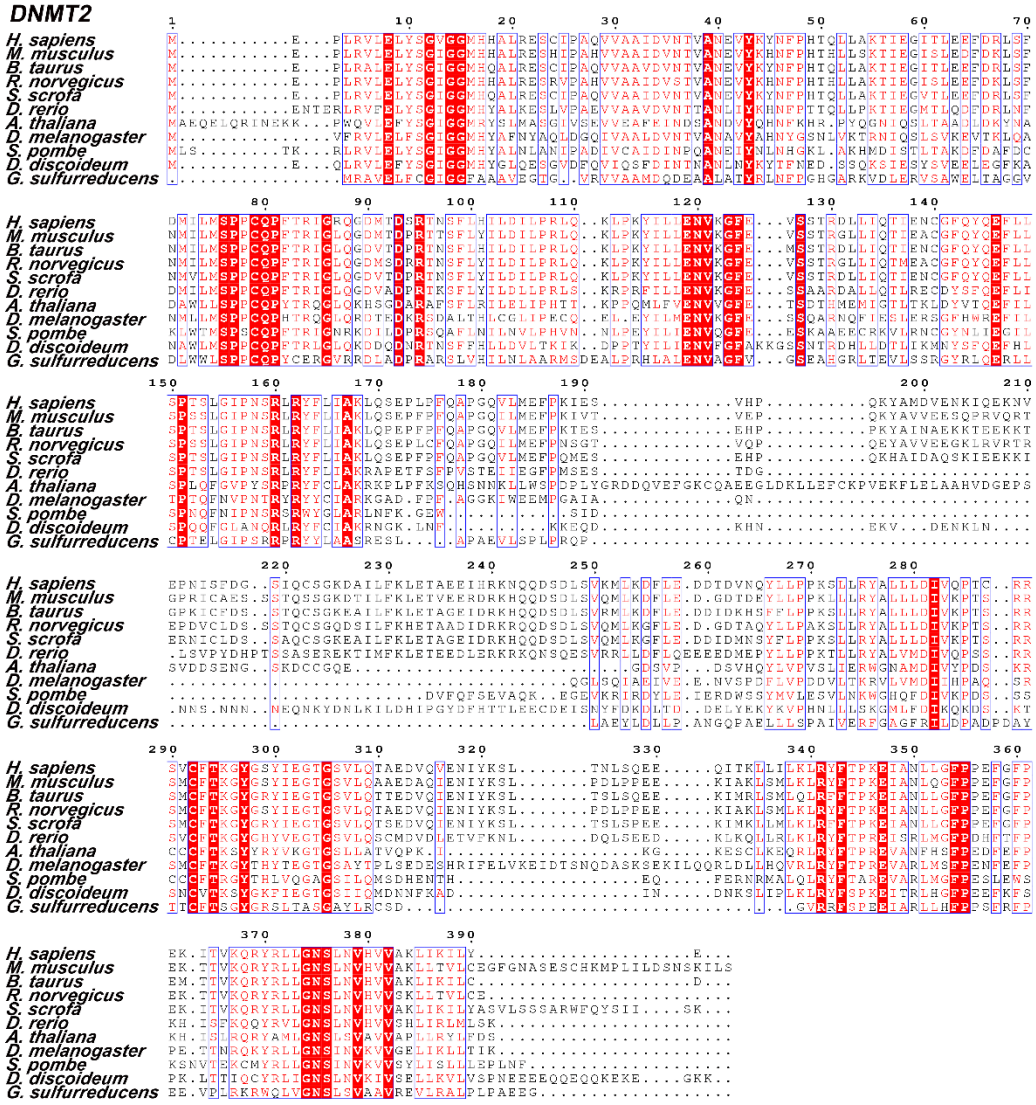
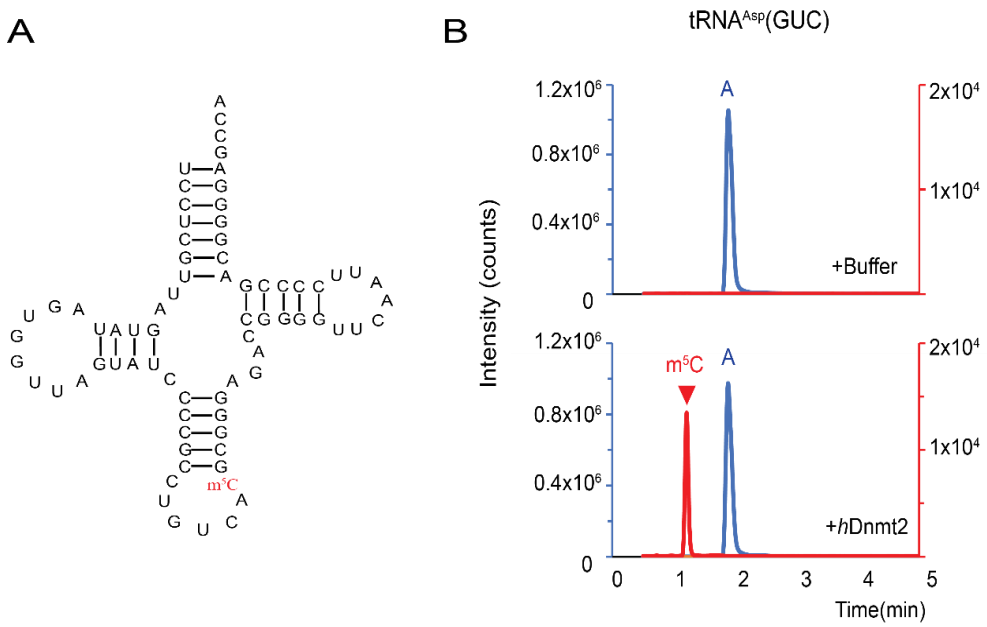
Supplementary Figures and Tables

