Dysregulated transmethylation leading to hepatocellular carcinoma compromises redox homeostasis and glucose formation

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ABSTRACT

Objective: The loss of liver glycine N-methyltransferase (GNMT) promotes liver steatosis and the transition to hepatocellular carcinoma (HCC). Previous work showed endogenous glucose production is reduced in GNMT-null mice with gluconeogenic precursors being used in alternative biosynthetic pathways that utilize methyl donors and are linked to tumorigenesis. This metabolic programming occurs before the appearance of HCC in GNMT-null mice. The metabolic physiology that sustains liver tumor formation in GNMT-null mice is unknown. The studies presented here tested the hypothesis that nutrient flux pivots from glucose production to pathways that incorporate and metabolize methyl groups in GNMT-null mice with HCC.

Methods: 2H/13C metabolic flux analysis was performed in conscious, unrestrained mice lacking GNMT to quantify glucose formation and associated nutrient fluxes. Molecular analyses of livers from mice lacking GNMT including metabolomic, immunoblotting, and immunochemistry were completed to fully interpret the nutrient fluxes.

Results: GNMT knockout (KO) mice showed lower blood glucose that was accompanied by a reduction in liver glycogenolysis and gluconeogenesis. NAD⁺ was lower and the NAD(P)H-to-NAD(P)⁺ ratio was higher in livers of KO mice. Indices of NAD⁺ synthesis and catabolism, pentose phosphate pathway flux, and glutathione synthesis were dysregulated in KO mice.

Conclusion: Glucose precursor flux away from glucose formation towards pathways that regulate redox status increase in the liver. Moreover, synthesis and scavenging of NAD⁺ are both impaired resulting in reduced concentrations. This metabolic program blunts an increase in methyl donor availability, however, biosynthetic pathways underlying HCC are activated.

Keywords Intermediary metabolism; Metabolic flux analysis; NAD⁺; Redox state; S-adenosylmethionine

1. INTRODUCTION

The incidence of liver cancer, of which hepatocellular carcinoma (HCC) is the primary form, is increasing in the United States [1–3]. This rise in newly diagnosed liver cancer is proposed to be, in part, the result of the high prevalence of metabolic diseases such as diabetes and obesity [1,2]. Dysregulated metabolism is an important contributor to the pathogenesis of cancer [4]. This metabolic reprogramming includes dysregulated nutrient uptake, metabolite mediated changes in cell function, and shifts in intracellular nutrient fluxes [5]. From the perspective of liver nutrient flux, experimental models suggest that impeding glucose production promotes HCC [6,7], while enhancing glucose production has the opposite effect [8,9]. It has been proposed that the reduction in hepatic glucose formation spares precursors for alternate metabolic and biosynthetic pathways that contribute to tumorigenesis [10,11]. Findings in humans with HCC support this hypothesis. For example, glucose production in response to gluconeogenic stimuli such as glucagon is diminished in patients with HCC [12]. Also, hypoglycemia has been reported as an early characteristic in a subset of individuals with HCC [13,14].

Of particular interest in the relationship between glucose control and HCC is the role of liver glycine N-methyltransferase (GNMT). GNMT, the most abundant liver methyltransferase, catalyzes a reaction whereby a methyl group from s-adenosylmethionine (SAM) is transferred to glycine forming sarcosine and s-adenosylhomocysteine (SAH) [15]. The main function of this enzyme is to maintain SAM homeostasis. In humans with HCC, liver GNMT can be greatly decreased [16,17]. Moreover, GNMT-null mice develop HCC by eight months of age [18–20] suggesting a causal role for impaired GNMT action and dysregulated SAM in tumorigenesis. We have previously shown that glucose formation is impaired in GNMT-null mice at 12 weeks of age prior to HCC [10]. This is associated with a dysregulated energy state in the liver which includes a reduction in NAD⁺ to NADH ratio influences citric acid cycle (CAC) and gluconeogenic reactions [22,23] such that critically low NAD⁺ availability would diminish glucose formation. Results also indicated that gluconeogenic
precursors were being diverted to pathways that counter the elevation
in SAM [10]. These SAM utilization pathways are biosynthetic in nature
and are pro-tumorigenic.
As noted above, we have previously shown that impaired glucose
formation in 12-week-old GNMT-null mice occurs prior to the
appearance of liver tumor formation. Given this, we hypothesized that
the reduced glucose production owing to a shift in precursor fate is key
in promoting and maintaining liver tumorigenesis in GNMT-null mice.
Here we directly test whether glucose formation is impaired in 44-
week-old GNMT knockout (KO) mice with HCC to further assess
whether this metabolic pathway underlies tumorigenesis in the
absence of GNMT. \(^{2}{H}/^{13}{C}\) metabolic flux analysis (MFA) of nutrients
was combined with metabolomic analysis in conscious, unrestrained
GNMT KO mice and wild-type (WT) littermates under post-absorptive
conditions. The results show the lack of GNMT reduces glucose pro-
duction from glycogen and gluconeogenic sources. Evidence from
molecular and metabolic analyses lead to the conclusion that
reduced glucose production is coupled to impaired NAD\(^+\) synthesis and
salvage. Results show a decline in NAD\(^+\) availability may diminish
glucose production directly and/or by redirecting flux of glucose pre-
cursors to pathways that regulate liver redox state and tumorigenesis.
These pathways include de novo glutathione synthesis and the pentose
phosphate pathway.

