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N-Myristoyltransferase 1 Is Essential in Early Mouse Development*

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***N*-Myristoyltransferase (NMT) transfers myristate to an amino-terminal glycine of many eukaryotic proteins. In yeast, worms, and flies, this enzyme is essential for viability of the organism. Humans and mice possess two distinct but structurally similar enzymes, NMT1 and NMT2. These two enzymes have similar peptide specificities, but no one has examined the functional importance of the enzymes *in vivo*. To address this issue, we performed both genetic and biochemical studies. Northern blots with RNA from adult mice and *in situ* hybridization studies of day 13.5 embryos revealed widespread expression of both *Nmt1* and *Nmt2*. To determine whether the two enzymes are functionally redundant, we generated *Nmt1*-deficient mice carrying a β-galactosidase marker gene. β-Galactosidase staining of tissues from heterozygous *Nmt1*-deficient (*Nmt1*+/−) mice and embryos confirmed widespread expression of *Nmt1*. Intercrosses of *Nmt1*+/− mice yielded no viable homozygotes (*Nmt1*−/−), and heterozygotes were born at a less than predicted frequency. *Nmt1*−/− embryos died between embryonic days 3.5 and 7.5. Northern blots revealed lower levels of *Nmt2* expression in early development than at later time points, a potential explanation for the demise of *Nmt1*−/− embryos. To explore this concept, we generated *Nmt1*−/− embryonic stem (ES) cells. The *Nmt2* mRNA could be detected in *Nmt1*−/− ES cells, but the total NMT activity levels were reduced by ~95%, suggesting that *Nmt2* contributes little to total enzyme activity levels in these early embryo cells. The *Nmt1*−/− ES cells were functionally abnormal; they yielded small embryoid bodies in *in vitro* differentiation experiments and did not contribute normally to organogenesis in chimeric mice. We conclude that *Nmt1* is not essential for the viability of mammalian cells but is required for development, likely because it is the principal *N*-myristoyltransferase in early embryogenesis.**

N-Myristoylation is a lipid modification of proteins that facilitates the targeting of proteins to membrane surfaces. The process is catalyzed by *N*-myristoyltransferase (NMT),[1] an enzyme that transfers myristate from myristoyl-coenzyme A to the amino group of an amino-terminal glycine (1). There is little doubt that this posttranslational modification is important for eukaryotic cells. *NMT* is essential for cell viability in *Saccharomyces cerevisiae* (2), *Candida albicans* (3), and *Cryptococcus neoformans* (4). *NMT* is also required for the viability of flies (5), worms (6, 7), trypanosomes (8), and *Leishmania* (8). Because its activity is essential, NMT has sparked interest as a target for antifungal, antiparasitic, and even anticancer therapy (9). *N*-Myristoylation is also required to produce infectious human immunodeficiency virus type 1, suggesting that NMT could be a potential anti-human immunodeficiency virus type 1 target (10, 11).

Mammals (*e.g.* human, mouse, rat, and cow) and other vertebrates (*e.g.* chicken, frog, and zebrafish) possess two NMTs, NMT1 and NMT2, which are products of distinct genes (www.ensembl.org/). Human NMT1 and NMT2 are 77% identical at the amino acid level and have similar selectivities for peptide substrates (12). The two mouse genes, *Nmt1* and *Nmt2*, encode enzymes that are >95% identical to the human enzymes at the amino acid level (12).

Why mammals and other higher organisms have two NMTs is not clear. Northern blots comparing the patterns of *Nmt1* and *Nmt2* expression have not yet been published for either mouse or human, and it is not known whether the two enzymes are functionally redundant *in vivo*. In this study, we sought to address this issue by creating and characterizing *Nmt1* knockout mice and by analyzing the biochemical and functional properties of an *Nmt1*-deficient cell line.

EXPERIMENTAL PROCEDURES

Nmt1-deficient Mice—A mouse embryonic stem (ES) cell line (XE400, strain 129/Ola) with an insertional mutation in *Nmt1* was created in a gene-trapping program, BayGenomics (baygenomics.ucsf.edu/). The gene-trapping vector, pGT1lxf, was designed to create an in-frame fusion between the 5′ exons of the trapped gene and a reporter, βgeo (a fusion of β-galactosidase and neomycin phosphotransferase II). *Nmt1* spans 13 exons on mouse chromosome 11. The insertional mutation in XE400 occurred in intron 3. Thus, the gene-trapped locus is predicted to yield a fusion transcript containing exons 1–3 of *Nmt1* and βgeo. The ES cells were injected into C57BL/6 blastocysts to create chimeric mice, which were bred with C57BL/6 mice to generate heterozygous (+/−)

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[1] The abbreviations used are: NMT, *N*-myristoltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ES, embryonic stem; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; E, embryonic day.

Nmt1-deficient mice. All mice had a mixed genetic background (~50% C57BL/6 and ~50% 129/Ola). The mice were weaned at 21 days of age, housed in a barrier facility with a 12-h light/dark cycle, and fed chow containing 4.5% fat (Ralston Purina, St. Louis, MO).

