Glycine N-Methyltransferase (GNMT) affects genetic stability by regulating DNA methylation and interacting with environmental carcinogens. To establish a Gnmt knockout mouse model, 2 lambda phage clones containing a mouse Gnmt genome were isolated. At 11 weeks of age, the Gnmt−/− mice had hepatomegaly, hypermethioninemia, and significantly higher levels of both serum alanine aminotransferase and hepatic S-adenosylmethionine. Such phenotypes mimic patients with congenital GNMT deficiencies. A real-time polymerase chain reaction analysis of 10 genes in the one-carbon metabolism pathway revealed that 5,10-methylenetetrahydrofolate reductase, S-adenosylhomocysteine hydrolase (Ahcy), and formiminotransferase cyclodeaminase (Ftcd) were significantly down-regulated in Gnmt−/− mice. This report demonstrates that GNMT regulates the expression of both Ftcd and Ahcy genes. Results from pathological examinations indicated that 57.1% (8 of 14) of the Gnmt−/− mice had glycogen storage disease (GSD) in their livers. Focal necrosis was observed in male Gnmt−/− livers, whereas degenerative changes were found in the intermediate zones of female Gnmt−/− livers. In addition, hypoglycemia, increased serum cholesterol, and significantly lower numbers of white blood cells, neutrophils, and monocytes were observed in the Gnmt−/− mice. A real-time polymerase chain reaction analysis of genes involved in the gluconeogenesis pathways revealed that the following genes were significantly down-regulated in Gnmt−/− mice: fructose 1,6-bisphosphatase, phosphoenolpyruvate carboxykinase, and glucose-6-phosphate transporter. Conclusion: Because Gnmt−/− mice phenotypes mimic those of patients with GNMT deficiencies and share several characteristics with GSD Ib patients, we suggest that they are useful for studies of the pathogenesis of congenital GNMT deficiencies and the role of GNMT in GSD and liver tumorigenesis. (HEPATOLOGY 2007;46:1413-1425.)
Glycine N-methyltransferase (GNMT), also known as a 4S polycyclic aromatic hydrocarbon binding protein, has multiple functions. In addition to acting as a major folate binding protein,\(^1\) it also regulates the ratio of S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH) by catalyzing sarcosine synthesis from glycine.\(^2\) We previously reported that GNMT is down-regulated in hepatocellular carcinoma.\(^3,4\) Results from a genetic epidemiological study indicate that Gnmt is a tumor susceptibility gene for liver cancer.\(^5\) In addition, we reported that GNMT binds benzo(a)pyrene and prevents DNA-adduct formation.\(^6\)

In mice, GNMT expression is regulated by growth hormone, with the hepatocytes of female mice having up to 8 times the expression level normally found in male mice.\(^7\) There have been three reports of pediatric patients (2 boys and 1 girl) with congenital GNMT deficiencies resulting from a missense mutation in the GNMT gene.\(^8,9\) All 3 children had hypermethioninemia, clinical symptoms mimicking chronic hepatitis,\(^8,9\) and mild elevations of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The livers of an Italian girl and her older brother had hepatomegaly at 4.7 and 9.7 years of age, respectively; the Gypsy boy had no hepatomegaly at 5 years.\(^8,9\) The girl had stunted growth and suffered from emotional instability. Although SAM levels in the plasma of all 3 children increased, no or very little elevation in the SAH levels of the following: Cbs, Ahcy, Ms, Mthfr, Gpc2, Cdh3, Mnd, Cysht, Cysteine beta-synthase (Cbs), methionine synthase (Ms); methylenetetrahydrofolate reductase (Mthfr); molybdenum cofactor (Moco); and adenosylhomocysteinase (Ahcy) were observed.\(^8,9\) Liver biopsies showed mild centrilobular fibrosis and some eosinophils in the girl patient and mild hydropic degeneration in the Gypsy boy.\(^8,9\) This background triggered our decision to generate a Gnmt knockout mouse model to study the role of GNMT in liver physiology.

