Methylation of tRNA^{Asp} by the DNA Methyltransferase Homolog Dnmt2

Mary Grace Goll,[1] Finn Kirpekar,[2] Keith A. Maggert,[3] Jeffrey A. Yoder,[4] Chih-Lin Hsieh,[5] Xiaoyu Zhang,[6] Kent G. Golic,[7] Steven E. Jacobsen,[6] Timothy H. Bestor[1]*

The sequence and the structure of DNA methyltransferase-2 (Dnmt2) bear close affinities to authentic DNA cytosine methyltransferases. A combined genetic and biochemical approach revealed that human DNMT2 did not methylate DNA but instead methylated a small RNA; mass spectrometry showed that this RNA is aspartic acid transfer RNA (tRNA^{Asp}) and that DNMT2 specifically methylated cytosine 38 in the anticodon loop. The function of DNMT2 is highly conserved, and human DNMT2 protein restored methylation in vitro to tRNA^{Asp} from Dnmt2-deficient strains of mouse, *Arabidopsis thaliana*, and *Drosophila melanogaster* in a manner that was dependent on preexisting patterns of modified nucleosides. Indirect sequence recognition is also a feature of eukaryotic DNA methyltransferases, which may have arisen from a Dnmt2-like RNA methyltransferase.

The DNA (cytosine 5) methyltransferases of the Dnmt1 and Dnmt3 families establish and maintain patterns of methylation at cytosine residues in flowering plants, deuterostomes, and a subset of protostomes (*1*). Proteins of the Dnmt2 family show all the sequence and structural characteristics of DNA methyltransferases (*2*–*5*) except for a putative nucleic acid binding cleft that cannot easily accommodate duplex DNA (*1*). Despite the sequence and structural affinities between Dnmt2 and authentic DNA methyltransferases, genomic methylation patterns are not measurably altered in Dnmt2-deficient mouse embryonic stem (ES) cells (*6*).

Localization experiments indicate that Dnmt2 does not have the properties expected of a DNA methyltransferase. Human DNMT2 protein (hDNMT2) is primarily localized to the cytoplasm of transfected mouse 3T3 fibroblasts (Fig. 1). The cytoplasmic localization of DNMT2 contrasts with the exclusively nuclear localization of Dnmt1 and Dnmt3 (*7*, *8*).

The biological function of Dnmt2 was evaluated in strains of mouse, *Arabidopsis thaliana*, and *Drosophila melanogaster* that lack Dnmt2 (Fig. 2). The mouse deletion allele excised amino acids 181 to 359; this region includes the highly conserved Cys-Phe-Thr-XX-Tyr-XX-Tyr (where X is any amino acid) motif unique to Dnmt2 homologs (*1*, *4*) and DNA cytosine methyltransferase motifs VIII and IX (Fig. 2A and fig. S1). The *MT2*/*Dnmt2* (hereafter *dDnmt2*) gene of *D. melanogaster* was disrupted by insertion of 28 base pairs of sequence that contained three in-frame stop codons and a +1 frameshift 5′ of the region that encodes motif IV (Fig. 2B), which is required for enzymatic activity (*9*). Wild-type *Dnmt2* sequence was not present in flies homozygous for this mutation (fig. S2). A strain of *A. thaliana* that contains a large *Agrobacterium* transferred DNA (T-DNA) insertion adjacent to exon 7 (Fig. 2C) was obtained from the Salk T-DNA collection (*10*). The introduction of deletion or truncating mutations in all three organisms causes loss of catalytic motifs that

[1]Department of Genetics and Development, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA. [2]Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark. [3]Department of Biology, Texas A&M University, College Station, TX 77843, USA. [4]Department of Molecular Biomedical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27606, USA. [5]Department of Urology and Department of Biochemistry and Molecular Biology, University of Southern California, Los Angeles, CA 90089, USA. [6]Howard Hughes Medical Institute (HHMI) and Department of Molecular, Cell, and Developmental Biology, University of California, Los Angeles, CA 90095, USA. [7]Department of Biology, University of Utah, Salt Lake City, UT 84112, USA.