Genotyping by Southern Blot and PCR—Genomic DNA (10–20 μg) from tail biopsies, yolk sacs, or embryos was digested with BglI and analyzed by Southern blot with an *Nmt1* probe located 3′ of the insertion site. The probe was amplified from mouse genomic DNA with primers 5′-GACCTGTAGTGAGGCCATCC-3′ and 5′-ATTCCACAGGGCAGTGCTAG-3′. The wild-type allele yielded a 17.5-kb band, and the mutant allele yielded a 6.2-kb band.

Blastocysts or cellular outgrowths from blastocysts were genotyped by PCR. The cells were lysed in 20 μl of PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 μg/ml gelatin, 0.45% Nonidet P-40, 0.45% Tween 20, and 200 μg/ml proteinase K) at 56 °C for 1 h and then at 95 °C for 15 min (13). The PCR contained primer A (5′-CCGAAGGTCCAGAGTTCAAA-3′) and primer B (5′-ATGCCTTTGGGTTGACTCAC-3′), which correspond to sequences upstream and downstream, respectively, of the insertion point, and primer C (5′-CCACAACGGGTTCTTCTGTT-3′), which is specific to pGT1lxf sequences. Genomic DNA (100 ng) and primers A, B, and C (50 ng each) were placed in 10× Titanium PCR buffer (Clontech) containing 0.2 mM deoxynucleoside triphosphates and 1.25 unit of *Taq* polymerase (Clontech) for 30 s at 95 °C. After enzymatic amplification for 35 cycles (1 min at 94 °C, 1 min at 68 °C, and 8 min at 72 °C), the PCR products were size-fractionated on a 1.5% agarose gel in 1× Tris acetate-EDTA buffer. Primers A and B amplify a 560-bp band from a wild-type allele; primers A and C amplify a 960-bp fragment from a mutant allele.

RNA Isolation and Northern Blot Analysis—Total RNA (25 μg) isolated from 50–150 mg of mouse tissue with TRIzol reagent (Invitrogen) was separated by electrophoresis on a 1% agarose/formaldehyde gel and transferred to a Nytran SuPerCharge membrane (Schleicher & Schuell, Keene, NH). Two different mouse multiple-tissue poly(A)$^+$ RNA blots (OriGene Technologies, Rockville, MD) and mouse embryo poly(A)$^+$ RNA blots (Clontech) were used to determine the distribution of *Nmt1* and *Nmt2* expression in adult mice and to examine *Nmt1* and *Nmt2* expression during embryogenesis. [α-^{32}P]dCTP-labeled cDNA probes were prepared with All-in-One random prime labeling mix (Sigma). Standard pre-hybridization, hybridization, and washing procedures were used. Bands were visualized by autoradiography (Hyperfilm ECL; Amersham Biosciences) and quantified by densitometry (Molecular Imager FX; Bio-Rad). Probes were prepared by PCR. The *Nmt1* protein-coding sequences were amplified from a mouse liver cDNA library (Clontech) with primers 5′-TGTCCGGCTCTCGCAACCCAAGATGGC-3′ and 5′-GGGATACACAGCCAGTGCCCAAA-3′; *Nmt2* coding sequences were amplified with primers 5′-AAATAGCCGCCGCGATGGCGGAGG-3′ and 5′-CTATGGACACCTTTAATCCCTCCC-3′. A cDNA probe for the 3′-untranslated region of *Nmt1* was amplified with primers 5′-AGTTGCCAGTGAGATTCTGGA-3′ and 5′-TCACGAGCTAGGTTTCAGCA-3′; an *Nmt2* untranslated region probe was amplified with primers 5′-AGGTGTCCAGGGACAGACTC-3′ and 5′-TTTTTCACTCAACTTGTGCTTTT-3′. A β-galactosidase cDNA probe was amplified from *Nmt1*+/− mouse liver RNA with primers 5′-TTTTCGATGAGCGTGGTGGTTATGC-3′ and 5′-GCGCGTACATCGGGCAAATAATATC-3′. A glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe (Ambion, Austin, TX) was used as a control.

In Situ Hybridization—Sectioning of mouse embryos was performed according to techniques described on the BayGenomics web site (baygenomics.ucsf.edu/), and *in situ* hybridization studies with ^{33}P-labeled *Nmt1* and *Nmt2* riboprobes were performed as described previously (14).