For this study, we isolated 2 lambda phage clones containing the mouse Gnmt gene. Fluorescence in situ hybridization (FISH) was used to identify the chromosomal location. We developed a Gnmt knockout mouse model and characterized the phenotypes of both male and female homozygous knockout (Gnmt\(^{-\text{/-}}\)) mice. The liver GNMT, SAM, SAH, serum homocysteine, and ALT concentrations were measured, and the genes involved in one-carbon metabolism pathways were analyzed. Because more than half of the Gnmt\(^{-\text{/-}}\) mice had abnormal glycogen accumulations in their livers, we also studied the expression levels of a panel of genes involved in glycogen metabolism. Those results indicated that several genes involved in either the one-carbon or glycogen metabolism pathway were down-regulated in Gnmt\(^{-\text{/-}}\) mice, thus suggesting a novel gene regulation mechanism via GNMT.

### Materials and Methods

**Mouse Gnmt Isolation and Characterization.** A C57BL/6-strain mouse placental genomic DNA library constructed in lambda phage FIX II (Stratagene, La Jolla, CA) was used to isolate Gnmt genomic clones. Human GNMT complementary DNA was used as a probe; hybridization procedures were performed according to standard protocols described previously.\(^10\) Phage clones 3-2 (Fig. 1A) and 5-3, containing Gnmt, were sequenced with a shotgun DNA sequencing procedure according to standard protocols.\(^11\) Phage clone 3-2 was digested with the NotI restriction enzyme and subcloned into pBluescrip II KS (Stratagene) to generate plasmid pSK-3-2.

**Chromosomal Localization.** The FISH analysis was performed according to steps described previously\(^12\) (for additional information, see the supplementary material).

**Animal Experimentation.** All mice were kept in a 12-hour light-dark-cycle room with water and standard mouse pellet chow. Liver lobes were separated and divided into 2 parts: 1 part was fixed in buffered formalin to perform hematoxylin and eosin (HE) stain and periodic acid-Schiff (PAS) stain pathological examinations, and the other was stored in liquid nitrogen for DNA, RNA, and protein analyses. All animal protocols were approved by the Institutional Animal Care and Use Committee of National Yang-Ming University.

**Messenger RNA (mRNA) Isolation and Reverse-Transcription Polymerase Chain Reaction (RT-PCR).** Samples from wild-type mice were obtained from whole embryos (7.5 and 9.5 days old), embryonic livers (12.5 and 13.5 days old), and the livers, kidneys, and brains of newborn mice. The methods of the RNA extraction and polymerase chain reaction (PCR) conditions are detailed in the supplementary material. The primer sequences are listed in Table 1.

**Generating Gnmt Knockout Mice.** A targeting vector was constructed and used to generate the Gnmt knockout mouse model. The methods are detailed in the supplementary material. At 4 weeks, 11 weeks, and 9 months of age, at least 3 mice in each group were sacrificed for different analyses. All the mice had been fasting for at least 8 hours before they were sacrificed.

**Real-Time PCR for Determining the Genes Expression Profile.** The primer sequences are listed in Table 1. Real-time PCR was used to determine the gene expression levels of the following: Gnmt; S-adenosylhomocysteine hydrolase (Ahcy); methionine synthase (Ms); cystathionine beta-synthase (Cbs); 5,10-methylenetetrahydrofolate reductase (Mthfr); molybdenum cofactor (Moco); dehydrogenase (oxidized nicotinamide adenine dinucleotide phosphate–dependent), molybdenetetrahydrofolate
cyclohydrolase, formyltetrahydrofolate synthase (Mthfd1); aldehyde dehydrogenase 1 family member L1 (Aldh1l1); 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (Atic); serine hydroxymethyltransferase 2 (Shmt2); 5,10-methylenetetrahydrofolate synthetase (Mthfs); formiminotransferase cyclodeaminase (Ftcd); glycogen synthase 2 (Gys2); glucose-6-phosphatase (G6Pase); glucose-6-phosphate transporter (G6PT); α-glucosidase (Gaa); amylo-1,6-glucosidase (Agl); branching enzyme 1 (Gbe1); glycogen phosphorylase (Pygl); phosphor-ylase kinase alpha 2 (Phka2); fructose 1,6-bisphosphatase (Fbp1); and phosphoenolpyruvate carboxykinase (PEPCK).
Complementary DNA was produced from hepatic RNA (5 μg). The procedures of real-time PCR analysis have been described.\(^{13}\)  

