Nuclear Localization of de Novo Thymidylate Biosynthesis Pathway Is Required to Prevent Uracil Accumulation in DNA*

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Received for publication, September 26, 2011, and in revised form, October 14, 2011. Published, JBC Papers in Press, November 4, 2011, DOI 10.1074/jbc.M111.307629

Background: S phase nuclei contain the thymidylate synthesis pathway.

Results: Mice overexpressing a Shm1 transgene exhibit elevated expression of SHMT1 and TYMS, impaired nuclear localization of the thymidylate biosynthesis pathway, and elevated uracil in DNA.

Conclusion: SHMT1 and TYMS localization to the nucleus is essential to prevent uracil accumulation in DNA.

Significance: SHMT1-mediated nuclear de novo thymidylate synthesis is critical for maintaining DNA integrity.

Uracil accumulates in DNA as a result of impaired folate-dependent de novo thymidylate biosynthesis, a pathway composed of the enzymes serine hydroxymethyltransferase (SHMT), thymidylate synthase (TYMS), and dihydrofolate reductase. In G1, this pathway is present in the cytoplasm and at S phase undergoes small ubiquitin-like modifier-dependent translocation to the nucleus. It is not known whether this pathway functions in the cytoplasm, nucleus, or both in vivo. SHMT1 generates 5,10-methylenetetrahydrofolate for de novo thymidylate biosynthesis, a limiting step in the pathway, but also tightly binds 5-methyltetrahydrofolate in the cytoplasm, a required cofactor for homocysteine remethylation. Overexpression of SHMT1 in cell cultures inhibits folate-dependent homocysteine remethylation and enhances thymidylate biosynthesis. In this study, the impact of increased Shm1 expression on folate-mediated one-carbon metabolism was determined in mice that overexpress the Shm1 cDNA (Shm1Tg mice). Compared with wild type mice, Shm1Tg mice exhibited elevated SHMT1 and TYMS protein levels in tissues and evidence for impaired homocysteine remethylation but surprisingly exhibited depressed levels of nuclear SHMT1 and TYMS, lower rates of nuclear de novo thymidylate biosynthesis, and a nearly 10-fold increase in uracil content in hepatic nuclear DNA when fed a folate- and choline-deficient diet. These results demonstrate that SHMT1 and TYMS localization to the nucleus is essential to prevent uracil accumulation in nuclear DNA and indicate that SHMT1-mediated nuclear de novo thymidylate synthesis is critical for maintaining DNA integrity.

Depletion of de novo DTMP synthesis caused by folate deficiency, antifolate inhibitors, or genetic disruption of the pathway results in deoxyuridine misincorporation into nuclear DNA leading to genome instability (1–3). De novo thymidylate biosynthesis is distinct from the other nucleotide synthesis pathways in that it is compartmentalized at the sites of DNA replication, namely the mitochondria (4) and nucleus (5, 6). The de novo thymidylate biosynthesis pathway requires three enzymatic activities: serine hydroxymethyltransferase (SHMT), which catalyzes the conversion of serine and tetrahydrofolate (THF) to form glycine and methyleneTHF; thymidylate synthase (TYMS), which catalyzes the conversion of methyleneTHF and dUMP to dTMP; and dihydrofolate (DHF). The cycle is completed by the conversion of DHF to THF in a NADPH-requiring reaction catalyzed by dihydrofolate reductase (DHFR) (Fig. 1). In mitochondria, the pathway is encoded by SHMT2, TYMS, and DHFR1 (4). Disruption of the pathway in Chinese hamster ovary cell mitochondria results in a glycine auxotrophy and elevated levels of uracil in mitochondrial DNA (4). In the nucleus, the de novo thymidylate biosynthesis pathway is encoded by SHMT1 and, to a lesser extent SHMT2, which are functionally redundant in supplying methyleneTHF, TYMS, and DHFR. TYMS is the only gene that is common to and essential for mitochondrial and nuclear thymidylate biosynthesis and is essential for early embryonic development (7). Nuclear thymidylate biosynthesis is cell cycle-regulated through the small ubiquitin-like modifier (SUMO)-dependent nuclear translocation of SHMT1, TYMS, and DHFR during S phase (5, 6, 8). Isolated intact nuclei exhibit de novo thymidylate synthesis activity, but this activity is lost in sonicated nuclei, suggesting that this pathway functions through a multienzyme complex and requires compartmentalization in an intact nucleus (8). It is not known whether thymidylate biosynthesis occurs in the cytoplasm. Shm1Tg mice are viable and fertile.

* This work was supported by Public Health Service Grant DK58144.
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4 The abbreviations used are: SHMT, serine hydroxymethyltransferase; AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; DHF, dihydrofolate; DHFR, DHF reductase; SUMO, small ubiquitin-like modifier; THF, tetrahydrofolate; TYMS, thymidylate synthase; ANOVA, analysis of variance.
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FIGURE 1. Folate-dependent de novo thymidylate biosynthesis. The de novo thymidylate biosynthesis pathway is comprised of SHMT1, SHMT2α, TYMS, and DHFR. During the S phase, these enzymes are sumoylated by Ubc9, which serves as a signal for nuclear import.