2. MATERIAL AND METHODS

2.1. Animals

Vanderbilt University Animal Care and Use Committee approved mouse
procedures. Male, mice with a global KO of GNMT and WT littermates
[10,24] had ad libitum access to food (PicoLab\textsuperscript{R} Laboratory Rodent
Diet 5LOD, Purina, Richmond, IN, USA) and water. The mice were
housed with natural soft cellulose bedding (BioFresh\textsuperscript{R} Comfort
Bedding, Ferndale, WA, USA) in a humidity- and temperature-
controlled room on a 12-hour light/dark cycle. Mice were studied at
44 weeks of age unless otherwise stated.

2.2. Body composition

Body composition was determined by using a mq10 nuclear magnetic
resonance analyzer (Bruker Corporation, Billerica, MA, USA) in 43-
week-old, male mice.

2.3. Surgical procedures

Mice receiving stable isotope infusions had vascular catheterization
surgeries performed as previously described [25] at 43 weeks of age.
Seven days of postoperative recovery was provided before stable
isotope infusion studies were completed. Mice were individually
housed during the postoperative recovery period.

2.4. Stable isotope infusions

Access to food and water was prevented during the first hour of the
light cycle. Three hours into the fast, the exteriorized ends of the
implanted catheters were connected to intravenous syringes via a two-
channel swivel. Following an hour acclimation, an 80 \(\mu\)l arterial
blood sample was obtained to determine natural isotopic enrichment of
plasma glucose. Venous infusions were performed as previously
described in conscious, unrestrained mice [10,26]. In brief, a bolus of
\(^{2}{H}_{2}\text{O} (99.9\%)-\text{saline solution was infused over the course of a 25-
minute period to achieve } 4.5\% \text{ enrichment of body water. } [6,6-^{2}\text{H}]\text{
glucose (99\% solubilized in the } ^{2}{H}_{2}\text{-O-saline bolus was delivered as a
prone (440 \(\mu\)mol kg\(^{-1}\)). Subsequently, a continuous } [6,6-^{2}\text{H}]\text{glucose (4.4
\(\mu\)mol kg\(^{-1}\cdot\text{min}^{-1}\) infusion was started. An intravenous
administration of } [^{13}\text{C}]\text{propionate (99\%, sodium salt) was delivered as
a primed (1.1 mmol kg\(^{-1}\)), continuous (0.055 mmol kg\(^{-1}\cdot\text{min}^{-1}\) infusion
starting two hours following the } ^{2}{H}_{2}\text{O bolus and } [6,6-^{2}\text{H}]\text{ glucose prime. From 90 to 120 min after initiating the } [^{13}\text{C}]\text{propionate bolus (7.5-8 h of fasting), 100 \(\mu\)l arterial samples were obtained every
10 min (four samples in total) and stored at } -20^\circ \text{ C. Following the final
arterial sample, mice were sacrificed via cervical dislocation. Tissues
were excised, freeze-clamped, and stored at } -80^\circ \text{ C. The eight-hour
timeline was performed to quantify fluxes under conditions identical to
prior studies in 12-week old male GNMT-null mice and WT littermates
[10]. Each stable isotope was purchased from Cambridge Isotope
Laboratories, Inc (Tewksbury, MA, USA). Unless otherwise stated, all
venous infusates were made with a 4.5\% \(^{2}\text{H}_{2}\text{O-enriched saline solution.

2.5. \(^{2}{H}/^{13}{C}\) metabolic flux analysis

Derivatization of plasma glucose, gas chromatography-mass spec-
 trometry analysis, and \(^{2}{H}/^{13}{C}\) metabolic flux analysis were completed
as previously outlined [10,26,27]. Briefly, mass isotopomer distributions
were determined from fragment ion ranges: methyloxime pentapi-
ropionate derivatization of glucose, \(m/z\) 145–149; aldonitrile pentapropi-onate derivatization of glucose, \(m/z\) 173–178, 259–266, 284–291,
and 370–379; di-\(O\)-isopropylidene propionate derivatization of glucose,
\(m/z\) 301–314. For each sample, estimates of fluxes were repeated 50
times from random starting values. A chi-square test (\(p = 0.05\)) with 34
degrees of freedom was performed to determine goodness-of-fit. Flux
estimates for each mouse are the average of values acquired from
samples at 90, 100, and 110 min after initiating the \([^{13}\text{C}]\text{propionate bolus. The serial sampling confirmed isotopic steady state was
achieved. The flux estimates were normalized to liver weight.

2.6. Blood and plasma analyses

Contour\textsuperscript{R} blood glucose meters (Ascensia Diabetes Care, Parsippany,
NJ, USA) were used to determine blood glucose concentration during
stable isotope infusion studies. Non-esterified fatty acids were
measured in plasma obtained at the 100-minute time point (seven hours
and 40 min of fasting) from mice undergoing the stable isotope infusions
via a colorimetric assay (NEFA C kit, Wako Chemicals USA Inc., Rich-
mond, VA, USA). Arterial insulin was measured from plasma samples
obtained at the 120-minute time point (eight hours of fasting) from mice
receiving stable isotope infusions as previously performed [28].