**Determining the Blood Biochemical Parameters and Hematological Analysis in Gnmt\(^{-/-}\) Mice.** The concentrations of plasma methionine were determined by tandem mass spectrometry. The serum homocysteine was measured with a high-performance liquid chromatograph equipped with fluorometric detection.\(^{14}\) The serum ALT and glucose were analyzed by tandem mass spectrometry. The serum triglyceride, uric acid, urea, alkaline phosphatase, total protein, phosphorus, and creatinine were analyzed with an Abbott Alcyon 300i (Abbott Laboratories, Ltd., United States). The white blood cell, neutrophil, lymphocyte, monocyte, eosinophil, basophil, red blood cell, and platelet counts were analyzed with an Abbott Alcyon 3700 cell counter (Abbott Laboratories).

### Results

**Mouse Gnmt Gene Characterization.** Two lambda phage clones (3-2 and 5-3, both containing mouse Gnmt genomic DNA) were isolated after the screening of more than 1 \(\times 10^4\) colonies from the mouse placental genomic DNA library with human GNMT complementary DNA as a probe. Shotgun DNA sequencing and a blast program were used to obtain full-length sequences [approximately 15 kilobases (kb)] of the 3-2 clone. Returns from a homology search using the Gene Blast program showed that the 3-2 clone contained the mouse Gnmt gene and a chromosome 17E probe. Shotgun DNA sequencing and a blast program were used to obtain full-length sequences [approximately 15 kilobases (kb)] of the 3-2 clone. Returns from a homology search using the Gene Blast program showed that the 3-2 clone contained the mouse Gnmt gene and a chromosome 17E probe.

**Chromosomal Gnmt Gene Localization.** The chromosomal localization of the mouse *Gnmt* gene was determined by FISH. Mouse metaphase chromosomes were cohybridized with a plasmid (pSK-3-2) DNA probe containing the *Gnmt* and a chromosome 17E probe.

### Table 1. Sequences of the Primers

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<tr>
<th>Gene</th>
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<td>Pex6</td>
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</tr>
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<td>GAPDH</td>
<td>TGATGTCGAGCTGACCTGCTG</td>
<td>ACTGAGGTGTTAGGGCACAGGCTGCTG</td>
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</table>
As shown in Fig. 1B, the mouse **Gnmt** gene was localized to chromosome 17C (green fluorescence).

**Gnmt and Pex6 Expression Profiles in Different Mouse Embryonic Stages and in Newborn Mouse Tissue.** We compared **Gnmt** and **Pex6** gene expression profiles at different embryonic stages and in organs of newborn mice. The RT-PCR results showed that **Pex6** transcripts could be detected in embryos as early as day 7.5 and that it was expressed throughout the embryonic development stages (Fig. 1C). Furthermore, **Pex6** was abundant in the livers, kidneys, and brains of newborn mice. In contrast, **Gnmt** transcripts were detected only during the last stage (day 13.5) of embryonic development (Fig. 1C, lane 4); these transcripts were found in the livers and kidneys of newborn mice but not in their brains.

**Generating a Gnmt Knockout Mouse Model.** A targeting vector (Fig. 2A) was constructed and used for transfer into embryonic stem cells via electroporation. Cells containing recombinant genomes were differentiated with a Southern blot analysis. As shown in lane 1 of Fig. 2B, the size of the BamHI (B)-BamHI DNA fragment decreased from 7.9 (wild-type allele) to 5.3 kb (recombinant allele). (C) Genotyping of **Gnmt** knockout mice by a polymerase chain reaction. By the **Gnmt** and neomycin specific PCR, the normal **Gnmt** allele yielded a 772-bp fragment, and the disrupted allele yielded a 409-bp fragment. (D) Expression of the GNMT protein confirmed by a western blot analysis. Each lane contains 10 μg of hepatic lysate. The GNMT molecular mass was 32 kDa, and GAPDH was used as an internal control. +/− indicates **Gnmt**+/− mice; +/+, **Gnmt** heterozygous mice; +/−, wild-type mice; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GNMT, glycine N-methyltransferase; and kb, kilobase.
SAM and SAH Concentrations in the Livers of Gnmt−/− Mice. At 11 weeks of age, male and female wild-type, Gnmt+/−, and Gnmt−/− mice (≥6 mice per group) were sacrificed for a phenotypic analysis. The SAM and SAH concentrations were detected with high-performance liquid chromatography (see the supplementary materials and methods). In comparison with wild-type mice of the same gender, the hepatic concentrations of SAM in Gnmt−/− mice significantly increased in both male and female mice (P < 0.05). In contrast, the hepatic concentration of SAM in Gnmt+/− mice was 2.8-fold lower than that in wild-type mice (Table 2), and the hepatic concentrations of SAH in male and female Gnmt−/− mice were similar to those in the wild-type mice. Accordingly, the SAM/SAH ratio increased 42-fold and 82-fold in the male and female Gnmt−/− mice, respectively (Table 2).