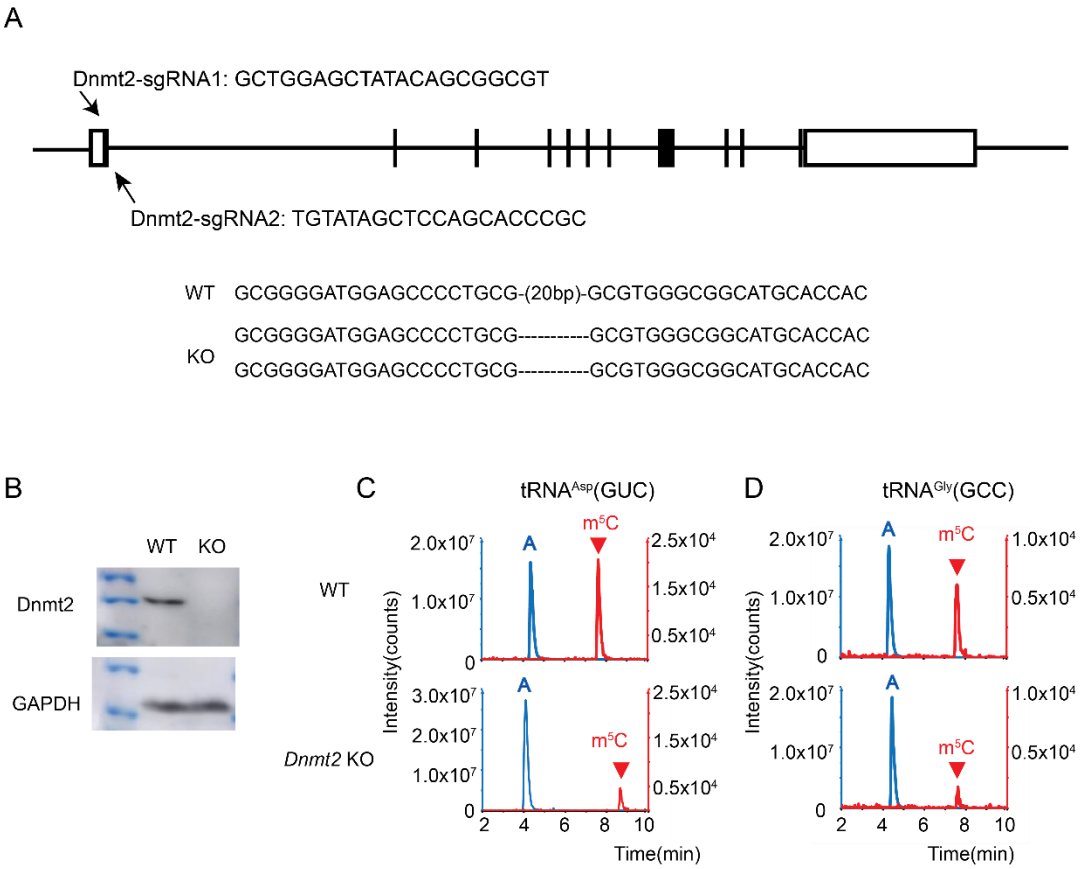


Supplementary Figure 1. Sequence alignment of Dnmt2s from different species. *H. sapiens*, *Homo sapiens*; *M. musculus*, *Mus musculus*; *B. taurus, Bos taurus*; *R. norvegicus*, *Rattus norvegicus*; *S. scrofa, Sus scrofa*; *D. rerio*, *Danio rerio*; *A. thaliana*, *Arabidopsis thaliana*; *D. melanogaster, Drosophila melanogaster*; *S. pombe*, *Schizosaccharomyces pombe*; *D. discoideum*, *Dictyostelium discoideum*; *G. sulfurreducens*, *Geobacter sulfurreducens*.