*To whom correspondence should be addressed. E-mail: THB12@Columbia.edu

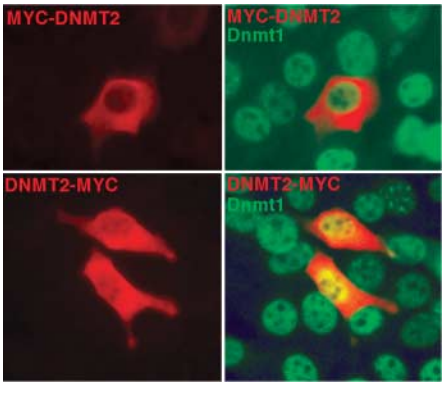


Fig. 1. Cytoplasmic localization of hDNMT2 in NIH3T3 cells. Cells were transiently transfected with hDNMT2 expression constructs that added an N- or C-terminal Myc epitope tag. Immunofluorescence shows localization of hDNMT2 (red) primarily in the cytoplasm, whereas Dnmt1 (green) is exclusively nuclear.

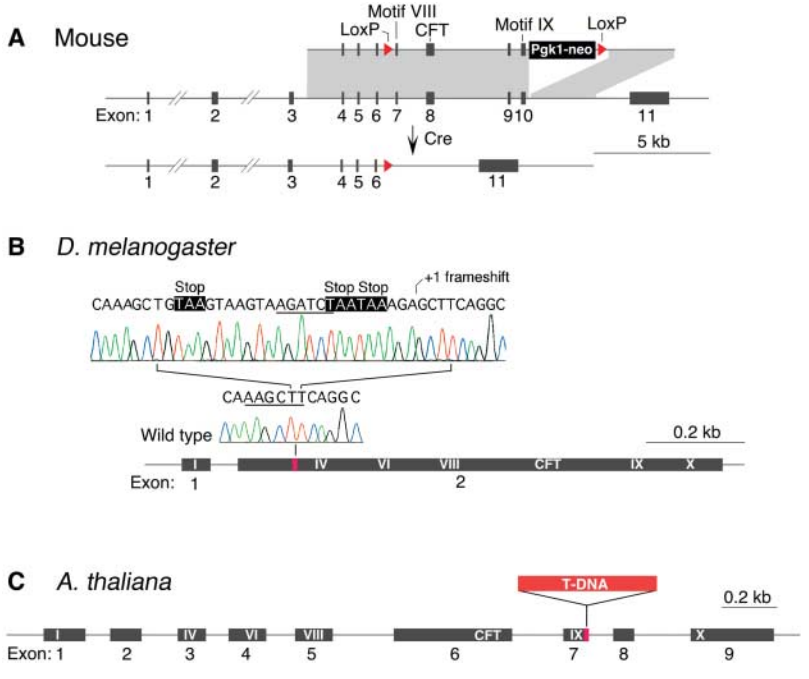
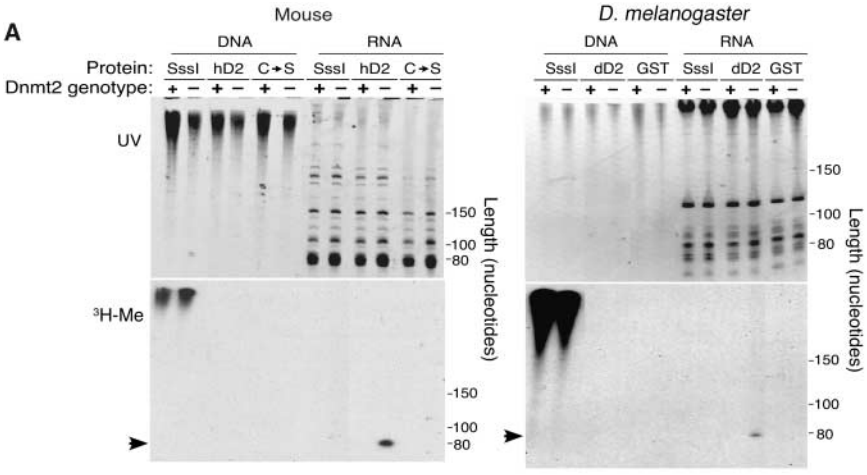