Serum Levels of Homocysteine and Methionine in Gnmt−/− Mice. The homocysteine levels remained unchanged across the different mouse groups. The methionine levels in the Gnmt−/− mice were 1.9-2.4-fold greater than those in the wild-type mice (Table 2).

Real-Time PCR Analysis of Genes Involved in the One-Carbon Metabolism Pathway. Real-time PCR analysis was used to analyze the mRNA levels in the following genes (involved in the one-carbon metabolism pathway) in both wild-type and Gnmt−/− mice: Gnm, Abcy, Ms, Cbs, Mtbfr, Mtbfr1, Aldh1l1, Atic, Shmt2, Mthfs, and Fted. In comparison with the wild-type mice, the mRNAs of Abcy, Mtbfr, and Fted were significantly down-regulated in both male and female Gnmt−/− mice (P < 0.05; Fig. 3). In terms of the gender difference in wild-type mice, the expression levels of Gnm, Cbs, and Mtbfr of male mice were significantly lower than those of female mice.

Liver Function of Gnmt−/− Mice. The total body weights of the Gnmt−/− and wild-type groups from the age of 1 week to the age of 9 months showed no significant difference (data not shown). For mice sacrificed at 4 weeks, 11 weeks, and 9 months, the mean liver weight/total body weight ratios for the Gnmt−/− mice were significantly higher than those for the wild-type mice, with 1 exception: 4-week-old male mice (Fig. 4A). This suggests hepatomegaly in the Gnmt−/− mice, in the females prior to the males. In terms of Gnmt+/− mice, the mean liver weight/total body weight ratios were slightly less than those of the wild-type mice at 11 weeks of age (Fig. 4A).

Table 2. Concentrations of Hepatic SAM and SAH and Levels of Serum Homocysteine and Methionine from Wild-Type and Gnmt Knockout Mice

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>Gnmt+/−</th>
<th>Gnmt−/−</th>
<th>Gnmt−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Total</td>
<td>Male</td>
</tr>
<tr>
<td>SAM (nmol/g of liver)</td>
<td>45.0 ± 23.4</td>
<td>52.4 ± 29.3</td>
<td>48.0 ± 24.6</td>
<td>18.0 ± 4.6</td>
</tr>
<tr>
<td>SAH (nmol/g of liver)</td>
<td>71.9 ± 26.2</td>
<td>64.2 ± 16.8</td>
<td>68.8 ± 22.2</td>
<td>77.1 ± 8.9</td>
</tr>
<tr>
<td>SAM/SAH</td>
<td>0.63</td>
<td>0.82</td>
<td>0.69</td>
<td>0.23</td>
</tr>
<tr>
<td>Homocysteine (µM)</td>
<td>5.7 ± 0.03</td>
<td>5.7 ± 0.04</td>
<td>5.6 ± 0.13</td>
<td>5.9 ± 0.10</td>
</tr>
<tr>
<td>Methionine (mg/dL)</td>
<td>0.74 ± 0.03</td>
<td>0.74 ± 0.01</td>
<td>0.75 ± 0.02</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND indicates not determined; SAH, S-adenosylhomocysteine; and SAM, S-adenosylmethionine.
The results from serum ALT level measurements show that at the age of 4 weeks, the mean ALT level in the female \textit{Gnmt}^{-/-} mice was significantly higher than that in the wild-type mice (P < 0.05), but there was no statistically significant difference between the male wild-type and \textit{Gnmt}^{-/-} mice (Fig. 4B). At 11 weeks of age, the mean ALT levels of both male and female \textit{Gnmt}^{-/-} mice were significantly higher than the levels of the wild-type mice (P < 0.05). At 9 months of age, the mean serum ALT levels were abnormal in both male and female \textit{Gnmt}^{-/-} mice; the differences between the wild-type and \textit{Gnmt}^{-/-} mice were not statistically significant (Fig. 4B).