SHMT1 activity can be limited by using nuclear de novo thymidylate biosynthesis and is also involved in homocysteine remethylation in the cytoplasm. Expression of the SHMT1 cDNA in cell cultures increases rates of de novo thymidylate biosynthesis, while impairing homocysteine remethylation (14). SHMT1 impairs folate-dependent homocysteine remethylation in the cytoplasm by binding and sequestering 5-methylTHF, the substrate for methionine synthase, thereby making it unavailable for the homocysteine remethylation cycle (Fig. 1) (3, 14). SHMT1 expression is regulated over a wide dynamic range in cell culture models by zinc chelators (15), ferritin (16), UV radiation (17), and vitamin A (18), but the effects of increased SHMT1 expression on one-carbon metabolism have not been examined in an animal model. Here, we have generated a transgenic mouse model that overexpresses SHMT1, Shmt1^tg^ mice, to determine the impact of elevated SHMT1 expression on homocysteine remethylation and de novo thymidylate synthesis in vivo. In this mouse model, increased SHMT1 expression impaired nuclear localization of the de novo dTMP synthesis pathway and markedly increased uracil accumulation in nuclear DNA, indicating an essential role for nuclear thymidylate biosynthesis in the maintenance of DNA integrity and suggests that de novo thymidylate biosynthesis does not occur in the cytoplasm at rates sufficient to prevent uracil misincorporation into DNA.

FIGURE 2. Generation of SHMT1 overexpressing mice. A, an inducible βgeo/Shmt1 expression vector was created to allow for Cre/lox activation of the Shmt1 transgene. The βgeo/Shmt1 expression cassette allows for detection and localization of the transgene promoter activity via the lacZ reporter gene and for conditional overexpression of functional SHMT1. Transgene activation is achieved when Shmt1^tg^ mice are mated to Cre-expressing mice, in this case, Cre expression was controlled by the CMV promoter. Cre expression results in the deletion of the βgeo reporter gene while simultaneously repositioning the Shmt1 transgene 3′ of the chicken β-actin promoter; we refer to mice expressing Cre and the transgene as Shmt1^tg^/^. B, Shmt1^tg^ genotyping in Cre^+^ Shmt1 wild type mice and Cre^−^ Shmt1^tg^/^-transgenic mice. The Shmt1 transgene is detected as a 215-bp PCR product, whereas the wild type (Wt) allele is detected as a 542-bp PCR product. The presence of the Cre transgene was detected as a 119-bp PCR product, and the Il2 internal control gene was detected as a 324-bp PCR product. C, Southern blot of genomic DNA demonstrating a single insertion of the Shmt1 transgene. Total nuclear DNA from tail snips was isolated from PCR verified Shmt1^tg^ and Shmt1^tg^ mice. The DNA was digested with BglII (lane 1, does not cut within the Z/EG vector), EcoR5 (lane 2, does not cut the Z/EG vector but cuts at position 418nt of the Shmt1 cDNA), or Xhol (lane 3, the site used for cloning that cuts 5′ and 3′ of the Shmt1 cDNA insert within the vector). Shmt1^tg^, Cre^−^ Shmt1 wild type mice; Shmt1^tg^, Cre^+^ Shmt1^tg^ mice.

EXPERIMENTAL PROCEDURES

Shmt1 Expression Vector—The Shmt1 Z/EG vector (Fig. 2A) was designed to express the βgeo reporter cassette under the
control of the chicken β-actin promoter. The Z/EG vector contains the chicken β-actin promoter with an upstream cytomegalovirus enhancer that drives the expression of a loxP-flanked βgeo cassette (lacZ-neomycin resistance fusion) and an internal ribosome entry sequence that enables the translation of an enhanced GFP (19). The murine Shmt1 cDNA (20) was cloned 3′ of the second loxP site and 5′ of the internal ribosome entry sequence-enhanced GFP into the XhoI site (Fig. 2A). Following Cre-mediated excision, the murine Shmt1 cDNA is located directly 3′ of the β-actin promoter.