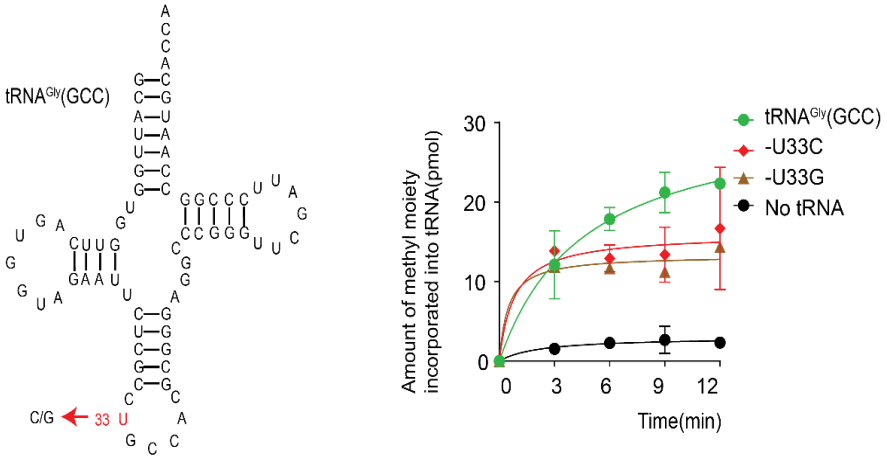
Supplementary Figure 2. Identification of the m^{5}C modification for tRNA^{Asp}(GUC) by hDnmt2 *in vitro*.

(A) The secondary structure of tRNA^{Asp}(GUC). (B) UPLC-MS/MS analysis of m^{5}C (Q1/Q3 =258.1/126.1) of tRNA^{Asp}(GUC) after incubation with or without hDnmt2.

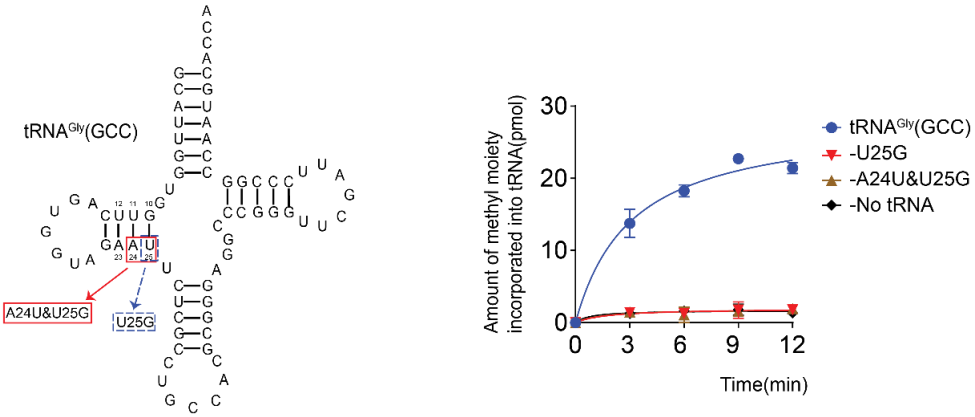


Supplementary Figure 3. Knockout of *Dnmt2* leads to a significant decrease in the abundance of m^{5}C on tRNA^{Gly}(GCC) and tRNA^{Asp}(GUC).

