Supporting Online Material for

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

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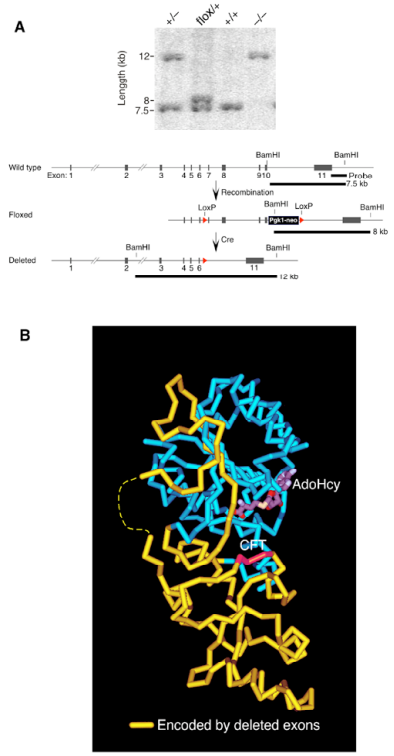
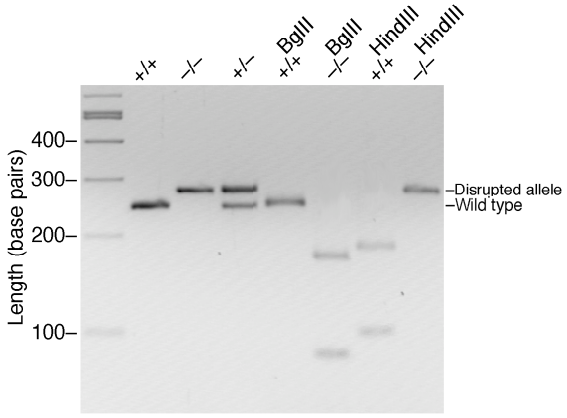
This PDF file includes:

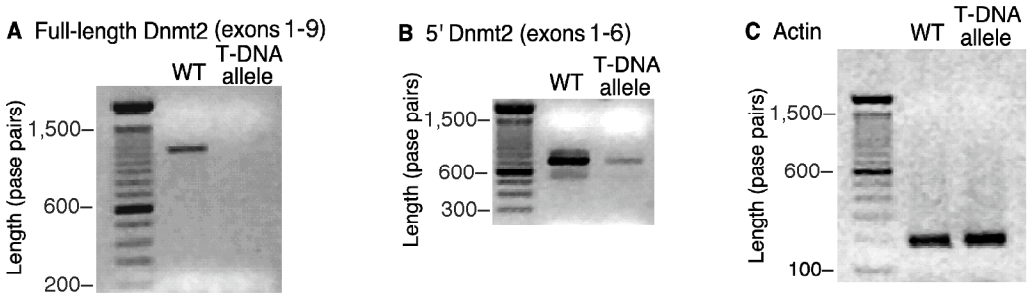
Supporting Online Material



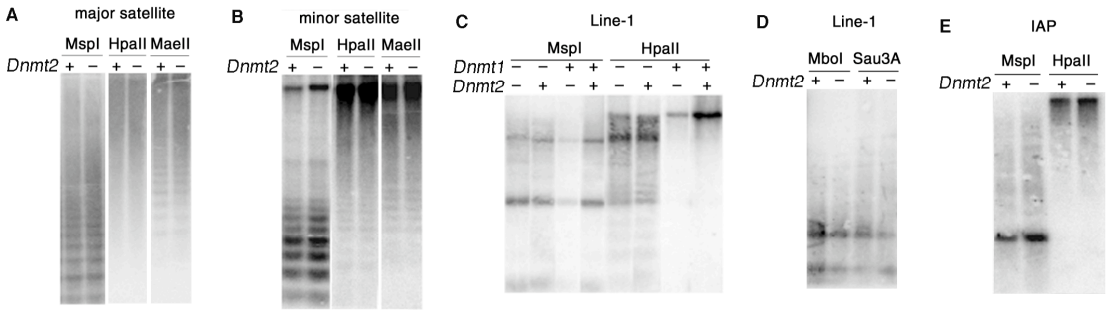
Supplementary Fig. S1. Confirmation of the genotype of *Dnmt2*$^{-/-}$ mice. (**A**) Southern blot showing excision of the targeted floxed *Dnmt2* gene in BamHI-digested genomic DNA. An additional BamHI site in the neomycin cassette results in an 8Kb fragment after introduction of loxP sites (flox). After exposure to Cre recombinase excision of the floxed allele results in loss of BamHI sites and a 12kb fragment. (**B**) Crystal structure of hDNMT2 in which the segment of the protein deleted by loxP recombination is highlighted in yellow; the CFTXXYXXY motif, which is also deleted, is shown in red.