Fig. 2. Mutant alleles of *Dnmt2* homologs in mouse, *D. melanogaster*, and *A. thaliana*. (**A**) loxP sites were introduced into the mouse *Dnmt2* gene by homologous recombination in ES cells, and the indicated deletion was induced by exposure to Cre recombinase (fig. S1). Roman numerals indicate DNA cytosine methyltransferase catalytic motifs. The conserved Cys-Phe-Thr-XX-Tyr-XX-Tyr motif diagnostic of Dnmt2 proteins is indicated by CFT. (**B**) Homologous recombination was used to introduce three in-frame stop codons and a +1 frameshift at the HindIII site in exon 2 of *D. melanogaster dDnmt2*. No wild-type *Mt2/dDnmt2* sequence was present in the homozygous fly stock (fig. S2). (**C**) A large T-DNA insertion 3′ of exon 7 of the *A. thaliana Dnmt2* gene allele truncates the mRNA (fig. S3).



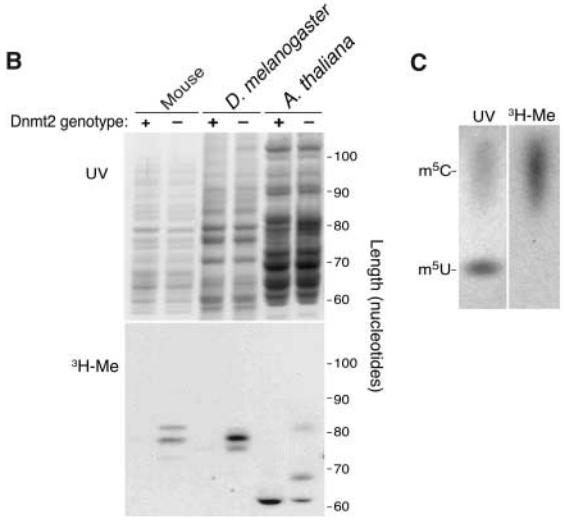


Fig. 3. DNMT2 methylates only a small RNA from DNMT2-deficent tissues. (**A**) Total DNA or RNA from wild-type or *Dnmt2*$^{-/-}$ mice was incubated with ^{3}H-AdoMet and either the bacterial DNA cytosine methyltransferase M.Sss1, His-tagged hDNMT2, or His-tagged hDNMT2 in which Cys^{79} in motif IV was substituted with serine. Nucleic acids were separated by electrophoresis, stained with ethidium bromide, and then prepared for fluorography. No evidence of methylation of DNA by hDNMT2 was seen, but a single small RNA was labeled (arrow) when that RNA originated from Dnmt2$^{-/-}$ mice. Total DNA or RNA from wild-type or *dDnmt2*$^{-/-}$ adult *D. melanogaster* was incubated with M.SssI, glutathione *S*-transferase (GST)-tagged *D. melanogaster* dDnmt2 purified from *E. coli*, or GST alone as on the left-hand gel. Only a small RNA was methylated (arrow). UV, ultraviolet. (**B**) High-resolution analysis of the Dnmt2 target RNA. hDNMT2 was incubated with ^{3}H-AdoMet and total RNA isolated from wild-type and *Dnmt2*$^{-/-}$ mice, *D. melanogaster*, and *A. thaliana*. An additional methylated band with a size of ~68 nucleotides present in RNA from *Dnmt2*$^{-/-}$ *A. thaliana* RNA is likely to represent an additional Dnmt2 substrate in flowering plants. The ~63-nucleotide band in the *A. thaliana* samples is a hDNMT2 substrate even when wild-type RNA is used, which suggests that this RNA species is not methylated in vivo. (**C**) Mouse *Dnmt2*$^{-/-}$ RNA was incubated with hDNMT2 and ^{3}H-AdoMet and hydrolyzed to nucleosides, which were resolved by thin-layer chromatography. The methylated product comigrated with 5-methyl cytosine (m^{5}C).

are required for enzymatic activity (Fig. 2 and figs. S1 to S3).