2.7. Tissue collection and analyses

Eight-hour fasted, non-catheterized mice were sacrificed via cervical
dislocation for analysis of liver metabolites, global DNA methylation,
immunoblotting, and immunohistochemistry. Liver (tumor and non-
tumor) was freeze-clamped within 20 s of sacrifice. Approximately
50 mg of liver tissue was sent to Human Metabolome Technologies
America, Inc. (Boston, MA, USA) for targeted ionic metabolite quanti-
fication via capillary electrophoresis mass spectrometry (CE-TOFMS
and CE-QqQMS) as previously described [29–32]. Hepatic tri-
acylglycerides and glycogen were measured as previously outlined [33].
A DNeasy\textsuperscript{R} Blood & Tissue Kit (Qiagen, Germantown, MD, USA) was
used to isolate liver DNA and 5-methylcytosine (5-mC) was measured
via an ELISA (Epigentek Group Inc, Farmingdale, NY, USA, p-1030).

2.8. Immunoblotting

Liver tissue lysate was prepared as previously described [10,27]. At 70
or 95 \(^\circ \text{C}, 20–40 \mu\text{g of protein from liver homogenate samples were
denatured and reduced. Liver proteins were separated using a NuPAGE
4–12\% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) and, subsequently,
transferred to a PVDF membrane. PVDF membranes were probed with the following antibodies: acetyl-CoA carboxylase (ACC; 3662, Lot 4, 1:1000 dilution), DNA (cytosine-5)-methyltransferase 1 (DNMT1; 5032, Lot 1, 1:1000 dilution), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Mm99999915_g1), and housekeeping gene, Rn18S (Mm03928990_g1). TaqMan™ gene expression assays (Thermo-FisherScientific, Waltham, MA, USA).

2.11. Statistical analysis
A student’s-t test or two-way repeated measures ANOVA were used to determine statistical differences (p < 0.05) followed by Bonferroni post hoc tests using SigmaStat™ software (Systat Software Inc., San Jose, CA, USA). Data are reported as mean ± SEM.

3. RESULTS

3.1. GNMT KO mice exhibit hepatomegaly
In a large, ad libitum-fed cohort of mice, KO mice showed a ~6% decline in body weight due to decreased adiposity (Table 1). An eight hour fast alleviated this decrease in a separate cohort of KO mice (Table 1). The fasting-induced weight loss may mask the body weight differences between genotypes. We cannot rule out that the difference in body weight may require a larger cohort to identify the small reduction in adiposity in KO mice. KO mice displayed a ~2.25-fold increase in liver weight and liver-to-body weight (Table 1). Arterial insulin and non-esterified fatty acids were similar between genotypes during post-absorptive conditions (Table 1).

3.2. GNMT KO mice display liver methionine cycle dysregulation and tumorigenesis
GNMT, a regulator of methionine cycle and SAM homeostasis (Figure 1A), was undetectable in KO mice (Figure 1B). Methionine cycle intermediates, methionine, SAM, and SAH were increased in the livers of KO mice (Figure 1C). Sarcosine, a product of the reaction catalyzed by GNMT, was reduced in KO mice (Figure 1C). Metabolites associated with homocysteine remethylation to methionine were also altered.

Table 1 — Biometric characteristics in glycine N-methyltransferase-null mice. Body composition data are from 43-week-old, male glycine N-methyltransferase knockout (KO) mice and wildtype (WT) littermates with free access to food and water. Data are mean ± SEM for n = 16–19 mice per genotype. In a separate cohort of mice (Liver Characteristics), body weight, liver weight, and liver-to-body ratio were determined in WT and KO littermates following an 8-hour fast. Data are for mean ± SEM for n = 7–8 mice per genotype. Arterial plasma non-esterified fatty acids were obtained from catheterized WT and KO littermates undergoing stable isotope infusions 7 h and 40 min following food and water withdrawal. Data are mean ± SEM for n = 6–9 mice per genotype. Arterial plasma insulin was obtained from catheterized KO and WT littermates undergoing stable isotope infusions 8 h following food and water withdrawal. Data are mean ± SEM for n = 7–8 mice per genotype. For fasting studies, food and water were withdrawn during the first hour of the light cycle. Liver characteristics and plasma measurements were collected for male mice at 44 weeks of age.

| Body Composition       | WT                          | KO                          |
|------------------------|-----------------------------|-----------------------------|
| Body Weight (g)        | 31.7 ± 0.5                  | 29.9 ± 0.4*                 |
| Lean Mass (%)          | 69.4 ± 0.7                  | 71.5 ± 0.4*                 |
| Fat Mass (%)           | 10.6 ± 0.7                  | 8.5 ± 0.3*                  |
| Liver Characteristics  |                             |                             |
| Body Weight (g)        | 30.4 ± 0.9                  | 30.5 ± 0.5                  |
| Liver Weight (g)       | 1.3 ± 0.0                   | 2.9 ± 0.2*                  |
| Liver-to-Body Weight % | 4.2 ± 0.1                   | 9.5 ± 0.5*                  |
| Plasma Hormones and Metabolites |             |                             |
| Plasma Insulin (ng/ml) | 0.48 ± 0.08                 | 0.45 ± 0.11                 |
| Plasma Non-Esterified Fatty Acids (mmol/L) | 0.72 ± 0.03 | 0.65 ± 0.06 |