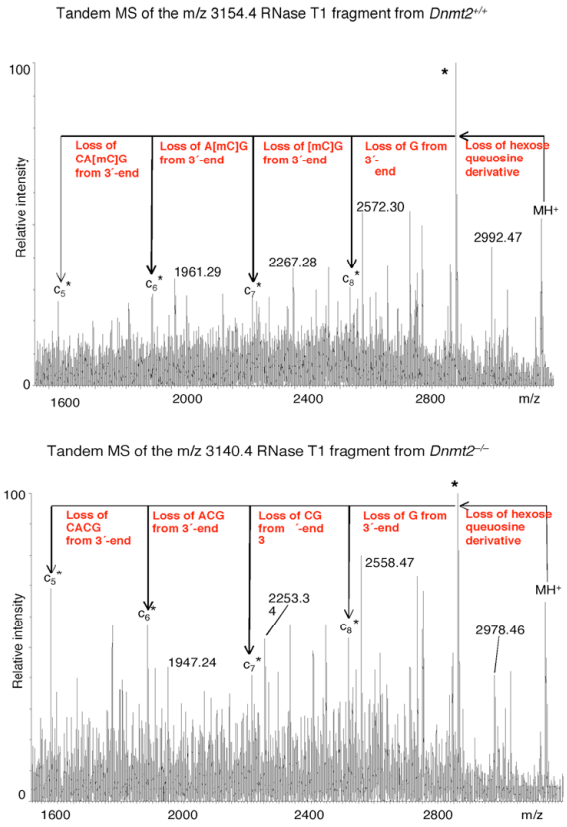
Supplementary Fig. S2. Confirmation of genotype of *dDnmt2*$^{-/-}$ *D. melanogaster.* (**A**) PCR analysis shows lack of wildtype *dDnmt2* sequence in mutant DNA. The wildtype allele contains a HindIII site, which was deleted after homologous recombination; the mutant allele contains a BglII site not present in the wildtype. The size and fragment patterns confirm the sequence shown in Fig. 2B and show that no wildtype *dDnmt2* sequence is present in the mutant flies used in this study.



Supplementary Fig. S3. Confirmation of Dnmt2 disruption in *A. thaliana* by RT-PCR (**A**) RT-PCR using primers in exons 1 and 9 of *A. thaliana* Dnmt2 indicate that full length Dnmt2 mRNA is not present. (**B**) RT-PCR using primers in exon 1 and 6 indicate that there is a severe decrease in Dnmt2 mRNA 5' of the T-DNA insertion. This truncated mRNA lacks coding sequence for DNA methyltransferase motif X. (**C**) RT-PCR control with primers complementary to the actin mRNA.