Generation of Shmt1tg Mice—Mice were maintained under specific pathogen-free conditions in accordance with standard use protocols and animal welfare regulations. All of the study protocols were approved by the Institutional Animal Care and Use Committee of Cornell University and conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Z/EG vector containing the Shmt1 cDNA was linearized with SfiI, which cuts 3′ of the enhanced GFP cDNA. Shmt1tg mice were generated by pronuclear injection of the linearized construct into FVB/N embryos at the Cornell University Transgenic Mouse Facility. Single site integration of the vector was confirmed in Shmt1tg founder mice by Southern blot analysis of purified tail DNA (Qiagen) (Fig. 2C). Total nuclear DNA was digested with BglIII, which does not cut within the Shmt1 cDNA or the vector; EcoR5, which cuts at nucleotide 418 of the Shmt1 cDNA but not in the vector; or XhoI, which was the cloning site for the Shmt1 cDNA insertion into the vector. The digests were run on a 1.5% agarose gel, transferred to nitrocellulose, and hybridized with a 32P-labeled probe generated from nucleotides 443–1233 of the Shmt1 cDNA but not in the vector; or XhoI, which was the cloning site for the Shmt1 cDNA insertion into the vector. The digests were run on a 1.5% agarose gel, transferred to nitrocellulose, and hybridized with a 32P-labeled probe generated from nucleotides 443–1233 of the Shmt1 cDNA using protocols described elsewhere (21). DNA isolated from the FVB/N-Tg(ACTB-LacZ/Shmt1)B1Stov (Shmt1tg) founder exhibited a high molecular band when digested with BglII or EcoR5. Digestion with XhoI resulted in a prominent band of 1.6 kb and a weaker smaller band, which were not observed in DNA isolated from wild type mice (Fig. 2C). The major 1.6-kb band approximates the size of the Shmt1 cDNA insert, whereas the smaller minor band likely reflects XhoI star activity. Southern blots were repeated over three generations without changes in the Southern banding pattern, indicating a stable activity. Southern blots were repeated over three generations without changes in the Southern banding pattern, indicating a stable activity.

To achieve cDNA activation, loxP sites were placed both 5′ and 3′ of the βgeo cassette. The Shmt1 transgene was activated by crossing male FVB/N-Tg(ACTB-LacZ/Shmt1)B1Stov (Shmt1tg) mice with female BALB/c-Tg(CMV-Cre)1Cgn/J (Cre; The Jackson Laboratory, Bar Harbor, ME) mice that were homozygous for the Cre transgene. Presence of the Cre allele was determined by PCR using the forward primer 5′-ACAGCCAGCTATCACCAGC-3′ and the reverse primer 5′-TTACATTGGTCCAGCCACC-3′, as described (The Jackson Laboratory). The forward and reverse primers 5′-CTAGGGCC-ACAGAATTGAAAGATCT-3′ and 5′-GTAGGTTGAAAATTCTAGCATCATCC-3′, respectively, were used for detection of the Il2 internal control gene, as described (The Jackson Laboratory) (Fig. 2B).

Diets—Mice used for characterization of protein overexpression and the nuclear dTMP synthesis assays were fed a standard rodent chow (Harlan Teklad LM-485) from weaning. Mice on the controlled diet study were randomly weaned at 3 weeks of age to a control diet (AIN93G containing 2 mg of folic acid/kg and 2.5 g of choline bitartrate/kg; Dyets, Inc., Bethlehem, PA) or a folate/choline-deficient diet (AIN-93G diet lacking folic acid and choline; Dyets, Inc.). The mice were maintained on the controlled diet for 5 or 32 weeks (until 8 or 35 weeks of age), as indicated in the results.

Determination of AdoMet and AdoHcy Concentrations—The animal feeding cycle was synchronized prior to tissue harvest to ensure AdoMet concentrations reflected homocysteine remethylation capacity with minimal contributions from dietary methionine. Food was removed 24 h prior to killing the animals. After 12 h, each animal was given one food pellet, and the animals were killed 12 h later by cervical dislocation. Tissues were harvested and immediately flash-frozen and stored at −80 °C until analysis. Frozen tissues were sonicated in 500 μl of 0.1 M NaAcO buffer (pH 6), and protein was precipitated by adding 312 μl of 10% perchloric acid to each sample. After vortexing, samples were centrifuged at 2000 × g for 10 min at 4 °C. AdoMet and AdoHcy were determined as described previously (14). AdoMet and AdoHcy values were normalized to total protein (22).

Immunoblotting—For tissues, total protein was extracted and quantified by the Lowry-Bensadoun assay (22). Tissue lysis was achieved by sonication in lysis buffer (2% SDS, 100 mm, dithiothreitol, 60 mm Tris, pH 6.8). For analysis of liver nuclei, pellet nuclei, purified as described below, were disrupted by boiling in SDS-PAGE loading buffer for 10 min, and protein concentrations were quantified.

Proteins (40 μg/well for tissues, 20 μg/well for nuclei) were separated on a 10% (nuclei) or 12% (tissue) SDS-PAGE gel. Proteins were transferred at 4 °C to an Immobilon-P polyvinylidene difluoride membrane (Millipore Corp.) using a Transblot apparatus (Bio-Rad). Following transfer, the membranes were blocked in 5% (w/v) nonfat skim milk in PBS for 1 h followed by overnight incubation in primary antibody at 4 °C. The membranes were washed with PBS containing 0.1% Tween 20 and then incubated overnight with the appropriate horseradish peroxidase-conjugated secondary antibody (below). The membranes were visualized using the SuperSignal® West Pico chemiluminescent substrate system (Pierce).