* p < 0.05 vs WT.
Figure 1: Methionine cycle dysregulation and pathological liver characteristics in glycine N-methyltransferase knockout mice. A: Schematic representation of select reactions, enzymes, and metabolites associated with one-carbon metabolism. Italicized metabolites were not measured. Enzymes are enclosed in boxes. B: Representative immunoblots of liver glycine N-methyltransferase (GNMT) from mice with a global deletion of GNMT (KO) and wildtype (WT) littermates. C: Liver metabolites related to the methionine cycle; methionine, S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), sarcosine, betaine, and N,N-dimethylglycine (n = 8–9 per genotype). D: Liver 5-methylcytosine relative to total DNA (5-mC; n = 8–9 per genotype). E: Liver S-adenosylhomocysteine (SAH) content. F-G: Liver albumin and α-fetoprotein mRNA (n = 9 per genotype). H: Percent Ki67 positive nuclei in livers with representative images (20X magnification; n = 8–9 per genotype). I: Percent F4/80 positive area per tissue area as determined by immunostaining with representative images (20X magnification; n = 8–9 per genotype). All data are from eight-hour fasted, male mice at 44 weeks of age. Data are mean ± SEM. *p < 0.05 vs. WT.
Specifically, betaine was increased and N, N-dimethylglycine was decreased in KO mice (Figure 1C). Liver global DNA methylation (5-mC) was reduced in KO mice (Figure 1D). The dysregulation of methionine cycle metabolism in KO mice was accompanied by liver nodules (Figure 1E). Liver albumin mRNA, a hepatocyte marker, was comparable between genotypes (Figure 1F). KO mice had a 20-fold increase in liver α-fetoprotein mRNA; an indicator of HCC (Figure 1G). Furthermore, GNMT KO mice showed increased liver cell proliferation as indicated by Ki67-positive nuclei (Figure 1H). Livers of KO mice also showed increased immune cell infiltration as suggested by a higher proportion of F4/80 per tissue area (Figure 1I).

3.3. Reduced liver glucose production in GNMT KO mice

This study tested the hypothesis that HCC resulting from loss of GNMT-mediated transmethylation was associated with lower liver glucose formation and associated fluxes (Figure 2A). Reduced arterial blood glucose was observed throughout the majority of the experiment in KO mice (Figure 2B). This was linked to a lower endogenous glucose production in KO mice (V_{EndoRa}, Figure 2C). Glycogenolysis, the flux of glycogen to glucose-6-phosphate (V_{PYGL}), was diminished in KO mice (Figure 2C). Total gluconeogenic flux (V_{Glucone}) in KO mice was lower due to a decline in gluconeogenesis from phosphoenolpyruvate (V_{Enol} Figure 2C). Despite a reduction in glycolysis and lower liver glycogen phosphorylase protein (Figure 2D) there were comparable liver glycogen levels between genotypes (Figure 2E). However, glycogen precursors, glucose-6-phosphate and UDP-glucose were lower in livers of KO mice (Figure 2G). GAPDH mRNA was higher in livers of KO mice (Figure 2F). In agreement with the decline in total gluconeogenesis, liver fructose-6-phosphate was reduced in KO mice (Figure 2G).

Figure 2: Lower fluxes related to endogenous glucose production in glycine N-methyltransferase knockout mice. A: A schematic representation of select metabolites and fluxes (highlighted in gray) from $^{2}$H/$^{13}$C metabolic flux analysis contributing to endogenous glucose production. B: A time course of fasting blood glucose concentration before, during, and after arterial sampling for $^{2}$H/$^{13}$C metabolic flux analysis in wildtype (WT) and glycine N-methyltransferase knockout (KO) mice (n = 8–9 per genotype). C: Model-estimated, metabolic fluxes normalized to liver weight (μmol liver wt$^{-1}$ min$^{-1}$) in mice for endogenous glucose production (V_{EndoRa}), flux from glycogen to glucose-6-phosphate (V_{PYGL}), flux from dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (V_{Aldo}), flux from glycerol to dihydroxyacetone phosphate (V_{GK}; hexose units), and flux from phosphoenolpyruvate to glyceraldehyde-3-phosphate (V_{Enol}; hexose units), n = 8–9 per genotype. D: Liver glycogen phosphorylase (PYGL) as determined by immunoblotting with a representative immunoblot (n = 7 per genotype). E: Liver glycogen concentration (mg g liver wt$^{-1}$ n = 8–9 per genotype). F: Liver glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA (n = 9 per genotype). G: Metabolites related to glucose production; glucose-6-phosphate, glucose-1-phosphate, UDP-glucose, fructose-6-phosphate, dihydroxyacetone phosphate, glyceraldehyde-3-phosphate, 2-phosphoglyceric acid, and phosphoenolpyruvic acid (nmol g liver wt$^{-1}$ n = 5–9 per genotype). All mice are males and 44 weeks of age. Data are mean ± SEM. *p < 0.05 vs. WT.
Figure 3: Diminished citric acid cycle-related fluxes in glycine N-methyltransferase knockout mice. A: A schematic representation of select metabolites and fluxes (highlighted in gray) from 2H/13C metabolic flux analysis associated with the citric acid cycle. B: Model-estimated, metabolic fluxes normalized to liver weight (μmol/g liver wt⁻¹/min⁻¹) in wildtype (WT) and glycine N-methyltransferase knockout (KO) mice for contribution of pyruvate kinase and malic enzyme to flux generating pyruvate (V_{PK+ME}), flux from non-phosphoenolpyruvate-derived, unlabeled anaplerotic substrate to pyruvate (V_{UAS}), anaplerotic flux as modeled by pyruvate to oxaloacetate (V_{PC}), cataplerosis modeled as oxaloacetate to phosphoenolpyruvate (V_{PCK}), flux from oxaloacetate and acetyl-CoA to citrate (V_{CS}), anaplerotic flux modeled as propionyl-CoA to succinyl-CoA (V_{PCC}), and flux from succinyl-CoA to oxaloacetate (V_{SDH}). n = 8–9 per genotype. C: Liver pyruvate carboxylase (PC) determined by immunoblotting and a representative immunoblot (n = 8 per genotype). D: Liver amino acids; leucine, isoleucine, valine, alanine, threonine, lysine, tyrosine, phenylalanine, aspartate, asparagine, glutamine, and histidine (nmol/g liver wt⁻¹; n = 8–9 per genotype). E: Liver citric acid cycle (CAC) and related metabolites; citric acid, cis-aconitic acid, succinic acid, fumaric acid, malic acid, lactic acid, pyruvic acid, and the lactic acid-to-pyruvic acid ratio (nmol/g liver wt⁻¹; n = 8–9 per genotype). All mice were males and 44-weeks-old. Data are mean ± SEM. *p < 0.05 vs. WT.
3.4. Lower citric acid cycle and associated fluxes in GNMT KO mice