Supplementary Fig S4. Representative Southern blots showing that DNA methylation patterns are unaffected in *Dnmt2*$^{-/-}$ mice. (**A-B**) Methylation sensitive Southern blots were performed on DNA from purified from wildtype and *Dnmt2*$^{-/-}$ mice after digestion with the methylation sensitive restriction enzymes HpaII (CCGG) or MaeII (ACGT) and the methylation insensitive HpaII isoschizomer MspI. Blots were probed for major satellite sequences (**A**) or minor satellite sequences (**B**). (**C**) Methylation sensitive Southern blot on DNA purified from *Dnmt2*$^{-/-}$ mice and *Dnmt2*$^{-/-}$ *Dnmt1*$^{N/N}$ mice digested with HpaII or MspI hybridized to a Line-1 probe (**D**) Methylation sensitive Southern blot of DNA from wildtype and *Dnmt2*$^{-/-}$ mice digested with the methylation sensitive enzyme Sau3A (GATC) and its methylation insensitive isoschizomer MboI hybridized to a Line-1 probe. (**E**) Methylation sensitive Southern blot with a probe to the Interstitial-A particle (IAP) transposon long terminal repeat (LTR) after digestion with HpaII or MspI.



Supplementary Fig. S5. Position 38 of $tRNA^{Asp}$ is modified by DNMT2. (A) Tandem MS of the m/z 3154.4 RNase T1 fragment from $tRNA^{Asp}$ purified from wildtype mouse tissues. Peaks corresponding to loss of 3' nucleotides are indicated as peaks C_5-C_8. Peaks corresponding to loss of 5' nucleotides are indicated by their m/z. Interpretation of all peaks is included in Table S2. (B) Tandem MS of the m/z 3140.4 RNase T1 fragment from $tRNA^{Asp}$ purified from *Dnmt2*$^{-/-}$ mouse tissues. The m/z of C_8 shows a difference corresponding to one methyl group between wildtype and *Dnmt2*$^{-/-}$ samples with loss of the 3' most G. The m/z of C_7 indicates loss of m^5C from the *Dnmt2*$^{+/+}$ sample and loss of C from the *Dnmt2*$^{-/-}$ sample .

From-To	M/Z calculated	Sequence	M/Z observed	Sequence by tandem MS
1-6	1953.20	PO_4-UCCUCG-PO_4	1967.24	PO_4-UCCUCG^m-PO_4
7-10	1287.16	UUAG-PO_4	1301.20	UUA^{m}G-PO_4
11-15	1616.21	UAUAG-PO_4	1616.22	UAUAG-PO_4
16-17	652.08	UG-PO_4	n.o.	-
18-18	346.05	G-PO_4	n.o.	-
19-20	652.08	UG-PO_4	n.o.	-
21-22	675.11	AG-PO_4	n.o.	-
23-30	2507.32	UAUCCCCG-PO_4	2507.33	UAUCCCCG-PO_4 (1)
31-34	**1262.16**	**CCUG-PO_4**	**3140.45**	**CCUXXCACG-PO_4+ hexose-queousine**
35-39	**1591.22**	**UCACG-PO_4**		

40-41	651.10	CG-PO_4	n.o.	-
42-42	346.05	G-PO_4	n.o.	-
43-43	346.05	G-PO_4	n.o.	-
44-45	675.11	AG-PO_4	n.o.	-
46-49	1285.19	ACCG-PO_4	1313.23	AC^mC^mG-PO_4
50-50	346.05	G-PO_4	n.o.	-
51-51	346.05	G-PO_4	n.o.	-
52-52	346.05	G-PO_4	n.o.	-
53-56	1263.15	UUCG-PO_4	1277.17	U^mUCG-PO_4
57-64	2507.32	AUUCCCCG-PO_4	2507.33	AUUCCCCG-PO_4 (1)
65-67	980.15	ACG-PO_4	980.15	Not performed
68-68	346.05	G-PO_4	n.o.	-
69-69	346.05	G-PO_4	n.o.	-
70-70	346.05	G-PO_4	n.o.	-
71-72	595.14	AG-OH	n.o.	-

Supplementary Table S1. T1 fragments identified from the purified Dnmt2 substrate RNA correspond to $tRNA^{Asp}$. The anticodon loop containing the Dnmt2 target cytosine is indicated in bold. RNAse T1 cleaves RNA 3' of G residues. T1 does not cleave at mannosylqueousine and as a result, predicted fragments 31-34 and 35-39 appear as a single oligonucleotide by mass spectrometry. n.o. indicates that the mass spectra of the T1 fragment was not obtained due to its small size. (1) These two fragments are isobaric. The tandem MS data suggested two species, one starting with a U and one with an A, but an otherwise identical fragmentation patterns. There was no evidence of a Dnmt2-dependent change of mass in these fragments. The sequence of mouse $tRNA^{Asp}$ appears in Fig 4D.