Strains of mouse, *D. melanogaster*, and *A. thaliana* homozygous for inactivating mutations in *Dnmt2* were viable, fertile, and morphologically indistinguishable from wild-type counterparts. The homozygous *Dnmt2* mutation did not modify the phenotype of mice homozygous for mutations in *Dnmt1*, nor did the homozygous *Dnmt2* mutation modify the phenotypes of *A. thaliana* homozygous for mutations in *drm1* and *drm2* (domains rearranged methyltransferases 1 and 2) or *cmt3* (chromomethylase 3) (*11*). Genomic methylation patterns were not detectably altered in Dnmt2-deficient mouse tissues (fig. S4).

We developed a combined biochemical and genetic approach to address the function of Dnmt2. Purified hDNMT2 was tested for its ability to transfer tritium-labeled methyl groups from the cofactor [^{3}H-*methyl*] S-adenosyl-L-methionine (^{3}H-AdoMet) to genomic DNA and RNA purified from wild-type or *Dnmt2*$^{-/-}$ mice. No DNA methylation was observed (Fig. 3A), but hDNMT2 specifically methylated a small RNA molecule when RNA from *Dnmt2*$^{-/-}$ mouse tissues was the substrate; wild-type RNA from mouse was not labeled (Fig. 3A). This implied that the small RNA molecule was the in vivo target of Dnmt2 and that wild-type RNA is methylated in vivo and cannot serve as substrate for methylation in vitro. Mutation of a key catalytic residue (Cys^{79} in motif IV) (*9*) abolished RNA methyltransferase activity (Fig. 3A). Purified *D. melanogaster* Dnmt2 protein (MT2, hereafter dDnmt2) did not methylate DNA but methylated a small RNA molecule only when that RNA was isolated from *Dnmt2*$^{-/-}$ flies (Fig. 3A).

Dnmt2 was found to have the same function in mammals, flowering plants, and dipteran insects. Purified hDNMT2 methylated one or two RNA molecules of ~80 nucleotides in length when RNA was isolated from *Dnmt2*-deficient mouse, *D. melanogaster*, or *A. thaliana* (Fig. 3, A and B). In all three organisms, hDNMT2 specifically methylated these RNAs only when the RNA was derived from Dnmt2-deficient tissues.

Inspection of the organization of functional groups within the putative active site of hDNMT2 (*4*) indicated that either cytosine or uracil might be the methylation target (*12*). The identity of the modified base was determined by thin-layer chromatography of nucleosides from hydrolysates of RNA labeled with hDNMT2 and ^{3}H-AdoMet. The product of hDNMT2 comigrated with authentic 5-methylcytidine and was clearly resolved from the 5-methyluridine standard (Fig. 3C).

The identity of the RNA substrate was determined by comparison of mass spectra of ribonuclease (RNase) T1 oligonucleotides from the Dnmt2 substrate purified from *Dnmt2*$^{-/-}$ and *Dnmt2*$^{+/+}$ mouse tissues. Matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry identified a single oligonucleotide that showed a difference of 14 atomic mass units when the wild-type and *Dnmt2*$^{-/-}$ samples were compared, which indicated the loss of a single methyl group (Fig. 4A). Mass spectrometry analysis of this oligonucleotide revealed a hypermodified hexose-queuosine base in the T1 fragment that contained the Dnmt2 target cytosine. Hexose-queuosine has been found in only tRNA^{Tyr} and tRNA^{Asp}, which contain galactosylqueuosine and mannosylqueuosine, respectively; in both cases, the hexose-queuosine is located in the wobble position of the anticodon (*13*, *14*). Tandem mass spectrometry on the T1 fragment that contained the Dnmt2 target cytosine yielded the sequence of the T1 oligonucleotide of the tRNA^{Asp} anticodon loop. Methylation was observed at cytosine 38 of wild-type tRNA^{Asp}, the second nucleotide 3′ of the anticodon, but this position was unmethylated in RNA from Dnmt2-deficient tissues (tables S1 and S2 and fig. S5).

Confirmation that the Dnmt2 target is tRNA^{Asp} was provided by Northern blot analysis of RNA from *Dnmt2*$^{-/-}$ and *Dnmt2*$^{+/+}$ tissues with probes to tRNA^{Asp}. In *Dnmt2*$^{-/-}$ mice, the tRNA substrate showed increased mobility relative to that of the wild type; the mass spectrometry data indicated that this mobility shift was the result of a loss of one methyl group.