For SHMT1 detection, sheep anti-mouse SHMT1 antibody (20) was diluted 1:10,000, and rabbit anti-sheep IgG secondary antibody (Pierce) was diluted 1:20,000. For TYMS detection, affinity-purified sheep anti-human T5 antibody (Abcam) was diluted 1:5000, and rabbit anti-sheep IgG secondary antibody (Pierce) was diluted 1:10,000. For DHFR detection, affinity-purified goat anti-human DHFR antibody (Santa Cruz Biotechnology) was diluted 1:1000, and mouse anti-goat IgG secondary antibody (Pierce) was diluted 1:5000. For lamin A detection, rabbit anti-lamin A (Santa Cruz Biotechnology) was diluted...
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1:500, and goat anti-rabbit IgG secondary antibody (Pierce) was diluted 1:20000. For detection of the loading control GAPDH, mouse anti-human GAPDH antibody (Novus Biologicals) was diluted 1:100,000 dilution, and goat anti-mouse IgG secondary antibody (Pierce) was diluted 1:10000.

Plasma and Tissue Folate Concentration—Folate concentration in plasma and liver was quantified using the Lactobacillus casei microbiological assay as described (14).

For quantification of nuclear folate levels, four livers were isolated from Shmt1wt+/+ and Shmt1tg+ mice and placed immediately in cold PBS at 5 °C containing 200 mM β-mercaptoethanol (Sigma) and 2% (w/v) sodium ascorbate (Sigma). Liver nuclei from four age-matched males for each genotype were combined and prepared as described under “Nuclear DNA.” Nuclear folate content was determined in two independent experiments.

Uracil Content in Nuclear DNA—Nuclear DNA was extracted from 25—50 mg of tissue using DNeasy Tissue and Blood Kit (Qiagen), including an incubation with RNase A (Sigma) and RNase T1 (Ambion) for 30 min at 37 °C. 10 mM NADPH (Sigma), 100 mM ATP, 200 mM GTP, 0.25 mM dTTP, 0.5 mM thymidine, 0.25 mM [15N2]uracil (Cambridge Isotopes) was added to each sample as an internal standard, and the sample was dried completely in a speed vacuum. 50 μl of water followed by 100 μl of isooctane were added to each sample. The samples were vortexed and centrifuged. Organic extraction of derived uracil was completed by the removal of the aqueous phase and analysis of the organic phase.

Analysis of uracil-3,5-bis(trifluoromethyl) benzyl bromide was carried out on a Shimadzu QP2010. 1 μl of sample or standard was analyzed in the splitless mode with a purge activation time of 1 min and split vent flow of 50 ml/min with an injection port temperature of 280 °C. Ultrapurity helium gas was used as carrier gas with a linear velocity of 55 ml/min. Separation of derived uracil was obtained by using an XT-5, 30 m, 0.25-mm inner diameter, 0.25-μm column (Restek), using the following temperature cycles for the oven: 100 °C for 1 min, ramping to 280 °C at 25 °C/min, holding for 5 min, ramping to 300 °C at 5 °C/min, and holding for 5 min. The interface temperature was held at 300 °C with an ion source temperature of 260 °C. Ionization was achieved using the NCi mode using methane as the reagent gas and monitoring for ions 337 m/z for uracil and 339 m/z for [15N2]uracil.

Nuclear de Novo Thymidylate Synthesis—Livers from age-matched male Shmt1wt+/+ and Shmt1tg+ mice fed a standard rodent chow diet (4—6 months of age, n = 12 per genotype) were dissected and placed immediately in cold PBS at 5 °C. Liver extracts for each genotype group were combined for nuclei purification. The nuclei were prepared using an iodoacetol gradient as previously described (8). De novo dTMP reactions were completed as previously described (8). In short, purified nuclei were suspended in 500 μl of nuclear assay buffer containing 5 mM NADPH (Sigma), 100 mM β-mercaptoethanol, 25 mM HEPES, pH 7.5, 50 mM sucrose, 5 mM MgCl2, 25 mM KCl, and 1 mM dUMP (Sigma) and quantified using a hemocytometer. 125 μl of assay buffer containing equal numbers of suspended nuclei were aliquoted into four 1.5-ml plastic tubes, and 8 μCi of [2,3-3H]-l-serine (Moravek Biochemicals) was added to each sample. The assay was conducted under three different experimental conditions: lysed nuclei, nuclei were lysed with sonication (Branson Sonifier 150) at 5 °C using two 10-s pulses at 10 watts separated by a 10-s resting interval; intact nuclei; or intact nuclei with SHMT1 inhibitor, intact nuclei incubated with aminomethylphosphonate (Sigma) added to a final concentration of 100 mM. The reactions were incubated for 12 h at 37 °C with shaking at 300 rpm. The nuclei were pelleted by centrifugation at 2000 rpm for 5 min, and the supernatant was collected and analyzed for radiolabeled thymidylate by HPLC. Sample preparation and HPLC was performed as previously described (23, 24). Fractions were collected, and tritium was quantified with a scintillation counter. The retention times of [2,3-3H]-l-serine (9 min) and [3H]thymidine (17 min) (Moravek Biochemicals) were verified prior to separation of the reaction mixtures. The data were normalized to the number of nuclei. All of the experiments were performed in duplicate.