The provision of gluconeogenic precursors is regulated by citric acid cycle (CAC) and related fluxes (Figure 3A). Flux of phosphoenolpyruvate to pyruvate (V\textsubscript{PK+ME}), was lower in KO mice (Figure 3B). The rate of unlabeled, non-phosphoenolpyruvate derived, anaplerotic substrates to pyruvate (V\textsubscript{UAS}; modeled as unlabeled lactate to pyruvate) was lower in KO mice (Figure 3B). Anaplerosis, modeled as flux from pyruvate to oxaloacetate (V\textsubscript{PC}) and flux from propionyl-CoA to succinyl-CoA (V\textsubscript{PCC}), were diminished in KO mice (Figure 3B). This was accompanied by a decline in pyruvate carboxylase (PC) protein (Figure 3C) and higher anaplerotic amino acids lysine and histidine (Figure 3D). Liver glutamine was lower in KO mice, suggesting that, despite reduced anaplerosis related to glucose production, glutaminolysis may be increased in KO mice (Figure 3D). KO mice displayed a reduction in cataplerotic flux (V\textsubscript{PCK}), modeled as metabolite flux from oxaloacetate to phosphoenolpyruvate (Figure 3B). A decline in CAC fluxes (V\textsubscript{CS} and V\textsubscript{SDH}) was also exhibited by KO mice (Figure 3B). This was associated with an increase in liver citric acid concentration (Figure 3E). Of note, while lactic acid and pyruvic acid were comparable between genotypes, the lactic acid-to-pyruvic acid ratio was increased in the livers of KO mice (Figure 3E).

3.5. Dysregulated NAD\textsuperscript{+} metabolism in livers of GNMT KO mice

The increased lactic acid-to-pyruvic acid ratio indicates dysregulated redox state in the livers of KO mice. Indeed, liver NAD\textsuperscript{+}, NADH, and the NAD\textsuperscript{+}-to-NADH ratio was reduced in KO mice (Figure 4B-D). Alterations at multiple metabolic nodes of NAD\textsuperscript{+} regulation may be involved in the perturbed NAD\textsuperscript{+} homeostasis (Figure 4A). In the absence of GNMT, there is an increase in the protein of other methyltransferases including DNMT1 (Supp Fig. A.1A), PEMT (Supp Fig. A.1B) and, most notably for NAD\textsuperscript{+} regulation, NNMT (Figure 4E). This increase constrains the elevated SAM at the expense of NAD\textsuperscript{+} salvage. NAD\textsuperscript{+} is an important substrate involved in the DNA damage response. Livers of GNMT KO mice displayed increased γH2AX (Figure 4F), higher PARP1 protein (Figure 4G), and lower ADP-ribose (Figure 4H). These markers indicate utilization of NAD\textsuperscript{+} in response to DNA damage is elevated. Also, de novo synthesis of NAD\textsuperscript{+} may be impaired as tryptophan was higher (Figure 4I), TDO protein was lower (Figure 4J), and negative regulator of TDO expression, URI, was higher (Figure 4K) in KO mice.

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**Figure 4:** Dysregulated NAD\textsuperscript{+} homeostasis in livers of glycine N-methyltransferase knockout mice. A: A schematic representation of select metabolites and enzymes associated with NAD\textsuperscript{+} synthesis, salvage, and utilization. B-D: Liver metabolites related to redox state in wildtype (WT) and glycine N-methyltransferase deficient (KO) mice; NAD\textsuperscript{+}, NADH, and the NAD\textsuperscript{+}-to-NADH ratio (nmol·g liver wt\textsuperscript{-1}; n = 8–9 per genotype). E: Liver nicotinamide N-methyltransferase (NNMT) as determined by immunoblotting with a representative immunoblot (n = 7–8 per genotype). F: Liver phosphorylated histone H2AX variant (γH2AX) as determined by immunoblotting with a representative immunoblot (n = 7–8 per genotype). G: Liver poly(ADP-ribose) polymerase 1 (PARP1) as determined by immunoblotting with a representative immunoblot (n = 8 per genotype). H: Liver ADP-ribose (nmol·g liver wt\textsuperscript{-1}; n = 9 per genotype). I: Liver tryptophan (nmol·g liver wt\textsuperscript{-1}; n = 8–9 per genotype). J: Liver tryptophan 2,3-dioxygenase (TDO) as determined by immunoblotting with a representative immunoblot (n = 7 per genotype). K: Liver unconventional prefoldin RPB5 interactor (URI) as determined by immunoblotting with a representative immunoblot (n = 6–7 per genotype). All data are from eight-hour fasted, male mice at 44 weeks of age. Data are mean ± SEM. *p < 0.05 vs. WT.
is important to note that a potential impairment in NAD$^+$ salvage due to increased NNMT occurs prior to tumorigenesis in GNMT KO mice (Supp Fig. A.2A). However, increased NAD$^+$ utilization and reduced de novo synthesis of NAD$^+$ coincide with tumorigenesis given that γH2AX (Supp Fig. A.2B), PARP1 (Supp Fig. A.2C), TDO (Supp Fig. A.2D), and URI (Supp Fig. A.2E) are comparable between genotypes at 12 weeks of age, prior to the appearance of HCC in GNMT KO mice.