Assay of Total NMT Activity—Total NMT activity levels were measured as described previously (15, 16). To produce [^{3}H]myristoyl-CoA, a mixture of 40 mM Tris-HCl, pH 7.4, 0.1 mM EGTA, 10 mM $MgCl_2$, 5 mM ATP, 1 mM lithium CoA, 1 μM [^{3}H]myristic acid (7.5 μCi), and 0.3 unit/ml *Pseudomonas* acyl-CoA synthetase (total volume, 200 μl) was incubated at 30 °C for 30 min. The efficiency of [^{3}H]myristate conversion to [^{3}H]myristoyl-CoA was generally >95%. Assays of total NMT activity contained a mixture of 40 mM Tris-HCl, pH 7.4, 0.5 mM EGTA, 0.45 mM β-mercaptoethanol, 1% Triton X-100, a phosphorylated, activated phosphoprotein 60-M (pp60src) peptide substrate (GSSKSKPKR, 500 μM), and a source of NMT in a total volume of 25 μl. The reaction was initiated by adding freshly generated [^{3}H]myristoyl-CoA, incubated at 30 °C for 30 min and terminated by spotting 15-μl aliquots of the incubation mixture onto P81 phosphocellulose paper discs and drying under a stream of warm air. The discs were washed in two changes of 40 mM Tris-HCl, pH 7.3, for 60 min. The radioactivity was quantified in 7.5 ml of Beckman Ready Safe Liquid Scintillation mixture in a Beckman liquid scintillation counter. One unit of NMT activity was defined as 1 pmol of myristoyl-peptide formed per minute.

β-Galactosidase Staining—Mice were anesthetized with avertin and perfusion-fixed with 4% paraformaldehyde. Tissues were harvested and fixed in 10% formalin for 4 h at 4 °C, immersed in 30% glucose for 16 h at 4 °C, and frozen in Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Torrance, CA) for sectioning. β-Galactosidase activity was assessed by incubating 10-μm-thick sections with 1.3 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) (Invitrogen) for 16 h at 37 °C. After counterstaining with eosin Y (Sigma), the sections were examined and photographed with an Eclipse 600 microscope (Nikon) equipped with a Spot RT Slider digital camera (Diagnostic Instruments, Burlingame, CA).

Isolation of Homozygous Nmt1-deficient ES Cells—*Nmt1*+/− ES cells were seeded onto a 100-mm gelatin-coated Petri dish in ES cell medium (baygenomics.ucsf.edu/) and grown for 24 h. A high concentration of G418 (5.0 mg/ml) was added, and the concentration was gradually increased to 12.0 mg/ml. After 7–10 days, single colonies were picked and grown in individual wells of a 96-well plate for 5–7 days. Cell lines were genotyped by both Southern blot and PCR. In parallel control studies, homozygous mutant ES cell lines were created from an ES cell line with a mutation in *Lmna* (the gene encoding prelamin A and lamin C).

Real-time PCR—Total RNA was isolated from wild-type blastocysts with the RNeasy Mini kit (Qiagen, Valencia, CA). The quality and quantity of the RNA were assessed with spectrometry and by examining the RNA on an ethidium bromide-stained 1% agarose gel. To generate first-strand cDNA, total RNA (1.0 μg) was reverse-transcribed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) at 25 °C for 10 min and then at 37 °C for 2 h. Ten ng of cDNA was used for each real-time PCR. Primer and probe sets from Applied Biosystems (*Nmt1*, Mm00500829_m1; *Nmt2*, Mm00476437_g1) were used to quantify *Nmt1* and *Nmt2* expression. A real-time PCR assay for *GAPDH* was used for normalization (Applied Biosystems). Real-time PCR was performed on the ABI Prism 7500 Sequence Detection System (Applied Biosystems). The thermal cycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15-s denaturation at 95 °C and 60-s annealing at 60 °C. All assays were performed in triplicate and normalized against *GAPDH*. The results are expressed as relative gene expression with the $2^{-\Delta\Delta C}$T method (17).

Analysis of Nmt1−/− ES Cells—The number of chromosomes in *Nmt1*−/− and the parental *Nmt1*+/+ ES cells was counted (18, 19). To determine whether the *Nmt1*−/− ES cells could differentiate into beating heart cells, *Nmt1*−/− ES cells were diluted in ES cell medium (25,000 cells/ml) and then pipetted in 20-μl droplets into a 96-well V-bottomed polypropylene plate (EK-21261; lids, EK26161; E&K Scientific Plates). The plates were flipped gently with one smooth motion and placed upside down in a 37 °C incubator with 7% CO_2 for 2–3 days. The plates were then flipped right-side up, and differentiation medium (1× Glasgow minimal essential medium/BHK21 medium, 2 mM glutamine, 1 mM sodium pyruvate, 1× nonessential amino acids, 20% fetal bovine serum, and a 1:1000 dilution of a β-mercaptoethanol stock solution (70 μl in 20 ml of water)) was added to each well (200 μl/well). The cells were grown right-side up at 37 °C for 3 days. On day 5, 100 μl of medium was removed and replaced with fresh medium. On day 7, the embryoid bodies were transferred to gelatin-coated tissue culture plates and cultured for 21 days; the medium was changed every 2–3 days. Differentiated *Nmt1*−/− embryoid bodies were photographed and analyzed with an AXIO Vert2000 microscope (Zeiss, Thornwood, NY). In addition to these *in vitro* differentiation experiments, *Nmt1*−/− ES cells were injected into C57BL/6 blastocysts to produce chimeric mice.