Metabolite Profile from Plasma—Total homocysteine, cystathionine, total cysteine, methionine, glycine, serine, α-aminobutyric acid, N,N-dimethylglycine, and N-methylglycine were assayed in mouse plasma by stable isotope dilution capillary gas chromatography-mass spectrometry as described previously (25, 26).

Statistical Analyses—Differences in genotype distribution were analyzed by the χ2 test. Differences between two groups were determined by Student’s t test. Differences among more than two genotypes were analyzed by two-way ANOVA and Dunnett’s post hoc test using the wild type genotype as the control group. Diet × genotype effects were analyzed by two-way ANOVA and Tukey’s Honestly Significant Difference post hoc test. The groups were considered significantly different when the p value ≤ 0.05. The data are presented as the mean ± S.E. All of the statistics were performed using JMP IN software, release 5.1.2.

RESULTS

Shmt1tg+ Mice Are Viable—Shmt1 transgene expression was activated by crossing Shmt1tg+ mice to Cre-expressing mice, which results in deletion of the βgeo cassette and relocation of the Shmt1 intronless transgene 3′ to the β-actin promoter, generating Shmt1tg+ mice (Fig. 2A; + denotes Cre positive). PCR-based genotyping results in the generation of a 542-bp PCR product for the wild type Shmt1 allele and a 215-bp PCR product for the transgene (Fig. 2B).

To determine whether Shmt1tg+ mice were viable upon activation of the transgene, the Shmt1tg+ genotype distribution was determined from crosses between BALB/c CMV-Cre homozygous male mice and FVB/N Shmt1tg+ female mice carrying one copy of the transgenic allele (Fig. 2C). A total of 226 F1 pups from 39 litters were examined (Table 1). The mean litter size was 5.8 pups, which approximates observed litter sizes for inbred FVB/N and BALB/c mice. The Shmt1 transgene was distributed as expected for Mendelian inheritance with a ratio of Shmt1wt+/+ to Shmt1tg+ mice of 97:129, and both sexes were
SHMT1 overexpressing mice are viable

SHMT1 overexpressing mice were crossed with Cre-expressing mice to activate expression of the Shmt1 transgene, and their progeny were genotyped. The expected genotype distribution was calculated based on a Mendelian distribution. Differences between observed and expected genotype distributions were analyzed by chi-squared analysis. p values ≤ 0.05 were considered significantly different. Shmt1\^tg\^cre\^, Cre\^, Shmt1\^wt\^, Cre\^, Shmt1\^tg\^ mice. NS, not significant.

| Genotype  | Observed genotype distribution | Expected genotype distribution |
|-----------|-------------------------------|-------------------------------|
|           | Male | Female | Total | Male | Female | Total |
| Shmt1\^tg\^ | 55   | 42    | 97    | 56.5 | 56.5   | 113   |
| Shmt1\^tg\^cre\^ | 70   | 59    | 129   | 56.5 | 56.6   | 113   |
| Total      | 125  | 101   | 226   | 113  | 113    | 226   |
| Number of litters observed | 39   |        |        |      |        |        |

Mean litter size (mean ± S.E.)

p value, observed versus expected genotype distribution

p value, observed versus expected sex distribution

Mean litter size = (5.8 ± 0.4) NS

Impact of SHMT1 Overexpression on Homocysteine Remethylation—SHMT1 binds 5-methylTHF in the cytoplasm, making it unavailable for the remethylation of homocysteine in cultured MCF-7 cells (14). Therefore increased SHMT1 expression is expected to result in decreased AdoMet levels and elevated homocysteine. Consistent with this expectation,
TABLE 2
Plasma and liver folate, liver S-adenosyl-methionine, S-adenosyl-homocysteine, S-adenosyl-methionine:S-adenosyl-homocysteine ratio, and uracil content in Shmt1 overexpressing mice at 32 weeks post-weaning

Differences between genotypes and diets were analyzed by Student’s t test. Nuclear DNA genotype \( \times \) diet effects were analyzed by two-way ANOVA using Tukey’s Honestly Significant Difference post-hoc analysis. The data represent the means \( \pm \) S.E. values. \( p \) values \( \leq 0.05 \) were considered significantly different, \( n = 4–7 \) group. Folate/choline-deficient Shmt1\(^{tg}\) mice are significantly different than control and folate/choline-deficient Shmt1\(^{wt}\) and control Shmt1\(^{tg}\) mice, \( p < 0.05 \), as analyzed by two-way ANOVA and Tukey’s Honestly Significant Difference post-hoc test Shmt1\(^{wt}\), Cre\(^{+}\) Shmt1 wild type mice; Shmt1\(^{tg}\), Cre\(^{+}\) Shmt1\(^{tg}\) mice. NS, not significant.