3.6. Altered pentose phosphate pathway metabolism in GNMT KO mice
While reduced NAD$^+$ availability impedes CAC fluxes and, consequently, gluconeogenesis, it may also perturb redox status so that precursors of glucose production are directed towards other pathways such as the pentose phosphate pathway to preserve reducing equivalent availability (Figure 5A). KO mice exhibited an increase in G6PDH protein (Figure 5B). Liver NADP$^+$ was reduced in KO mice (Figure 5C). NADPH was comparable between genotypes (Figure 5D); however, the NADP$^+$-to-NADPH ratio was lowered in KO mice (Figure 5E). Liver 6-phosphogluconic acid was diminished in KO mice (Figure 5F). Ribulose-5-phosphate (R5P) was elevated (Figure 5G) and the G6P-to-R5P ratio (Figure 5H) was reduced in KO mice. Together, these data provide support for increased flux through the oxidative phase of the pentose phosphate pathway in the absence of GNMT.

3.7. Elevated transsulfuration and glutathione (GSH) synthesis in KO mice
NADPH is an important reducing equivalent for maintaining GSH levels. In livers of KO mice where NADPH availability may be challenged, the elevated SAM may promote the de novo synthesis of GSH via higher transsulfuration (Figure 6A). Transsulfuration-related metabolites are altered in livers of KO mice. Serine is reduced (Figure 6B), cystathionine is increased (Figure 6C), and cysteine trends towards being elevated (Figure 6D) in KO mice. Also, KO mice showed a decline in glycine (Figure 6F) and increase in GSH (Figure 6G). The elevated GSH was associated with increased glutathione synthetase protein (Figure 6K). Importantly, GSH synthesis utilizes gluconeogenic precursors and may contribute to the decline in glucose formation.

3.8. Liver steatosis is absent in GNMT KO mice at 44 weeks of age
The altered redox state has implications for metabolic systems beyond glucose production, including lipogenesis. URI target, glutaryl-CoA dehydrogenase (GCDH), was reduced in KO mice (Supp Fig. A.3A). This may contribute to the lower acetyl-CoA concentration in KO mice (Supp Fig. A.3B). GNMT-deficient mice exhibit elevated liver malonyl-CoA and triacylglycerides prior to the appearance of HCC at 12 weeks of age [10]. However, at 44 weeks of age, when liver tumor nodules are present, malonyl-CoA and triacylglycerides are comparable between genotypes (Supp Figure A.3C and D). Of note, the normalization of liver steatosis in 44-week-old KO mice may also be the result of lower de novo lipogenesis given ATP-citrate lyase and fatty acid synthase are reduced (Supp Figure A.3E and G). A persistent elevation in polyamine turnover from 12- to 44 weeks of age may also limit triglyceride accumulation by using acetyl-CoA for polyamine catabolism (Supp Fig. A.4).

4. DISCUSSION
Studies in humans and mutant mouse models highlight a role for GNMT insufficiency in the pathogenesis of HCC [16–19]. Our previous work identified that liver glucose production from multiple sources was...
A reduction in liver glucose production has been theorized impaired in 12-week-old GNMT-null mice prior to the appearance of HCC [10]. Moreover, results show livers of KO mice at 44 weeks of age display a more pronounced dysregulation of NAD+ synthesis of GSH. Furthermore, these changes in nutrient handling are coupled to lower liver NAD+ availability. The results shown here in GNMT KO mice extend our previous work by showing that the reduction in endogenous glucose production (V_glycogenolysis and increased glycogenolysis (V_glycogenolysis) as determined by immunoblotting with a representative immunoblot (n = 7–8 per genotype). K: Liver glutathione synthetase (GSS) as determined by immunoblotting with a representative immunoblot (n = 7 per genotype). All data are from eight-hour fasted, male mice at 44 weeks of age. Data are mean ± SEM. *p < 0.05 vs. WT. 4.1. Glucose formation in the livers of KO mice Blood glucose was reduced in postabsorptive KO mice with HCC. This adds to prior work in GNMT-null mice studied before the appearance of HCC [10,38,39]. Here we show the lower blood glucose concentration adds to prior work in GNMT-null mice studied before the appearance of HCC [10,38,39]. The present study showed that this is due in part to lower glycogen phosphorylase. Liver glycogen was not significantly elevated in 44-week-old KO mice as previously reported in 11–12-week old GNMT-deficient mice. This may be due to a limitation in glycogen precursors, glucose-6-phosphate and UDP-glucose, which were lower in KO mice. Additionally, the impaired glycogenolysis and the absence of elevated glycogen could be linked to increased use of glucose-6-phosphate in the pentose phosphate pathway (detailed below). Concurrent with a reduction in glycogenolysis, was a decline in endogenous glucose production (V_Ende). Impaired glycogenolysis and increased glycogen have been reported in GNMT-deficient mice at time points prior to liver tumor formation [10,39,40]. The present study showed that this is due in part to lower liver glycogen phosphorylase. Liver glycogen was not significantly elevated in 44-week-old KO mice as previously reported in 11–12-week old GNMT-deficient mice. This may be due to a limitation in glycogen precursors, glucose-6-phosphate and UDP-glucose, which were lower in KO mice. Additionally, the impaired glycogenolysis and the absence of elevated glycogen could be linked to increased use of glucose-6-phosphate in the pentose phosphate pathway (detailed below). Concurrent with a reduction in glycogenolysis, was a decline in gluco-
anaplerosis, cataplerosis, and CAC-related fluxes. Total cataplerosis ($V_{CAT}$) and anaplerotic fluxes ($V_{ANAP}$ and $V_{ANAP}$) were reduced in KO mice. Regulators of anaplerosis including a reduction in CAC fluxes ($V_{CGN}$ and $V_{CGN}$) PC protein, and unlabeled anaplerotic substrate flux to pyruvate ($V_{UAS}$) may mediate the slowing of metabolite entry into the CAC. The metabolic fluxes were estimated from the mass isotopomer distribution of circulating glucose and normalized to liver weight; which is ~2.25-fold greater in GNMT KO mice. Given this, the conclusion that glucose formation and associated nutrient fluxes are reduced in GNMT KO mice assumes that the proportion of liver weight that is made up of hepatocytes is comparable between genotypes and that these cells produce glucose. Albumin mRNA, a hepatocyte marker, was comparable in livers of KO mice. It is important to consider that KO mice showed ~20-fold higher liver z-fetoprotein mRNA. Alpha-fetoprotein is expressed in hepatocyte precursor cells such as oval cells [44,45]. In fact, increased proliferation of oval cells contributes to tumor formation and the pathogenesis of HCC in GNMT-null mice [15,46]. Thus, it is reasonable to conclude that a similar or greater proportion of hepatocyte lineage cells are present in livers of KO mice. These cells, however, exhibit more heterogeneity in hepatocyte maturation.