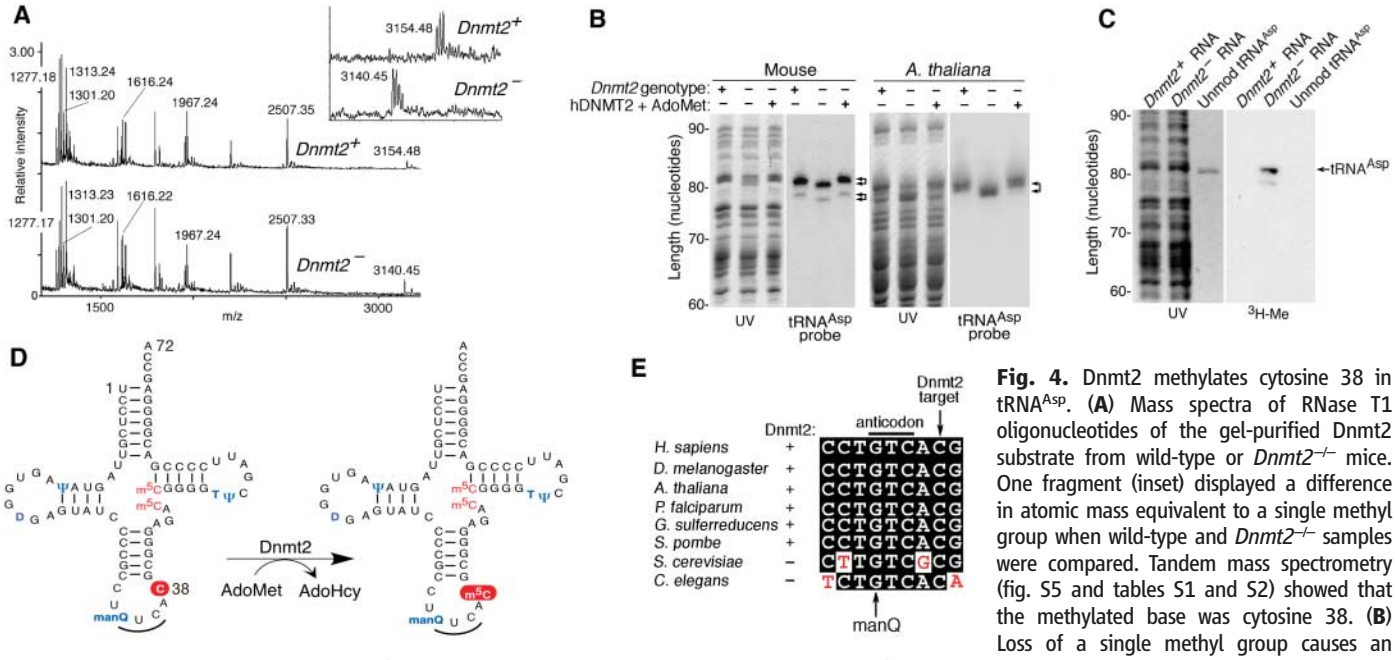


Fig. 4. Dnmt2 methylates cytosine 38 in $tRNA^{Asp}$. (**A**) Mass spectra of RNase T1 oligonucleotides of the gel-purified Dnmt2 substrate from wild-type or *Dnmt2*$^{-/-}$ mice. One fragment (inset) displayed a difference in atomic mass equivalent to a single methyl group when wild-type and *Dnmt2*$^{-/-}$ samples were compared. Tandem mass spectrometry (fig. S5 and tables S1 and S2) showed that the methylated base was cytosine 38. (**B**) Loss of a single methyl group causes an increase in electrophoretic mobility of $tRNA^{Asp}$. Electrophoresis and Northern blot analysis with $tRNA^{Asp}$ probes from mouse and *A. thaliana* confirmed that the target of Dnmt2 is $tRNA^{Asp}$. Identical results were obtained with probes complementary to the 3′ and 5′ ends of $tRNA^{Asp}$. Double arrows indicate the methylation-dependent mobility shift. Dnmt2 in *A. thaliana* has one additional target of ~68 nucleotides in addition to $tRNA^{Asp}$. (**C**) Methylation of $tRNA^{Asp}$ is dependent on preexisting modifications. Unmodified $tRNA^{Asp}$ produced by in vitro transcription was inactive as a hDNMT2 substrate. (**D**) Summary of pattern of modifications of $tRNA^{Asp}$. The mass spectrometry data show that Dnmt2 is required for methylation of position 38 in the anticodon loop of $tRNA^{Asp}$; formation of m^5C at positions 48 and 49 is independent of Dnmt2. Anticodon bases are underlined; mannosylqueuosine is indicated as manQ; other modified nucleosides are shown in blue; pseudouridine is indicated as Ψ; m^5C, in red. (**E**) Sequence alignment of the anticodon loops of $tRNA^{Asp}$. The sequence is invariant among organisms that contain Dnmt2, but base substitutions have occurred at positions adjacent to the target cytosine in $tRNA^{Asp}$ of *S. cerevisiae* and *C. elegans*, whose genomes do not contain a *Dnmt2*-related gene.