| Diet                      | Shmt1 genotype | Plasma folate | Liver folate | AdoMet | AdoHcy | AdoMet:AdoHcy | Liver uracil |
|---------------------------|----------------|---------------|--------------|--------|--------|--------------|--------------|
| AIN-93G                   | Shmt1\(^{wt}\) | 36.3 \(\pm\) 7.7 | 43.1 \(\pm\) 2.3 | 0.7 \(\pm\) 0.2 | 0.4 \(\pm\) 0.1 | 2.1 \(\pm\) 0.4 | 0.1 \(\pm\) 0.0 |
| AIN-93G minus folate & choline | Shmt1\(^{wt}\) | 46.8 \(\pm\) 5.8 | 51.3 \(\pm\) 3.4 | 0.3 \(\pm\) 0.2 | 0.2 \(\pm\) 0.2 | 1.2 \(\pm\) 0.0 | 0.2 \(\pm\) 0.0 |
|             | Shmt1\(^{tg}\) | 7.4 \(\pm\) 0.2 | 36.1 \(\pm\) 2.6 | 0.7 \(\pm\) 0.1 | 0.6 \(\pm\) 0.1 | 1.2 \(\pm\) 0.2 | 0.3 \(\pm\) 0.0 |
|             |                | 5.5 \(\pm\) 1.5 | 34.0 \(\pm\) 5.8 | 0.4 \(\pm\) 0.0 | 0.6 \(\pm\) 0.1 | 0.9 \(\pm\) 0.3 | 2.3 \(\pm\) 0.7 |

\( p \) value, diet effect
\( p \) value, genotype effect
\( p \) value, Diet \( \times \) genotype effect

FIGURE 4. Hepatic nuclear SHMT1, TYMS, and DHFR protein content and de novo dTMP biosynthesis in SHMT1 overexpressing mice. A, immunoblotting for SHMT1, TYMS, and actin (loading control) from whole liver extracts from Cre-activated Shmt1 wild type (Shmt1\(^{wt}\)) and Shmt1\(^{tg}\) mice. B, nuclei were isolated from Shmt1\(^{wt}\) and Shmt1\(^{tg}\) mouse liver, and the capacity to convert dUMP and [2,3-\( ^{3}H \)]-\( S \)-serine to \( [^{3}H] \)dTMP was determined in reactions that contained sonicated nuclei, intact nuclei, or intact nuclei incubated with 100 \( \mu \)M aminomethyl phosphonate, an SHMT1 inhibitor (8). De novo thymidylate biosynthesis activity was normalized to that of Shmt1\(^{wt}\) intact nuclei, which was assigned an arbitrary value of 1.0. The reactions were performed in duplicate, and the experiment was repeated twice. We did not observe a run-dependent difference; therefore data from the two replicate experiments were combined. The data are presented as the means \( \pm \) S.E. C and D, immunoblotting for SHMT1, TYMS, and DHFR was performed on hepatic nuclei isolated from Shmt1 null (C only) (3) and Shmt1\(^{wt}\) and Shmt1\(^{tg}\) mice. Lamin A was used as a nucleus-specific loading control. E, immunoblotting of cytoplasm-restricted GAPDH confirmed the purity of hepatic nuclei.

hepatic AdoMet and AdoMet:AdoHcy ratio, which is indicative of the cellular methylation capacity, were significantly lower in Shmt1\(^{tg}\) mice at 32 weeks post-weaning (Table 2). AdoHcy was unaffected by SHMT1 overexpression. We did not observe any genotype \( \times \) diet effects on AdoMet, AdoHcy, or the AdoMet:AdoHcy ratio.

The impact of SHMT1 overexpression on homocysteine- and folate-related metabolites in the plasma of male and female mice was determined at 5 weeks post-weaning. We did not observe a significant effect of SHMT1 overexpression on any of the metabolites queried because of insufficient power to detect significant differences. A significant sex effect on plasma homocysteine, methionine, cysteine, and cystathionine was observed. Female mice exhibited increased plasma homocysteine and cysteine and decreased cystathionine and methionine relative to male mice. Plasma serine tended to be decreased in
TABLE 3
Plasma metabolic profile of SHMT1 overexpressing mice at 5 weeks post-weaning

Differences between sexes, diets, and genotypes were analyzed by Student’s t test. Genotype × diet effects were analyzed by two-way ANOVA using Tukey's Honestly Significant Difference post-hoc analysis. The data are presented as the means ± S.E. values. p values ≤ 0.05 were considered significantly different. n = 3 males and n = 3 females/diet/genotype group. Shmt1tg, Cre−/− Shmt1 wild type mice; Shmt1tg, Cre+ Shmt1tg mice. NS, not significant.