4.2. Redox state in livers of KO mice
Glucose production and associated intermediary fluxes are closely linked to redox potential. Elevated cytosolic NADH and/or NADH/NAD$^+$ are inhibitory for gluconeogenesis from lactate [22,23]. Moreover, NAD$^+$ is a required cofactor in CAC reactions and the downstream generation of ATP required for gluconeogenesis. KO mice showed a decline in liver NAD$^+$, NADH, and the NAD$^+$-to-NADH ratio. These changes in redox parameters may impede gluconeogenesis in KO mice. The measured NAD$^+$-to-NADH does not distinguish between cell compartmentation. However, the lactate-to-pyruvate ratio is an indicator of the cytosolic NADH-to-NAD$^+$ ratio [47]. In GNMT KO mice, liver lactic acid relative to pyruvic acid was higher. Interestingly, the reduction in NAD$^+$ was more substantial than NADH. Metabolic processes independent of gluconeogenesis could prevent a similar decline in oxidized and reduced NAD$^+$. For example, the mRNA of the glycolytic enzyme, GAPDH, was elevated in GNMT KO mice. An increase in GAPDH action and/or glycolysis may impede the decline in NADH relative to NAD$^+$. Alternatively, we have previously shown respiration to be impaired in hepatocytes from 12-week-old GNMT KO mice [10]. A reduction in oxidative phosphorylation could blunt the lowering of liver NADH in GNMT KO mice at 44 weeks of age. The redox phenotype in KO mice is linked to the dysregulation of pathways associated with NAD$^+$ provision and utilization. Lower liver NAD$^+$ and its precursor, nicotinamide (NAM), are observed in KO mice as early as 12 weeks of age [10]. Here we show NNMT protein to be elevated in livers of KO mice prior to HCC at 12 weeks of age and in 44-week-old mice when HCC is present. NNMT transfers a methyl group from SAM to NAM forming 1-methylnicotinamide and SAH [48]. The increase in NNMT has been proposed to be a compensatory mechanism to prevent elevated liver SAM [10,21]. Indeed, liver SAM is reduced in NNMT-deficient mice when NNMT substrate, NAM, is not limiting [21]. However, overexpression of NNMT does not lower liver SAM in WT mice [49]. Thus, the ability of NNMT to control liver methyl donor balance is of importance only under conditions where it is positioned to be a predominant methyltransferase such as the case in mice with NNMT-deficient livers [49]. There is limited evidence to suggest that NNMT is a primary regulator of liver NAM homeostasis. Antisense and adenoviral knockdown of NNMT did not increase liver NAM and NAD$^+$ in mice [50,51]. However, NNMT-null mice display an increase in plasma NAM under high-fat fed conditions [52]. It is important to note that high-fat feeding lowers liver NNMT levels [53]. Thus, it is hypothesized that the elevated NNMT lowers NAM and, subsequently, compromises NAD$^+$ salvage under conditions of NNMT insufficiency. Intriguingly, supplementing the drinking water of NNMT-null mice with NAM prevents liver steatosis that precedes HCC in this mouse model [21]. Enhancing NAD$^+$ provision may also inhibit HCC NNMT-null mice.