Tandem MS of the m/z 3154.4 RNase T1 fragment from Dnmt2$^{+/+}$ tRNA

M/Z	Interpretation	Comment
3154.45	MH^+	Singly protonated parent ion for the tandem MS experiment.
2992.47	Hexose loss from MH^+	Loss of 162 is well-known diagnostic signal for hexose loss in tandem MS of carbohydrates.
2877.41	Loss of queuosine-derivative from MH^+	Loss of 277 corresponds to the dominating fragmentation site for queuosine derivatives [1]. Marked * in the spectrum.
2572.30	y_8*	Loss of C nucleotide from the 5´-end of the *-marked species.
2532.32	c_8*	Loss of G nucleotide from the 3´-end of the *-marked species.
2267.28	y_7*	Loss of CC di-nucleotide from the 5´-end of the *-marked species.
2213.24	c_7*	Loss of [C+methyl]G di-nucleotide from the 3´-end of the *-marked species. Reveals the Dnmt2 methylation site.
1961.29	y_6*	Loss of CCU tri-nucleotide from the 5´-end of the *-marked species.
1884.20	c_6*	Loss of A[C+methyl]G tri-nucleotide from the 3´-end of the *-marked species.
1579.15	c_5*	Loss of CA[C+methyl]G tri-nucleotide from the 3´-end of the *-marked species.

Tandem MS of the m/z 3140.4 RNase T1 fragment from Dnmt2$^{-/-}$ tRNA

M/Z	Interpretation	Comment
3140.43	MH^+	Singly protonated parent ion for the tandem MS experiment.
2978.46	Hexose loss from MH^+	Loss of 162 is well-known diagnostic signal for hexose loss in tandem MS of carbohydrates.
2863.57	Loss of queuosine-derivative from MH^+	Loss of 277 corresponds to the dominating fragmentation site for queuosine derivatives [1]. Marked * in the spectrum.
2558.47	y_8*	Loss of C nucleotide from the 5´-end of the *-marked species.
2518.31	c_8*	Loss of G nucleotide from the 3´-end of the *-marked species.
2253.34	y_7*	Loss of CC di-nucleotide from the 5´-end of the *-marked species.
2213.35	c_7*	Loss of CG di-nucleotide from the 3´-end of the *-marked species. Loss is [C+methyl]G in Dnmt2+/+ sample

1947.24	y_6*	Loss of CCU tri-nucleotide from the 5´-end of the *-marked species.
1884.31	c_6*	Loss of ACG tri-nucleotide from the 3´-end of the *-marked species.
1579.20	c_5*	Loss of CACG tri-nucleotide from the 3´-end of the *-marked species.

Supplementary Table S2. Interpretation of spectra from Fig. S5.

Materials and Methods

Disruption of Dnmt2 Genes

The mouse genomic library 129SVter DNA in λFIXII (Stratagene) was screened with a Dnmt2 cDNA probe and a clone encoding exons 4 through 11 was identified. The synthetic oligo GGTACCATAACTTCGTATAATGTATGCTATACGAAGTTATGTTTAAACGGTACC containing a loxP site was introduced into the unique KpnI site upstream of exon 7. A second LoxP site and the neomycin cassette from plasmid pLTNL (gift of Thomas Ludwig) was introduced into the AflII site downstream of the exon 10. Initial screening of the mouse ES cells to confirm proper integration of the portion of the targeting cassette was performed by Southern blot using a 460 bp PCR probe amplified using primers GAACTCACACGGGCATTGTA and GGCTCATGATTAGTCTGCTCAA. Proper targeting of the 5' loxP site was confirmed in 3' positive clones by PCR using primers AGAAGCCTGTGGCTTTCAGT and CCCTACAATCGTTTATTTTCCAA. Properly targeted heterozygous ES cells were injected into blastocysts and chimeric mice identified. After germline transmission, heterozygous mice were crossed to mice expressing Cre recombinase under the *hsp70* promoter. Proper recombination between LoxP sites was assessed by Southern blotting and mice were crossed to homozygosity. PCR amplification and direct sequencing of the product confirmed excision of the antibiotic resistance cassette and *Dnmt2* exons 7-10.