| Metabolite                  | Sex      | C         | FCD       | Shmt1tg, Cre−/− | Shmt1tg, Cre+ | p value of model effect |
|----------------------------|----------|-----------|-----------|----------------|---------------|------------------------|
| Homocysteine (μM)          | Both     | 5.6 ± 0.7 | 7.0 ± 0.7 | 7.8 ± 1.3      | 9.9 ± 1.0     | 0.009                  |
|                           | Male     | 4.8 ± 0.7 | 5.5 ± 0.2 | 6.6 ± 2.3      | 8.4 ± 0.6     | 0.10                   |
|                           | Female   | 6.4 ± 1.0 | 8.4 ± 0.3 | 9.0 ± 1.1      | 11.4 ± 1.6    | 0.03                   |
| Cystathionine (nm)         | Both     | 1697 ± 337| 1687 ± 334| 1552 ± 189     | 1555 ± 70     | NS                     |
|                           | Male     | 1979 ± 399| 2235 ± 483| 1931 ± 181     | 1655 ± 83     | NS                     |
|                           | Female   | 1414 ± 202| 1140 ± 159| 1173 ± 54      | 1455 ± 89     | NS                     |
| Cysteine (μM)              | Both     | 171 ± 29  | 200 ± 20  | 214 ± 19       | 219 ± 15      | <0.0001                |
|                           | Male     | 109 ± 21  | 158 ± 8   | 178 ± 15       | 192 ± 14      | 0.01                   |
|                           | Female   | 232 ± 4   | 242 ± 12  | 251 ± 18       | 246 ± 12      | NS                     |
| Methionine (μM)            | Both     | 39.6 ± 5.0| 29.3 ± 3.9| 40.9 ± 8.8     | 31.4 ± 1.3    | 0.03                   |
|                           | Male     | 49.9 ± 3.7| 34.6 ± 5.8| 48.1 ± 18      | 32.4 ± 1.6    | NS                     |
|                           | Female   | 29.3 ± 2.2| 24.1 ± 3.7| 33.7 ± 1.1     | 30.4 ± 2.2    | NS                     |
| α-Aminobutyric Acid (μM)   | Both     | 6.8 ± 1.7 | 5.0 ± 0.5 | 4.5 ± 0.6      | 4.2 ± 0.3     | NS                     |
|                           | Male     | 5.8 ± 1.4 | 4.6 ± 0.4 | 5.0 ± 1.2      | 4.4 ± 0.5     | NS                     |
|                           | Female   | 7.8 ± 3.3 | 5.4 ± 1.0 | 4.0 ± 0.3      | 4.0 ± 0.3     | NS                     |
| Glycine (μM)               | Both     | 312 ± 42  | 286 ± 22  | 270 ± 17       | 289 ± 20      | 0.0006                 |
|                           | Male     | 383 ± 59  | 322 ± 32  | 291 ± 23       | 327 ± 6       | NS                     |
|                           | Female   | 240 ± 12  | 249 ± 9   | 249 ± 22       | 250 ± 20      | NS                     |
| Serine (μM)                | Both     | 161 ± 21  | 136 ± 11  | 146 ± 18       | 134 ± 9       | <0.0001                |
|                           | Male     | 203 ± 17  | 158 ± 8   | 174 ± 25       | 151 ± 6       | NS                     |
|                           | Female   | 119 ± 10  | 113 ± 8   | 119 ± 16       | 117 ± 9       | NS                     |
| Dimethylglycine (μM)       | Both     | 7.8 ± 1.7 | 7.1 ± 0.7 | 6.0 ± 0.7      | 6.2 ± 0.8     | 0.004                  |
|                           | Male     | 5.0 ± 0.1 | 5.6 ± 0.4 | 5.0 ± 0.7      | 4.6 ± 0.2     | NS                     |
|                           | Female   | 10.5 ± 2.6| 8.6 ± 0.6 | 7.0 ± 1.1      | 7.8 ± 0.5     | NS                     |
| Methylglycine (μM)         | Both     | 2.9 ± 0.5 | 2.1 ± 0.2 | 1.4 ± 0.1      | 2.0 ± 0.3     | NS                     |
|                           | Male     | 2.3 ± 0.5 | 2.3 ± 0.3 | 1.4 ± 0.1      | 1.6 ± 0.1     | 0.02                   |
|                           | Female   | 3.3 ± 1.0 | 1.8 ± 0.2 | 1.3 ± 0.1      | 2.4 ± 0.7     | NS                     |

Shmt1tg mice, an effect driven by samples from male mice (Table 3). The folate/choline-deficient diet was associated with increased plasma homocysteine and cysteine and decreased methylglycine (Table 3). We did not observe any significant genotype × diet effects on any of the queried metabolites.

DISCUSSION

Our study demonstrates that nuclear localization of the de novo dTMP synthesis pathway is essential to prevent uracil accumulation in nuclear DNA and thereby maintain genome stability and confirms previous studies demonstrating that SHMT1 expression is an important determinant of uracil accumulation in DNA. The results demonstrate unequivocally that restricting the de novo dTMP synthesis pathway to the cytoplasm results in elevated uracil accumulation into nuclear DNA.