In addition to salvage processes, NAD$^+$ is synthesized de novo from tryptophan via the kynurenine pathway [54]. De novo synthesis of NAD$^+$ is primarily localized to the liver in vivo [55]. Here we show livers of KO mice exhibited an increase in tryptophan and a reduction in TDO, the hepatic enzyme catalyzing the conversion of tryptophan to N' formylkynurenine [56]. This strongly implies that reduced NAD$^+$ synthesis via the kynurenine pathway may be facilitated by the increase in liver URI observed in KO mice. Mouse models overexpressing and insufficient in URI show a reduction and increase in liver NAD$^+$, respectively [57]. Evidence suggests that URI reduces NAD$^+$ by

Figure 7: Schematic representation of hypothesized metabolic programming contributing to the development of HCC in livers of GNMT KO mice.
inhibiting aryl hydrocarbon receptor- and/or estrogen receptor-mediated expression of kynurenine pathway regulators such as TDO [57]. The URI gain-of-function in mice also invokes liver DNA damage and tumorigenesis that is prevented by supplementing the mouse diet with NAD⁺ precursor nicotinamide riboside [57]. An increase in liver URI may hold translational implications. URI is increased in humans with HCC and the magnitude of the increase in expression is associated with poor survival [57]. Beyond the kynurenine pathway, the higher URI potentially impacts liver lipid synthesis. At 12 weeks of age, GNMT-deficient mice are characterized by liver triacylglyceride accumulation and higher levels of enzymes involved in de novo lipogenesis [10]. Interestingly, here we show 44-week old KO mice no longer display liver steatosis. This is associated with a reduction in the protein of enzymes involved in de novo lipogenesis. It is notable that these enzymes are lower in livers of URI-overexpressing mice [57,58]. Results indicate that diminished liver NAD⁺ is the product of accelerated NAD⁺-consuming enzymes, including PARPs. PARPs transfers ADP-ribose from NAD⁺ to acceptor proteins to form ADP-ribose polymers [59]. PARP action is a substantial contributor to NAD⁺ catabolism in the cell [55,60]. In the liver, pharmacological inhibition of PARP elevates NAD⁺ [61,62]. PARP-mediated poly(ADP-ribose)ylation are involved in the control of many processes including the DNA damage response [59]. The NAD⁺-consuming action of PARPs is profound in response to genotoxic stresses that damage DNA [55,63–65]. This is owing to the increase in PARP catalytic activity upon binding to DNA strand breaks [60,66]. Liver PARP1 was higher in livers of 44- but not 12-week old KO mice. This is accompanied by an increase in DNA damage response marker, γH2AX. Thus, the lower NAD⁺ in KO mice with HCC may be related to genomic insult.

4.3. Diversion of glucose precursors towards pathways regulating redox potential in KO mice

An alternative fate to glucose production for glucose-6-phosphate is the oxidative phase of the pentose phosphate pathway [67]. The oxidative pentose phosphate pathway is an important source of intracellular NAPDH for reductive biosynthetic reactions and redox homeostasis [68,69]. Liver NAD⁺ and NADPH were lower and unchanged, respectively, in KO mice. This created a lower NAD⁺-to-NADPH ratio which was associated with an increase in liver G6PDH protein. G6PDH is a key controller of flux through the oxidative pentose phosphate pathway and is routinely observed to be higher in HCC models with elevated pentose phosphate pathway flux [70]. Furthermore, livers of KO mice showed a decrease in the G6P-to-R5P ratio; an indicator of increased flux through the pentose phosphate pathway [71]. Generating reducing potential via the pentose phosphate pathway to mitigate oxidative stress is protective for many cancer types [67]. The enhanced need for antioxidant defense may accelerate flux through the pentose phosphate pathway in the liver of this mouse model. While NADPH is a cofactor in the reduction of GSSG to GSH, GSH is also generated de novo via two reactions catalyzed by glutamate–cysteine ligase and GSS [72,73]. Liver glutamate-cysteine ligase subunits (GCLC and GCLM) and reactants (glutamate and cysteine) were similar between genotypes. The increase in cysteine did not reach statistical significance, however, cystathionine was higher and serine was lower in KO mice. This suggests increased flux and cysteine provision through the transsulfuration pathway in response to elevated SAM. GSS protein and GSH in the livers of KO mice were higher. Results indicate increased de novo synthesis of GSH downstream of higher transsulfuration in the livers of KO mice. A greater formation of GSH diverts gluconeogenic precursors (cysteine and glutamate) away from glucose formation in KO mice. Given the positive association between GSH and cell proliferation [73], it is reasonable to further hypothesize that the increased GSH promotes HCC in KO mice.

5. CONCLUSION

Metabolic reprogramming supports tumor formation. As such, metabolic nodes and pathways that are pivotal to this reprogramming may be effectively targeted for cancer therapeutics. GNMT KO mice with HCC exhibit a reduction in glucose formation from glycogen and gluconeogenic sources. Precursors for glucose are diverted to alternative biosynthetic pathways that can contribute to tumorigenesis. These include the pentose phosphate pathway and de novo GSH synthesis. The shift in metabolic fluxes are associated with a reduction in NAD⁺ and the NAD⁺-to-NADH ratio. The perturbation in NAD⁺ may be a product of a blockade in the kynurenine pathway, competition for NAM, and/or consumption of NAD⁺. It can be concluded from these studies that accelerating glucose formation directly and/or indirectly via NAD⁺ homeostasis holds potential to combat HCC in GNMT-null mice.

AUTHOR CONTRIBUTIONS

CCH and DHW designed the experiments. CCH, FDJ, ZW, and MG contributed to data acquisition. CCH analyzed and interpreted data. CCH drafted the manuscript. All authors contributed to editing the manuscript and approved the manuscript for publication.

CONFLICT OF INTEREST

No conflicts of interest, financial or otherwise, are declared by the authors.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2019.02.006.

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