RESULTS

Nmt1 and *Nmt2* are expressed in a wide variety of adult mouse tissues, as judged by Northern blots (Fig. 1). *In situ* hybridization studies on mouse embryos at embryonic day (E) 13.5 revealed widespread expression of *Nmt1* in multiple tissues, for example in the liver, lung, and neural tube (Fig. 2). *Nmt2* was also expressed in these same tissues, although the level of expression was consistently lower (Fig. 2).

To determine whether both enzymes are essential in mammals, we produced *Nmt1*+/− mice with a mutant ES cell line containing an insertional mutation in intron 3 of *Nmt1* (Fig. 3*A*). A PCR genotyping approach was developed (Fig. 3*B*), which was particularly useful for genotyping blastocysts. The mutation introduced a new BglI site, facilitating Southern blot

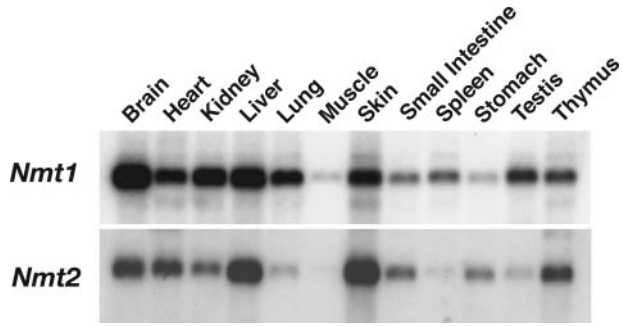

FIG. 1. ***Nmt1* and *Nmt2* expression in adult mice, as judged by Northern blots.** A mouse multiple-tissue poly(A)$^+$ RNA blot (loading normalized to β-actin) was hybridized with an *Nmt1* cDNA probe and an *Nmt2* cDNA probe. Northern blot patterns with coding sequence and untranslated region probes were identical.

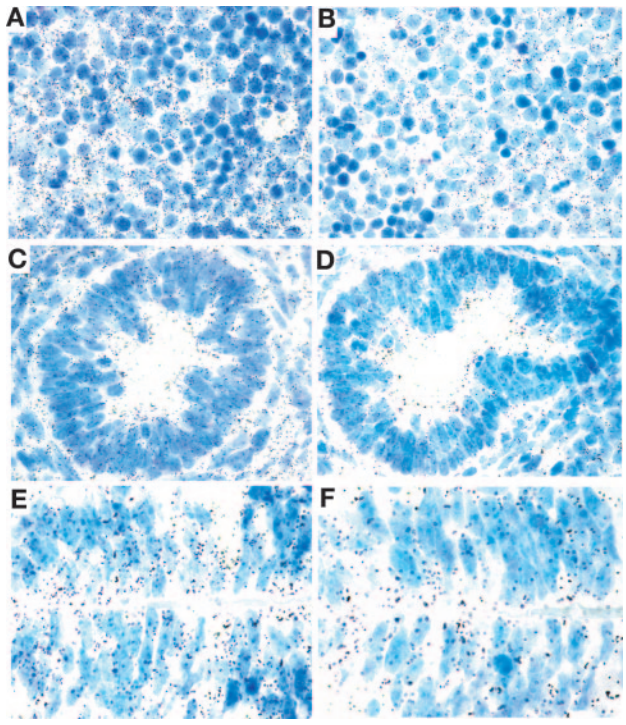

FIG. 2. **Patterns of *Nmt1* and *Nmt2* expression in E13.5 mouse embryos, as judged by *in situ* hybridization with ^{33}P-labeled *Nmt1* and *Nmt2* riboprobes.** The *small black grains* indicate gene expression; the *blue color* represents the counterstain. *A*, *Nmt1* expression in liver in a wild-type embryo. *B*, *Nmt2* expression in liver. *C*, *Nmt1* expression in lung in a wild-type embryo. *D*, *Nmt2* expression in lung. *E*, *Nmt1* expression in the neural tube in a wild-type embryo. *F*, *Nmt2* expression in the neural tube. In control experiments, sense probes yielded no signal.

genotyping (Fig. 3*C*). As predicted, the insertional mutation resulted in the production of an *Nmt1*-βgeo fusion transcript (Fig. 3*D*). *Nmt1* mRNA levels in *Nmt1*+/− mice were approximately one-half of those in wild-type controls. Full-length *Nmt1* transcripts were undetectable in *Nmt1*−/− ES cells (Fig. 3*E*). Because NMT is an essential enzymatic activity in yeast (2), we predicted that *Nmt2* would be expressed in the *Nmt1*−/− ES cells. Indeed, *Nmt2* expression was detectable by Northern blot after a long exposure (36 h) (Fig. 3*E*).

Nmt1+/− mice were normal in terms of weight, growth rate, and appearance; we observed no postnatal deaths in these mice. X-gal staining of tissues from adult *Nmt1*+/− mice revealed widespread expression of *Nmt1* (Fig. 4). In the brain, β-galactosidase expression was largely confined to neurons and was prominent in the Purkinje cells of the cerebellum and the pyramidal neurons of the CA1 region of the hippocampus. The facial nucleus in the medulla and the gray matter of the spinal cord also stained intensely. β-Galactosidase expression was identified in hepatocytes, renal tubular cells, and cardiac myocytes.