D. melanogaster Dnmt2 mutant flies were created by introducing the oligonucleotide AGCTGTAAGTAAGTAAGATCTAATAAAG into the endogenous HindIII site 3' of motif IV according to (*2*). The resulting truncation results in a protein of 67 amino acids. Genotyping of flies was achieved by PCR across the boundary of the targeted HindIII site using primers GTTTATGCGCACAATTACGG and GAAACCCTTGACGTTTTCCA. PCR products were gel purified and sequenced in both directions. No evidence of any wild type *dDnmt2* sequence was found in mutant flies homozygous for the mutant allele.

The *A. thaliana* T-DNA insertion mutant documented polymorphism SALK_1366 was obtained from the *Arabidopsis* information resource (TAIR) under accession number 1005061271 (*3*). RT-PCR primers were ATGGCGGAACAAGAATTACAGA and TCAAGAATCGAATAGATACCGAA for full length Dnmt2 mRNA and ACACTACTTGGCTCGCCATCAA and ATGGCGGAACAAGAATTACAGA for exons 1-6.

Protein Expression and Purification

Human RGS6XHis tagged Dnmt2 was expressed and purified as in (*4*). *Drosophila* dDnmt2 cDNA was obtained from Open Biosystems (cat EDM1133-6873739) and was cloned into pGEX2T in frame with an N-terminal GST tag. *Drosophila* dDnmt2 protein was expressed in *E. coli* (McrBC deficient strain ER2488) and soluble recombinant protein was purified on GST agarose (Molecular Probes) according to manufacturers instructions.

Immunocytochemistry

NIH3T3 cells were transiently transfected with the use of lipofectamine (Invitrogen). Expression plasmids consisted of hDNMT2 fused to an N or C terminal MYC epitope tag. Expression required sequences to be cloned downstream of the γ-globin intron 2. At 24 h post transfection cells were fixed with formaldehyde and viewed by

immunoflourescence using the anti MYC antibody 9E10 (Biosource) and polyclonal rabbit anti-Dnmt1 antibody (anti-PATH52) (4, 5) as primary antibodies.

Methyltransferase assay

5 μg of RNA or DNA purified from mouse liver was incubated with 40 ng of purified protein, 1 uCi [^{3}H] S-Adenosyl-L-methionine (15 Ci mmol^{-1}; Amersham) and 5 units of RNaseIN (Promega). Samples were incubated 2 h at 37° in 20mM Tris-HCl (pH 7.4), 0.5 mM DTT, 2 mM EDTA, 50 mM KCL, 5% glycerol. After methylation reactions were complete, DNA was subjected to digestion with MboI to reduce fragment size. Samples were phenol extracted, ethanol precipitated, resuspended in formamide, and run on a 12% denaturing poly acrylamide gel. Fluorographic detection of tritium signal was achieved by soaking the gel in 2 M sodium salicylate in 45% methanol-10% acetic acid for 1 h. Gels were dried and exposed to Biomax film (Kodak) at –70° C. For *D. melanogaster*, experimental conditions were the same as above except that 2 μg of DNA or 20 μg of total RNA from freshly eclosed adult flies was used as substrate. M. SssI was purchased from New England Biolabs (NEB).

