

50 pg of $^{15}N_6$ uracil to samples and uracil standards as an internal control. Data is represented as picograms of uracil per micrograms of mtDNA, relative to 0mM glycine 0mM serine.

Mitochondrial mass: The number of mitochondria per cell was quantified by the Citrate Synthase Activity Assay Kit (Invitrogen) according to manufacturer's instructions. Citrate synthase activity was normalized to protein concentration from whole cell extracts.

SHMT2 siRNA knockdown: SHMT2 was knockdown in HeLa cells with a Nucleofector (Lonza) using two different SHMT2 siRNA constructs designed by the vendor (Qiagen, Hs-SHMT2-8 5'-CGGCTACATGTCTGACGTCAA-3' and Hs-SHMT2-10 5'-CCCAGCCAACCTGGCCGTCTA-3' correspond to shRNA 1 and 2 respectively), according to manufacturer's instructions. SHMT2 knockdown was validated by Western Blot.

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Conflict of interest:

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions:

JRA, MSF, and PJS designed the research and wrote the manuscript. JRA conducted the research. All authors analyzed the results and approved the final version of the manuscript.

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CHAPTER 4: Future Directions and Unanswered Questions

Chapter 2 -MPV17

Deficiency in MPV17 is associated with hepatic mtDNA depletion syndrome (MDS). MPV17 function is unknown, but it has been postulated to be a mitochondrial transport protein; *in vitro* studies demonstrated that purified recombinant MPV17 "opens" and "closes" under certain conditions such as changes in membrane potential and pH (1). These variables must be taken into account when designing experiments to test the transporter hypothesis. In chapter two, we showed that lack of MPV17 expression in HeLa cells induces depletion of mitochondrial folate pools by ~43%. Mitochondria from MPV17 knockdown cells can still synthesize dTMP and incorporate it into mtDNA, but the supply of dTMP insufficient to sustain mtDNA synthesis, and therefore these cells require access to cytosolic dTMP pools for DNA replication. Based on these results, MPV17 was postulated to be a mitochondrial dTMP (and probably dUMP) transporter. Additional experiments must be performed to test this hypothesis.

Our results also showed that mitochondrial purine pools were reduced in MPV17 knockdown cells. Others have shown that mitochondrial dGTP, but not dATP, levels are reduced in mouse liver with reduced MPV17 (2). It is not clear if MPV17 expression affects both dATP and dGTP or just dGTP pools in human cells. Both mitochondrial pyrimidine nucleotide carriers, PNC1 and PNC2 (SLC25A33 and SLC25A36) (3,4), transport GTP into the mitochondria, but with a lower affinity than for primary substrates (UTP and CTP respectively). If MPV17 is in fact, a dTMP transporter, it is possible that MPV17 transports GTP as well, and this should be investigated.

To test the hypothesis that MPV17 functions as a mitochondrial dTMP transporter, a protocol adapted from Gonzalez-Vioque et. al. 2011 (5) may be used. Fresh mitochondria from MPV17 KD and control stable lines could be isolated and incubated with [^{3}H]-labeled nucleotides as described in Antonenkov, Isomursu et al. 2015 (1). After incubation, mitochondria could be pelleted and washed until

washes have no traces of radioactive labels or near background. Washed mitochondria would be then lysed to release the [^{3}H-]-label, which would be quantified in a scintillation counter. ^{3}H counts would be normalized to total protein.

Suppressed MVP17 expression in HeLa cells reduced mitochondrial folate levels. Most of the 1C units for cytosolic and nuclear FOCM come from mitochondrial serine metabolism (~70%) (6). Are mitochondrial and nuclear *de novo* dTMP biosynthesis in MPV17-deficient cells prioritized over other folate-dependent endpoints, such as *de novo* purine biosynthesis? It is relevant to determine if- and how- a reduction in mitochondrial folate pools induced by MPV17 may affect cytosolic/nuclear 1C units and FOCM. Our data shows that neither nuclear *de novo* dTMP biosynthesis capacity or nuclear uracil misincorporation were affected by the expression of MPV17, suggesting that lack of MPV17 does not impair folate-dependent nuclear *de novo* dTMP biosynthesis.

Chapter 3 –SHMT2

Deficiency in serine hydroxymethyltransferase is not associated with MDS, but mice lacking SHMT1 show sign of disturbed dTMP biosynthesis. *SHMT2* (mitochondrial and nuclear), but not *SHMT1* (cytosolic/nuclear), is ubiquitously expressed. SHMT2α activity accounts for only ~25% of nuclear dTMP synthesis capacity in *Shmt1*$^{-/-}$ mice (7). It is not clear how *de novo* dTMP biosynthesis is regulated in tissues that do not express SHMT1. It is possible that the contributions of individual SHMT isoforms vary among cell types. It may be useful to further investigate the individual contributions by SHMT isoforms to the multiple dTMP pools. Quantification of dTMP nucleotides for each compartment is feasible.

Equally important, the biological relevance of having multiple *de novo* dTMP biosynthesis pathways must be determined. It is unclear how much crosstalk there is among nuclear, cytosolic, and mitochondrial dTMP pools. The dTMP biosynthesis capacity should be explored under the following conditions: lack of SHMT2 (mitochondria), perturbed SUMOylation of SHMT1/SHMT2α (cytoplasm),

and lack of SHMT1 and SHMT2α (nucleus, regulated by cell cycle). There are empirical reasons to believe that there is limited crosstalk between nuclear and mitochondrial dTMP pools. Nuclear dTMP biosynthesis is cell cycle dependent. It implies that if the nucleus shares its dTMP pool with mitochondria, it might only happen during S phase. Nuclear *de novo* dTMP biosynthesis is believed to occur at the replication fork (8), which would make sharing nuclear and mitochondria pools more challenging. If mitochondrial dTMP pools were to be shared with the nucleus, it might not be sufficient to sustain both nuclear and mitochondrial dTMP pools since mice lacking SHMT1 have compromised nuclear *de novo* dTMP synthesis (7,9). If there is any crosstalk, the cytosolic dTMP pool is most likely to be the one shared with other compartments, but further investigation is needed.

Nuclear *de novo* dTMP synthesis requires SUMOylation of dTMP biosynthesis enzymes as well as assembly of a complex to the nuclear lamina. Mitochondrial isoforms of these enzymes already reside inside the mitochondria matrix and inner membrane. SUMOylation of mitochondrial proteins is important for mitochondria morphology and function (10-13). If SUMOylation of nuclear dTMP enzymes is required as a nuclear transport signal, it is possible that SUMOylation is not needed for mitochondrial dTMP synthesis; however, this possibility should be tested. Similarly, it is not known if mitochondrial dTMP biosynthesis enzymes form a complex like their nuclear counterparts. More research is required to understand the underlying mechanisms and regulation of mitochondrial *de novo* dTMP biosynthesis and how it affects mtDNA integrity in the context of mtDNA-related disorders.

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