Because the specificities of NMT1 and NMT2 for peptide substrates are similar (12) and because the expression patterns of *Nmt1* and *Nmt2* are similar in adult mice and E13.5 embryos (Figs. 1 and 2), we predicted that the two genes would play entirely redundant roles and that *Nmt1*−/− mice would be viable and phenotypically normal. This prediction was not upheld. In genotyping 247 offspring of *Nmt1*+/− intercrosses by Southern blot, we identified 121 *Nmt1*+/+ mice, 126 *Nmt1*+/− mice, and no *Nmt1*−/− mice. Interestingly, *Nmt1*+/− mice were born less frequently than predicted by Mendelian genetics ($p < 0.01$, χ^2 test). Thus, heterozygous *Nmt1* deficiency adversely affected survival.

Additional genotyping revealed a complete absence of *Nmt1*−/− embryos at E7.5, E8.5, E11.5, E15.5, and E17.5 (data not shown), indicating that *Nmt1* is essential for development. Not surprisingly, β-galactosidase was expressed at high levels in multiple tissues of *Nmt1*+/− embryos at E13.5, including the central nervous system, heart, lung, and liver (Fig. 5*A*). Strong β-galactosidase expression was also identified in embryos at E6.5 (data not shown). Although *Nmt1*−/− embryos were absent at E7.5, we identified 5 *Nmt1*−/− blastocysts (E3.5) among 31 that were genotyped. When placed in culture, both *Nmt1*−/− and *Nmt1*+/+ blastocysts yielded cellular outgrowths (Fig. 5*B*).

One potential explanation for the demise of *Nmt1*−/− embryos is that *Nmt1* is the "main NMT" early in mouse development. In line with this concept, *Nmt1* expression was 6.76 ± 0.37-fold higher in wild-type 3.5-day mouse embryos (blastocysts) than *Nmt2* expression, relative to *GAPDH*, as judged by TaqMan real-time PCR. In addition, *Nmt1* expression was higher at E7 than at E11 and E15, as judged by Northern blot analysis (Fig. 6). *Nmt2* expression was relatively low at E7 and then increased gradually (Fig. 6).

To further explore the issue of *Nmt1* expression in early embryo cells, we isolated *Nmt1*−/− ES cells from *Nmt1*+/− ES cells by selection in high concentrations of G418 (20). The *Nmt1*−/− cells, like the parental *Nmt1*+/− ES cells, had a normal chromosome count ($n = 40$). Based on the Northern blot studies, we predicted that the majority of the NMT activity in these early embryonic cells might result from the expression of *Nmt1* rather than *Nmt2*. If so, one would expect to observe much lower levels of enzymatic activity in *Nmt1*−/− cells than in *Nmt1*+/+ ES cells. Indeed, this was the case (Fig. 7). NMT activity levels were intermediate in *Nmt1*+/− cells (Fig. 7).

To assess the functional capabilities of *Nmt1*−/− ES cells, we assessed their ability to differentiate into beating cardiac myocytes in embryoid bodies *in vitro*. *Nmt1*−/− ES cells yielded embryoid bodies (Fig. 8*A*) that stained brightly for β-galactosidase (Fig. 8*B*). However, the embryoid bodies and the area of beating heart cells within them were smaller with *Nmt1*−/− ES cells than with *Nmt1*+/+ ES cells (Fig. 8, *C* and *D*).

Because *Nmt1* is obviously required for mouse development, we suspected that the *Nmt1*−/− ES cells, when injected into blastocysts, might be defective in populating the tissues of the chimeric mice. The mice ($n > 40$) generated from blastocysts that had been injected with *Nmt1*−/− ES cells exhibited minimal evidence of chimerism, as judged by coat color. Moreover, β-galactosidase staining of 1-day-old pups from the injected blastocysts revealed a complete absence of β-galactosidase expression in the brain, lung, and liver (tissues that normally exhibit strong β-galactosidase staining in *Nmt1*+/− mice). However, the injected blastocysts did indeed yield chimeras because we identified β-galactosidase staining in enterocytes in