Incubation of *Dnmt2*$^{-/-}$ RNA with hDNMT2 and AdoMet restored the wild-type mobility (Fig. 4B). In mouse, the $tRNA^{Asp}$ probe hybridized to two bands of similar mobility, both of which showed a modification-dependent mobility shift (Fig. 4B). These bands are likely to represent populations of differentially modified $tRNA^{Asp}$; heterogeneity of tRNA modification patterns (*15*) and methylation-dependent shifts in electrophoretic mobility of tRNAs (*16*) have been observed. Northern blot analysis of RNA from *A. thaliana* with a $tRNA^{Asp}$ probe revealed a single band that had a Dnmt2-dependent mobility shift, which confirmed that $tRNA^{Asp}$ is the Dnmt2 target in mouse and *A. thaliana* (Fig. 4B). Cytosine 38 had been previously reported to be methylated in $tRNA^{Asp}$ from mouse and *Xenopus laevis* (*14*, *17*) (Fig. 4B). Unmodified $tRNA^{Asp}$ produced by in vitro transcription was not a substrate for DNMT2 (Fig. 4C), which suggests that methylation is guided to cytosine 38 by other modifications; mannosylqueuosine is likely to be involved, because it is unique to $tRNA^{Asp}$.

Analysis of $tRNA^{Asp}$ sequences showed complete conservation of the anticodon loop in species whose genomes encode Dnmt2 homologs, but the $tRNA^{Asp}$ anticodon loops in *Caenorhabditis elegans* and *Saccharomyces cerevisiae*, which lack Dnmt2 homologs, have diverged (Fig. 4E). The bacterium *Geobacter sulferreducens* contains a Dnmt2 homolog, and the $tRNA^{Asp}$ anticodon loop of this organism is identical to that of Dnmt2-containing eukaryotes. These findings indicate coevolution of Dnmt2 and the anticodon loop of $tRNA^{Asp}$. The strong conservation of Dnmt2 across divergent taxa indicate that it is under positive selection and suggests that Dnmt2 increases fitness under unidentified sources of stress or has an incremental effect on fitness that is not apparent under laboratory conditions.

The data shown here indicate that Dnmt2 methylates an RNA, even though the sequence and the order of catalytic motifs of Dnmt2 are characteristic of DNA rather than RNA methyltransferases (*18*, *19*). Methylation of $tRNA^{Asp}$ by Dnmt2 requires information beyond the RNA sequence, and structural information is involved in target selection by other RNA methyltransferases (*20*, *21*). Indirect sequence recognition is also a feature of eukaryotic DNA cytosine methyltransferases (*1*), whereas bacterial restriction methyltransferases have innate sequence specificity that determines the structure of genomic methylation patterns in these organisms [reviewed in (*22*)]. This raises the possibility that eukaryotic DNA cytosine methyltransferases were derived from an ancestral Dnmt2-like RNA methyltransferase rather than prokaryotic restriction DNA methyltransferases, which could explain the profound differences in mechanisms of target selection by DNA cytosine methyltransferases of prokaryotes and eukaryotes (*1*).

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Materials and Methods
Figs. S1 to S5
Tables S1 and S2

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