\textbf{Pathological Findings for \textit{Gnmt}^{-/-} Mice.} The overall appearances of the liver organs from both wild-type and \textit{Gnmt}^{+/+} mice were relatively normal at the age of 11 weeks (Fig. 5A,B), whereas male and female \textit{Gnmt}^{-/-} mice of the same age had enlarged livers (Fig. 5C,D). Multiple gray-whitish nodules approximately 1 mm long were found in the livers of 6 of 7 male \textit{Gnmt}^{-/-} mice (Fig. 5C). HE staining of histological mouse liver sections revealed no abnormalities in the \textit{Gnmt}^{+/+} mice (Fig. 5F,J). However, we observed coagulative necrosis, sinusoidal dilatation, and congestion in the male \textit{Gnmt}^{-/-} mice (Fig. 5G,K). Degenerative changes and perinuclear vacuoles were observed in the intermediate zones of livers in 5 of 7 female \textit{Gnmt}^{-/-} mice (Fig. 5H,L).

PAS staining was used to examine glycogen storage in mouse liver tissue. In comparison with the male and female wild-type mice (Fig. 5M,N), abnormal storage patterns were found in 3 of 7 (42.9%) male and 5 of 7 (71.4%) female \textit{Gnmt}^{-/-} mice (Fig. 5O,P). Glycogen accumulation was more severe in the female \textit{Gnmt}^{-/-} mice. Glycogen was present in the cytoplasm and sinusoids of livers of female \textit{Gnmt}^{-/-} mice (Fig. 5P) and in the cytoplasm but not the sinusoids of livers of male \textit{Gnmt}^{-/-} mice (Fig. 5O). No pathological abnormalities were noted in the following organs of \textit{Gnmt}^{-/-} mice: the heart, spleen, lungs, pancreas, stomach, kidneys, and small and large intestines. In addition, no abnormal glycogen storage was found in either the hearts or kidneys of the \textit{Gnmt}^{-/-} mice (data not shown).

At the age of 9 months, HE staining of histological sections of mouse livers showed degenerative changes and perinuclear vacuoles in the intermediate zones of male and female \textit{Gnmt}^{-/-} mice (Fig. 5Q,S). PAS staining showed that two-thirds of both male and female \textit{Gnmt}^{-/-} mice had abnormal accumulations of glycogen in their livers (Fig. 5R,T).

\textbf{Hematology and Analysis of the Blood Biochemical Parameters of \textit{Gnmt}^{-/-} Mice.} As shown in Fig. 6A, hematological examinations demonstrated that the \textit{Gnmt}^{-/-} mice had significantly lower numbers of white blood cells, neutrophils, and monocytes than the wild-type mice (n = 12 for each group, P < 0.05). Although the \textit{Gnmt}^{-/-} mice had lower numbers of lymphocytes, basophils, and eosinophils than the wild-type mice, the differences were not statistically significant (P = 0.101, 0.478, and 0.078, respectively; Fig. 6A). We also compared the following blood biochemical parameters between \textit{Gnmt}^{-/-} and wild-type mice: the concentrations of serum glucose, cholesterol, triglyceride, uric acid, urea, alkaline phosphatase, total protein, phosphorus, and creatinine. The results showed that the \textit{Gnmt}^{-/-} mice had significantly lower glucose levels (P < 0.05) and higher cholesterol levels (P < 0.01) than the wild-type mice (Fig. 6B). Although the serum triglyceride was lower in the
Gnmt−/− mice, the difference was not statistically significant. All levels of the other blood biochemical parameters in the Gnmt−/− mice were within the normal range (data not shown).