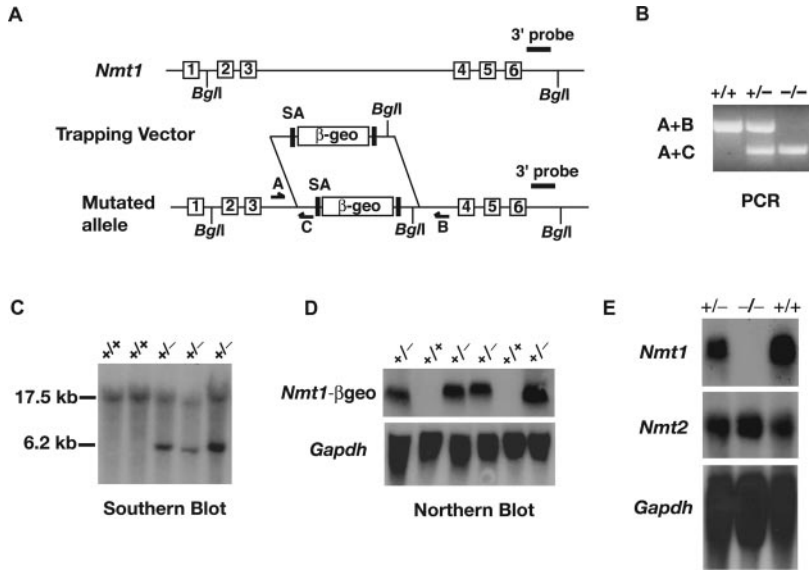

FIG. 3. **Insertional mutation in *Nmt1*.** *A*, schematic of the mutation in *Nmt1*. *Numbered boxes* represent exons. The mutant allele yields an in-frame fusion transcript with exons 1–3 of *Nmt1* and βgeo. BglI sites and sites for oligonucleotide primers A, B, and C are indicated. *SA*, splice acceptor. The location of Southern blot probe is also shown. *B*, genotyping of ES cells by PCR. *C*, Southern blot of genomic DNA from *Nmt1*+/+ and *Nmt1*+/− mice. *D*, Northern blot of RNA from tissues of *Nmt1*+/− mice, hybridized with a β-galactosidase probe, revealing the *Nmt1*-βgeo fusion transcript. *E*, Northern blot of RNA from *Nmt1*+/+, *Nmt1*+/−, and *Nmt1*−/− ES cells hybridized with *Nmt1*, *Nmt2*, and *GAPDH* probes.

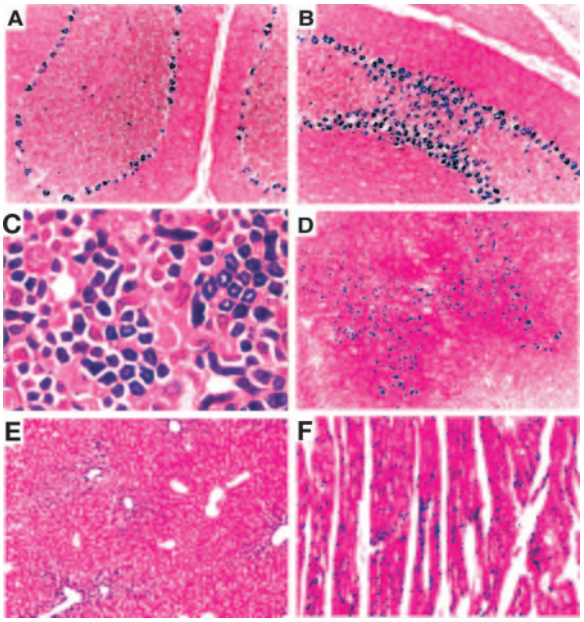

FIG. 4. **β-Galactosidase expression in tissues of adult *Nmt1*+/− mice.** *A*, cerebellum. *B*, hippocampus. *C*, kidney. *D*, spinal cord. *E*, liver. *F*, heart.

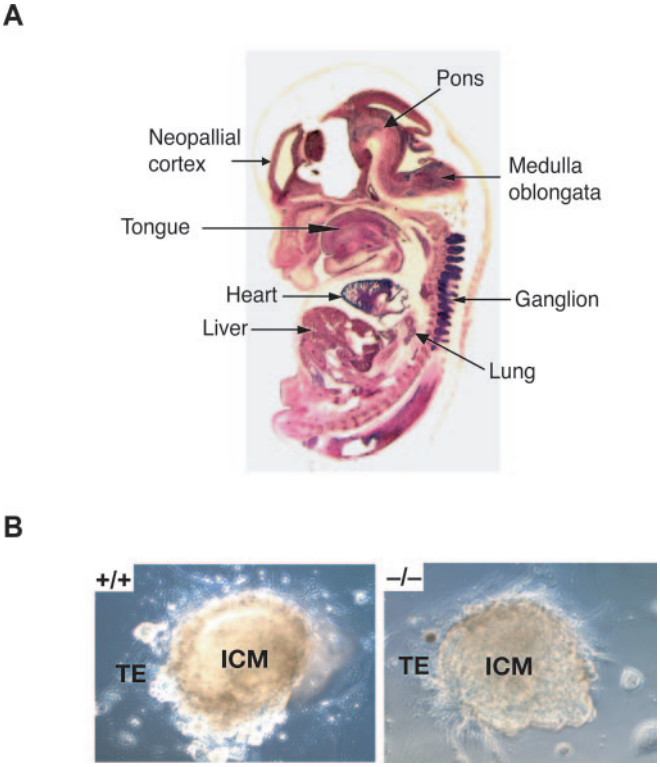

FIG. 5. ***Nmt1* and mouse development.** *A*, β-galactosidase staining of an *Nmt1*+/− embryo at E12.5. *B*, *Nmt1*+/+ and *Nmt1*−/− blastocysts that were placed in Petri dishes and allowed to grow. *ICM*, inner cell mass; *TE*, trophectoderm.

every pup that was examined ($n = 8$) (Fig. 9). In parallel control experiments, high G418 selection was used to produce euploid ES cells that were homozygous for a mutant *Lmna* allele. *Lmna* is not essential for embryonic development (21). Unlike the *Nmt1*−/− ES cells, the homozygous *Lmna* ES cells consistently yielded 80–90% chimeric mice.