Thin layer chromatography

Total RNA from *Dnmt2*$^{-/-}$ mice was incubated with hDNMT2 and ^{3}H-AdoMet for 2 h. RNA was then passed over an RNA QuickSpin column (Roche). Nucleosides were generated by incubation first with P1 nuclease (100 μg/ ml) in 30 mM sodium acetate pH 5.3, 0.1 mM $ZnCl_2$ at 60° C for 1h, followed by 5 units calf alkaline phosphatase (NEB) for 1 h at 37° C in the provided buffer. Mononucleosides were separated by thin layer chromatography on cellulose plates developed in isobutyric acid-ammonium hydroxide-0.1 M EDTA (100: 60: 1.6) (*5*). Ribothymidine standard (Mann Research laboratories) was a kind gift of B. Erlanger (Columbia University). 5-methylcytidine was purchased from Sigma. RNA was visualized by UV shadowing and the position of the standards was documented, tritium signals on the same plate were then visualized by fluorography according to (*6*).

Mass spectrometry

Total RNA purified from wild type and *Dnmt2*$^{-/-}$ mouse tissues was run on a denaturing polyacrylamide gel for 22 h and visualized by ethidum bromide staining. The single band that shows a change in mobility between wild type and *Dnmt2*$^{-/-}$ RNA was excised and eluted in 2 M ammonium acetate. The digestion mixture contained approximately 1 pmol/μl Dnmt2 substrate tRNA, 50 mM 3-hydroxypicolinic acid and 100 u/μl RNase T1 (USB). Digestion was performed for 4 hours at 37° C. These digestion conditions produced almost exclusively 2´-3´ cyclic phosphate digestion products. Samples for mass spectrometry were prepared by mixing 1 μl of tRNA digestion mixture with 0.7 μl of 0.5 M 3-hydroxypicolinic acid in 50% acetonitrile and approximately 0.1 μl of ammonium-loaded cation exchange beads, after which the sample was left to air-dry at room temperature. MALDI mass spectrometry was performed on a Perseptive Voyager STR MALDI instrument detecting positive ions in reflector Time of Flight mode. Spectrum processing was done with the producer-supplied software using internal calibration. Tandem mass spectrometry was done on a Micromass MALDI Q-TOF Ultima instrument in positive ion mode using the same sample preparation as above. Details may be found in (*7*).

Northern Blots

Total RNA was run on a 12% denaturing polyacrylamide gel at 800V for 22h and stained with ethidium bromide. RNA was transferred to a nylon membrane by semidry electrophoretic transfer. Membranes were hybridized and washed using end labeled probes as in (*8*), except washes were carried out at 55° C. Probe sequences were as follows: mouse $tRNA^{Asp}$ probes, CTCCCCGTCGGGGAATTGAA and GATACTACCACTATACTAACGAGGA; *A. thaliana* $tRNA^{Asp}$ probe: ATACTTACCACTATACTACAACGAC, *D. melanogaster*: GATACTAACCACTATACTATCGAGGA and CTCCCCGACGGGGAATTGAA.

In vitro transcription of mouse $tRNA^{Asp}$

The $tRNA^{Asp}$ DNA template was created using the primers TGGCGCCCCGTCGGGGAATTGAACCCCGGTCTCCCGC and AAGCTTAATACGACTCACTATAGCCTCGTTAGTATAGTGGT and a template oligonucleotide corresponding to positions 1 to 52 of mouse $tRNA^{Asp}$. PCR replaces T at position 1 of $tRNA^{Asp}$ with a G to allow for T7 transcription and position 71 A with a C to maintain the hairpin structure of tRNA as in (*9*). Reactions were treated with DNAse and run on a denaturing polyacrylamide gel with size standards. The largest T7 transcript, corresponding to full-length $tRNA^{Asp}$ was gel purified, eluted and tested for its ability to accept a methyl group from ^{3}H-AdoMet as described previously.

Southern Blot Probes

An oligo probe corresponding to the sequence AACAGTGTATATCAATGAGTTACAATGAG was used to probe minor satellite sequences. For the major satellite, a 250 bp insert was excised from pMR196 (*10*). The L1 5-UTR probe was amplified according to (*11*). The IAP probe was as described in (*12*).

References

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