Real-Time PCR Analysis of Genes Involved in Glycogen Metabolism. Results from a pathological examination revealed that Gnmt−/− mice had abnormally high glycogen accumulations in their livers, so we used...
real-time PCR to analyze the expression levels for genes linked with various types of glycogen storage disease (GSD): Gys2, G6Pase, G6PT, Gaa, Pygl, Fbp1, and PEPCK were lower in 9-month-old Gnmt−/− mice; the differences between Gnmt−/− and wild-type mice were not statistically significant (Fig. 7B).

**Discussion**

In this study, we used FISH to demonstrate that the mouse Gnmt gene is localized in the chromosome 17C region (Fig. 1B). This finding is consistent with data in the National Center for Biotechnology Information mouse genome project database (http://www.ncbi.nlm.nih.gov/genome/guide/mouse/). In addition, we found very different mRNA expression profiles of Gnmt and Pex6 during various mouse embryonic stages and in the organs of newborn mice. Pex6 transcripts were detected as early as day 7.5, whereas Gnmt transcripts were not detected until day 13.5. This suggests an absence of interaction between Gnmt and Pex6 at the transcriptional level.

We measured hepatic SAM and SAH concentrations and found that the mean SAM concentration in the
Gnmt−/− mice was 71-fold higher than that in the wild-type mice; the SAH concentration was the same in the two mouse types (Table 2). Previously, Luka et al. established a Gnmt−/− mouse model and found that compared with the wild-type mice, the Gnmt−/− mice had a 55-fold higher level of hepatic SAM and a significantly lower level of SAH. In their report, they did not mention the age, gender, or number of the mice that they used for the analysis. Therefore, it is difficult to compare the two models because of the limited information. However, we analyzed the hepatic SAH levels of 9-month-old mice (3 in each group) and found no significant differences between Gnmt−/− and wild-type mice (data not shown). In a previous report describing patients with congenital GNMT deficiencies, their hepatic SAM levels were 22-40 times higher than the reference value, and the SAH levels remained constant. In this study, the SAM/SAH ratios were 42 and 67 for male and female Gnmt−/− mice, respectively, at 11 weeks of age, and these ratios are compatible with that noted in a 5-year-old boy with a congenital GNMT deficiency. Hypermethioninemia has previously been reported in patients with congenital GNMT deficiencies, with serum levels of homocysteine remaining unchanged. This is similar to what we observed in our Gnmt−/− mice (Table 2). Therefore, we suggest that our Gnmt−/− mouse model is useful for the study of the pathogenesis of congenital GNMT deficiencies.

In this study, we found that the mean hepatic concentration of SAM in Gnmt+/− mice was about one-third of that in wild-type mice, and the SAM/SAH ratio was reduced from 0.69 to 0.23 (Table 2). This suggests an overcompensation mechanism in hepatocytes that reacts to a reduced GNMT level. SAM is an essential metabolite in all cells. It has been proposed that SAM acts as an intracellular control switch that regulates essential hepatic functions such as regeneration, differentiation, and the sensitivity of this organ to injury. It has been reported that in methionine adenosyltransferase 1A (MATIA)−/− mice, the hepatic SAM and glutathione levels decrease, and the mice develop steatohepatitis and hepatomegaly. Therefore, the hepatic SAM level needs to be maintained within a certain range, and a deficiency or excess can lead to an abnormality.

We used real-time PCR to analyze the expression levels of genes involved in the one-carbon metabolism pathway. Our results showed that the mRNA of Abyc, Mthfr, and Ftcd was down-regulated significantly in both male and female Gnmt−/− mice (Fig. 3). As shown in Fig. 8A, Abyc catalyzes the hydrolytic process of SAH to homocysteine. Homocysteine removal is essential because the equilibrium of the Abyc catalytic reaction strongly favors SAH formation. Under normal conditions, the homocys-
tein turnover rate is sufficient for favoring SAH hydrolysis; this is an important point because SAH is a potent inhibitor of most methyltransferases.\textsuperscript{18,19} AHCY enzyme activity inhibition in rat hepatocytes results in the intracellular accumulation of SAH and the inhibition of SAM-dependent methylation.\textsuperscript{20} Because AHCY is thought to play a pivotal role in transmethylation reaction control via the regulation of intracellular homocysteine and SAH levels, Abcy down-regulation may play an important role in maintaining the SAH level in the livers of Gnmt\textsuperscript{−/−} mice.