DISCUSSION

The fact that *N*-myristoylation of proteins is important in eukaryotes is beyond dispute. Yeast, fungi, flies, worms, trypanosomes, and *Leishmania* cannot survive without the enzyme that carries out this protein modification (2–5, 8). However, in mammals as well as several other vertebrates, two distinct *N*-myristoyltransferases exist (12). Although the two human enzymes have similar peptide specificities and kinetic properties (12), it has remained mysterious whether the enzymes are entirely redundant or whether one enzyme might play a unique, vital role *in vivo*. In the current study, we provide new insights into that issue, demonstrating that *Nmt1* is essential for mouse development. Homozygous *Nmt1*-deficient mice died between 3.5 and 7.5 days of embryonic development. This result was unexpected, not just because of the similar properties of the two enzymes (12), but also because the tissue-specific expression patterns of the enzymes are similar, as judged by Northern blots and *in situ* hybridization studies of mouse embryos (Figs. 1 and 2). Interestingly, only about one-half of the heterozygous *Nmt1*-deficient mice survived. The demise of significant numbers of heterozygous knock-out mice has been observed previously with mutations in other essential genes, for example *Apob* (22) and *Cul-4A* (13).

The *Nmt1* knock-out mice were generated with an ES cell line containing an insertional mutation in intron 3 of *Nmt1*. A theoretical concern with gene trap mutations is that the splicing

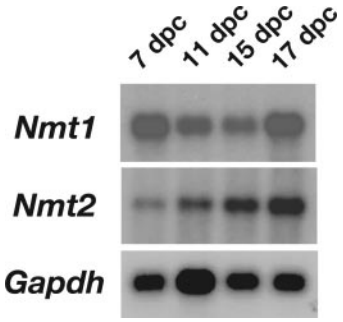

FIG. 6. ***Nmt1* and *Nmt2* expression during embryonic development.** A mouse poly(A)$^+$ RNA blot showing *Nmt1* and *Nmt2* expression at different points during embryogenesis. The blot was hybridized with *Nmt1, Nmt2*, and *GAPDH* probes.

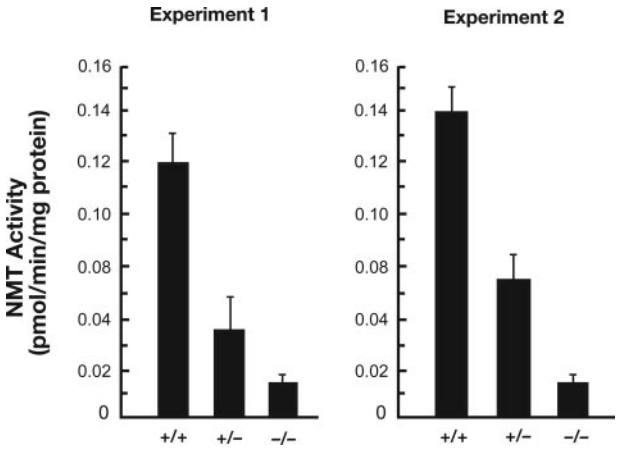

FIG. 7. **Total NMT activity levels in *Nmt1*+/+, *Nmt1*+/−, and *Nmt1*−/− ES cells.** Assays of total NMT activity in cellular extracts were performed with an activated phosphoprotein 60-M (pp60src) peptide substrate (GSSKSKPKR). Two experiments, performed with independently prepared cellular extracts, are shown.

machinery might "splice around" the entire insertion, leading to the production of low levels of a wild-type transcript. In the case of the mutant *Nmt1* ES cell line, this did not occur, because we were unable to find any full-length *Nmt1* transcripts in *Nmt1*−/− ES cells by Northern blot, even on long exposures. The insertion of the gene trap vector led to the production of a fusion transcript (consisting of a small segment of *Nmt1* and βgeo), which could be detected by Northern blot. The production of the *Nmt1*-βgeo fusion protein was fortunate because it made it possible for us to assess *Nmt1* expression in tissue sections with X-gal stains. The fusion protein would not be predicted to retain any NMT enzymatic activity, inasmuch as it would lack important functional domains of NMT, for example regions that bind the protein substrate and myristoyl-CoA (23, 24).