Shmt1tg mice demonstrated a 2–4-fold increase in SHMT1 and TYMS protein in tissues that normally express SHMT1, namely the liver, kidney, and gastrointestinal tract (Fig. 3). No significant differences in genotype distribution among Cre-activated F1 wild type or transgenic pups were observed (Table 1), indicating that the achieved level of SHMT1 overexpression had no impact on embryo survival in dams fed a standard rodent chow diet. It will be of interest to determine the effect of a folate deficient diet on Shmt1tg embryo development, because we have demonstrated folate-responsive neural tube defects in Shmt1 heterozygous null mice, which was attributed to reduced de novo thymidylate synthesis (10).

Shmt1+/− mice have been reported to exhibit increased uracil content in hepatic nuclear DNA, reduced levels of nuclear SHMT1 and TYMS, and consequent decreased capacity for de novo thymidylate synthesis (3, 9). In this study, contrary to expectations, uracil content in hepatic nuclear DNA was increased in Shmt1tg mice compared with wild type mice, which was exacerbated markedly by a folate- and choline-deficient diet. Indeed, hepatic nuclear DNA uracil content in Shmt1tg mice fed the control diet was comparable with that observed in Shmt1+/− mice (3, 9). Compared with wild type mice, Shmt1tg mice exhibited a 4-fold increase in total hepatic SHMT1 and TYMS protein expression, whereas nuclear localization of SHMT1 and TYMS was reduced by ~75%, resulting in a 50% reduction in dTMP synthesis in isolated nuclei from folate-replete mice (Fig. 4). Although Shmt1tg have severalfold increased expression of SHMT1 and TYMS compared with Shmt1+/− mice, they both exhibit decreased levels of nuclear SHMT1 and TYMS protein, reduced nuclear thymidylate synthesis, and elevated uracil in DNA compared with wild type mice. Collectively, these results indicate that nuclear localization of the de novo thymidylate biosynthesis pathway is essential for maintaining sufficient thymidine nucleotide pools for DNA replication.

The mechanism by which SHMT1 and TYMS accumulation in the nucleus is inhibited in Shmt1tg mice is not known. Elevated expression of SHMT1 in the cytoplasm may impair sumoylation of SHMT1 and/or TYMS or prevent their translocation into the nucleus. Interestingly, the accumulation of SHMT1 and TYMS, but not DHFR, in the nucleus is coordinated and dependent on SHMT1 expression.

Similar to our previous studies, in which SHMT1-dependent expression of TYMS was observed in colon and embryonic tissue (9, 10), the present study provides additional evidence for the coregulation of these two proteins. Shmt1+/− mice were shown to have ~50% SHMT1 protein content, which was con-
comitant with a reduction in TYMS (9). Interestingly, SHMT1, TYMS, and thymidine kinase 1 protein expression were also responsive to folate deficiency as demonstrated by their increased expression in mice fed the folate/choline-deficient diet (9). Microarray analysis did not indicate significant transcriptional changes to TYMS expression in Shmt1<sup>-/-</sup>, suggesting that their coregulation occurs post-transcriptionally (9). We also have not queried the effect of SHMT1 expression on serum one-carbon metabolites, because our study was underpowered to detect significant differences.

We previously demonstrated that the common human L474F variant impairs the UBC9-SHMT1 interaction and consequently SHMT1 sumoylation and translocation to the nucleus (5). The impact of this SHMT1 variant on TYMS overexpression did not have a major impact on serum one-carbon metabolites, because our study was underpowered to detect significant differences.

We also have not queried the effect of SHMT1 expression on nuclear localization and nuclear distribution of SHMT1 (5). The impact of this SHMT1 variant on TYMS expression influences TYMS levels is actively being investigated.

This study also confirms that increased SHMT1 expression plays a modest role in the regulation of homocysteine methylation in the cytoplasm. SHMT1 is a 5-methylTHF binding protein (27), and therefore overexpression of SHMT1 was anticipated to reduce hepatic methionine and AdoMet synthesis by sequestering cytoplasmic 5-methylTHF. Consistent with this expectation, SHMT1 overexpression decreased hepatic AdoMet, which resulted in a reduced AdoMet:AdoHcy ratio (Table 2). SHMT1 overexpression did not have a major impact on serum one-carbon metabolites, because our study was underpowered to detect significant differences.

We previously demonstrated that the common human L474F variant impairs the UBC9-SHMT1 interaction and consequently SHMT1 sumoylation and translocation to the nucleus (5). The impact of this SHMT1 variant on TYMS localization and nuclear de novo thymidylate biosynthesis capacity is unknown. The reduction of nuclear de novo dTMP synthesis capacity in the Shmt1<sup>1G-e</sup> mouse provides a unique experimental model of the human L474F SHMT1 variant and permits mechanistic studies that investigate and validate reported epidemiological associations of this variant with lung cancer and cardiovascular risk (11–13).

Acknowledgments—We acknowledge Martha Field, Anna Beaudin, Sylvia Allen, and Rachel Slater for technical assistance.

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