MTHFR catalyzes the conversion of 5,10-methyltetrahydrofolate to 5-methyltetrahydrofolate (Fig. 8A).\textsuperscript{21} According to Wagner et al.,\textsuperscript{22} 5-methyltetrahydrofolate inhibits GNMT enzyme activity. Jencks et al.\textsuperscript{23} found that SAM inhibits MTHFR, thereby reducing the supply of methyl groups originating from the one-carbon pool. Accordingly, Mthfr down-regulation in Gnmt\textsuperscript{−/−} mice may result from the absence of GNMT activity and the accumulation of SAM.

The intermediate metabolism enzyme FTCD links histidine catabolism with folate metabolism. FTCD also catalyzes the folate-dependent degradation of N-formimino glutamic acid to form 5,10-methylene tetrahydrofolate, glutamate, and ammonia. These reactions are the final 2 steps in the pathway responsible for L-histidine degradation (Fig. 8A).\textsuperscript{24} Our study is the first demonstration that GNMT can regulate Fticl gene expression. Further studies are needed to determine the underlying mechanism.

Clinically, the Italian girl and her brother with a congenital GNMT deficiency had hepatomegaly at the ages of 4.7 and 9.7 years, respectively. However, the Gypsy boy with a GNMT deficiency did not have hepatomegaly at the age of 5 years.\textsuperscript{8,9} The ALT and AST values for these 3 patients were also higher than the reference values.\textsuperscript{8,9} We observed hepatomegaly in 4-week-old female Gnmt\textsuperscript{−/−} mice but not in male Gnmt\textsuperscript{−/−} mice (Fig. 4A). In addition, the mean serum level of ALT in female Gnmt\textsuperscript{−/−} mice was significantly higher than that in the wild-type mice (P < 0.05); the difference in the serum ALT levels between male Gnmt\textsuperscript{−/−} and wild-type mice was not statistically significant (Fig. 4B). This suggests that the absence of GNMT in the liver may induce liver cell damage and that the effects start earlier in the female mice.

Pathological examinations revealed degenerative changes and perinuclear vacuoles in the intermediate zones of female Gnmt\textsuperscript{−/−} mouse livers (Fig. 5H, L). Focal necrosis and sinusoid congestion were observed in male Gnmt\textsuperscript{−/−} mouse livers (Fig. 5G, K). In addition, PAS staining demonstrated that a higher percentage of female Gnmt\textsuperscript{−/−} mice (71.4%) than male Gnmt\textsuperscript{−/−} mice (42.9%) had abnormal glycogen accumulations. All Gnmt\textsuperscript{−/−} mice had been fasted for 8 hours before being sacrificed. Previously, Aida et al.\textsuperscript{7} reported that in mouse livers, Gnmt expression is much lower in male mice than in female mice and that this expression is regulated by a growth hormone.\textsuperscript{7} We will continue to confirm the diverse responses of liver pathology between male and female Gnmt\textsuperscript{−/−} mice.

GSDs, characterized by abnormal inherited glycogen metabolism in the liver, muscle, and brain, are divided into types 0 to X.\textsuperscript{25} We compared the mRNA levels of a panel of genes responsible for various types of GSDs in wild-type and Gnmt\textsuperscript{−/−} mice. Our results showed that the mRNA levels of G6PT, Gaa, and Pygl were significantly lower in the Gnmt\textsuperscript{−/−} mice in general and in those with abnormal glycogen accumulations (Figs. 7A and 8B). The down-regulation of G6PT results in a glucose 6-phosphate accumulation that inactivates PYGL.\textsuperscript{26} Little is known about the interaction between G6PT and GAA. Raben et al.\textsuperscript{27} generated Gaa\textsuperscript{−/−} mice that started to accumulate glycogen in cardiac and skeletal muscle lysosomes by 3 weeks of age, and this was followed by a progressive increase. At 8-9 months of age, these mice developed obvious signs of muscle deterioration and had waddling gaits.\textsuperscript{27} Neither abnormal glycogen accumulation in the heart and kidneys nor muscle wasting was observed in Gnmt\textsuperscript{−/−} mice. We speculate that G6PT down-regulation is responsible for the GSD phenotype that we observed in our Gnmt\textsuperscript{−/−} mice.