Why would *Nmt1* be essential for development, given that a similar gene (*Nmt2*) exists within the genome? It seems unlikely that *Nmt1* is simply required for cell viability, as it is in yeast, given that we had no difficulty in isolating *Nmt1*−/− ES cells. Those ES cells that expressed *Nmt2* retained the capacity to differentiate into enterocytes in chimeric mice. A more attractive explanation is that *Nmt1* is simply "the main Nmt enzyme" early in embryonic development. By Northern blot, *Nmt2* expression was lower at E7 than at later time points, whereas *Nmt1* expression was strong at E7. In addition, *Nmt2* was expressed at far lower levels than *Nmt1* in wild-type blastocysts (E3.5), as judged by real-time PCR. Also, total enzymatic activity levels were quite low in the *Nmt1*−/− ES cells, suggesting that *Nmt2* contributes minimally to enzyme activity levels in those cells. The *Nmt1*−/− ES cells created within this study were functionally defective, lacking the capacity to contribute to the formation of organs in which *Nmt1* is normally expressed at high levels (*e.g.* brain, liver, and lung).

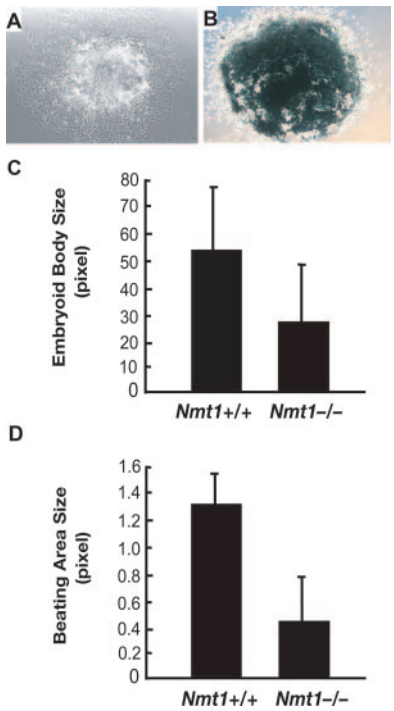

FIG. 8. **Differentiation of *Nmt1*−/− ES cells *in vitro*.** *A*, embryoid body from *Nmt1*−/− ES cells. *B*, β-galactosidase staining of an *Nmt1*−/− embryoid body. Strong β-galactosidase staining was observed throughout the embryoid body, including in the beating heart cells. *C*, size of embryoid bodies from *Nmt1*−/− ES cells and the parental *Nmt1*+/+ ES cells. $p < 0.0001$ (*t* test). *D*, size of beating heart cells in *Nmt1*+/+ and *Nmt1*−/− embryoid bodies. $p < 0.0001$.

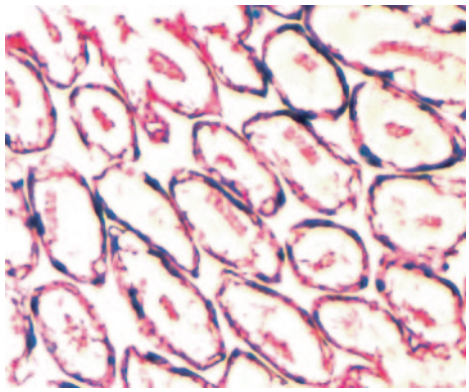

FIG. 9. ***Nmt1*−/− ES cells contribute to the formation of intestinal epithelial cells in chimeric mice.** *Nmt1*−/− ES cells were injected into blastocysts; pups were sacrificed at 1 day of age, sectioned, and stained for β-galactosidase. In each mouse ($n = 8$), strong β-galactosidase staining was observed in enterocytes. Some staining was also observed in the tubular cells of the kidney.

The ES cell clone used to create the *Nmt1* knock-out mice was produced by BayGenomics, one of the gene-trapping programs that form the International Gene Trap Consortium (www.igtc.org.uk/). Thus far, the International Gene Trap Consortium lists ~27,000 mutant ES cell lines covering ~32% of the ~30,000 mouse genes (25). Within BayGenomics, we have documented that the likelihood of trapping a particular mouse gene is directly correlated to the size of the gene and its level of expression in ES cells.[2] Thus, large genes and genes expressed

[2] B. Conklin, A. Nord, W. Skarnes, and S. G. Young, unpublished observations.

at high levels in ES cells are more likely to be trapped than small genes and genes that are expressed at low levels in ES cells. The preference for larger genes makes perfect sense, inasmuch as they provide a greater opportunity for the random gene-trapping insertional event. Trapping of highly expressed genes also makes sense because the generation of a drug-resistant ES cell colony depends on the expression of βgeo, which is driven by the promoter of the trapped gene. Of note, *Nmt2* is ~5 kb longer than *Nmt1*, so by that criterion, one would expect *Nmt2* to be trapped by the International Gene Trap Consortium more often than *Nmt1*. However, as we showed, NMT activity levels are quite low in *Nmt1*−/− cells, suggesting that *Nmt1* might normally be expressed more highly in ES cells than *Nmt2*. If so, one would predict that *Nmt1* would have been trapped by the IGTC much more frequently than *Nmt2*. Indeed, this has been the case: *Nmt1* has been trapped 12 times, whereas *Nmt2* has never been trapped.

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