Patients with G6PT deficiencies (GSD Ib) suffer from retarded growth, hypoglycemia, hepatomegaly, nephromegaly, hyperlipidemia, hyperuricemia, lactic academia, neutropenia, and myeloid dysfunction.\textsuperscript{28} The phenotypes of the G6PT\textsuperscript{−/−} mouse model developed and mimicked those of GSD Ib patients.\textsuperscript{29} We found that the Gnmt\textsuperscript{−/−} and G6PT\textsuperscript{−/−} mice shared the following characteristics: hypoglycemia, hepatomegaly, increased cholesterol, glycogen accumulation, and neutropenia, whereas growth retardation, nephromegaly, hyperuricemia, and increased triglyceride levels were not observed in the Gnmt\textsuperscript{−/−} mice. With respect to the rate-limiting enzymes involved in the gluconeogenesis pathway (FBP1 and PEPCK), results from a real-time PCR analysis demonstrated that both genes were down-regulated in the Gnmt\textsuperscript{−/−} mice with abnormal glycogen accumulations (Fig. 7A). Because of the down-regulation of both Fbp1 and PEPCK, the serum glucose levels of our Gnmt\textsuperscript{−/−} mice were significantly lower than those of the wild-type mice.

Previously, Schalinske’s group\textsuperscript{30-32} conducted a series of experiments and showed that rat hepatic GNMT activity can be up-regulated by vitamin A, glucocorticoid, and streptozotocin-induced diabetes. In addition, the
treatment of streptozotocin-induced diabetic rats with insulin prevented the induction of GNMT. This suggests that GNMT may play a role in the regulation of gluconeogenesis and glycolysis pathways. In this study, we strengthen this idea by the observation of hypoglycemia and glycogen accumulation in the Gmnt−/− mice.

Glycogen retention upon fasting is one of the most important markers of a preneoplastic lesion in the liver. In a rat liver tumor model, both clear and eosinophilic cells form multiple foci during the preneoplastic stages of hepatocarcinogenesis. The accumulation of glycogen in neoplastic nodules suggests that they originate from foci that store excess glycogen; patients suffering from type I GSD frequently develop hepatic tumors. We previously reported that the expression of GNMT is down-regulated in hepatocellular carcinoma. A genetic epidemiological study has identified GNT as a tumor susceptibility gene for liver cancer; we therefore hypothesize that GNMT down-regulation is an early event during the initiation stage of hepatocarcinogenesis. Interactions between GNMT and environmental carcinogens will be determined in the near future with Gmnt−/− mice challenged with various carcinogens.

In summary, hepatomegaly, hypermethioninemia, and significantly higher levels of serum ALT and hepatic SAM were observed in our Gmnt−/− mice. The phenotypes mimic those of patients suffering from GNMT deficiencies. In addition, hypoglycemia, increased serum cholesterol, and significantly lower numbers of white blood cells, neutrophils, and monocytes were observed in the Gmnt−/− mice. The phenotypes share several characteristics with patients with congenital G6PT deficiencies. We suggest that this animal model is useful for studies of the pathogenesis of congenital GNMT deficiencies and the role of GNMT in glycogen metabolism and in liver tumorigenesis.

Acknowledgment: We thank Dr. Chung-Yung Chen from the Genomic Center of National Yang-Ming University for the sequencing of our phage clones; Dr. Teh-Yung Chou, and Dr. Ting-Yao Chen for their helpful discussions on pathological examinations of liver specimens; Dr. John T. Kung from the genome-wide mutant mouse animal model core facility of the National Research Program on Genomic Medicine; Mr. Yi-Cheng Wang from the Department of Food Science and Biotechnology at National Chung Hsing University for the determination of SAM, SAH, and homocysteine; and members of the Division of Preventive Medicine of the Public Health Institute at National Yang-Ming University for their technical support.

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