(A) Schematic diagram of *Dnmt2* and target sites of mutations introduced by the CRISPR-Cas9 system in HEK293T cells. Shaded and open boxes indicate coding regions and untranslated regions of exons, respectively. Lines indicate introns. For hDnmt2, two sgRNA sequences for targeting the first exon are noted. Sequences of both alleles of *Dnmt2* in the KO cell line are aligned. Deleted nucleotides are indicated as dashed lines. (B) The western blotting to show the knockout efficiency of *Dnmt2*. UPLC-MS/MS analysis of m^{5}C (Q1/Q3 =258.1/126.1) of tRNA^{Asp}(GUC) (C) and tRNA^{Gly}(GCC) (D), which were isolated from wild-type and *Dnmt2* knockout 293T cell lines.



Supplementary Figure 4. U33 is not recognized by hDnmt2.
The capacity of tRNA^{Gly}(GCC)-U33C and -U33G mutants to be methylated by hDnmt2. Error bars represent the standard errors of three independent experiments.



Supplementary Figure 5. D-stem is involved in the substrate recognition of hDnmt2.

The capacity of tRNA^{Gly}(GCC)-U25G and -A24U&U25G to be methylated by hDnmt2. Error bars represent the standard errors of three independent experiments

Supplementary Tables

Supplementary Table 1. The PCR primers used for each genes in the plasmid construction.

Genes	plasmid	Primer forward	Primer reverse
Human	pET28a	CGCGGATCCATGGAGCCCCTGCG	CCGCTCGAGTTCATATAAGATTT
Dnmt2		GGTGCTGGAGCTATACA	TGATTAGTTTAGCTACTAC
M. musculus	pET28a	AGCAAATGGGTCGCGGATCCATG	TGGTGGTGGTGGTGCTCGAGAG
Dnmt2		GAACCTCTGCGTGTCCT	ATAGAATCTTGGAATTAC
ADAT2	pRSFduet	ACAGCCAGGATCCGAATTCGGAG	CATTATGCGGCCGCAAGCTTTCA
		GCGAAGGCGGCACCCAA	AGATTTCTGACATTCCT
ADAT3	pRSFduet	AAGAAGGAGATATACATATGATCC	GTTTCTTTACCAGACTCGAGCTA
		TCTGCTCCCGTCTCTG	CGTGTCGGGGTCCAGCC

Supplementary Table 2. The sequences of thirteen C38-containing tRNAs.

tRNAs	Sequence
$tRNA^{Ala}$(AGC)	GGGGAAUUAGCUCAAGUGGUAGAGCGCUUGCUUAGC ACGCAAGAGGUAGUGGGAUCGAUGCCCACAUUCUCC ACCA
$tRNA^{Ala}$(UGC)	GGGGAUGUAGCUCAGUGGUAGAGCGCAUGCUUUGCA CGUAUGAGGCCCCGGGUUCAAUCCCCGGCAUCUCCA CCA
$tRNA^{Asp}$(GUC)	UCCUCGUUAGUAUAGUGGUGAGUAUCCCCGCCUGUC ACGCGGGAGACCGGGGUUCGAUUCCCCGACGGGGAG CCA
$tRNA^{Glu}$(CUC)	UCCCUGGUGGUCUAGUGGUUAGGAUUCGGCGCUCUC ACCGCCGCGGCCCGGGUUCGAUUCCCGGUCAGGGAA CCA
$tRNA^{Glu}$(UUC)	UCCCAUAUGGUCUAGCGGUUAGGAUUCCUGGUUUUC ACCCAGGUGGCCCGGGUUCGACUCCCGGUAUGGGAA CCA
$tRNA^{Gly}$(GCC)	GCAUUGGUGGUUCAGUGGUAGAAUUCUCGCCUGCCA CGCGGGAGGCCCGGGUUCGAUUCCCGGCCAAUGCAC CA
$tRNA^{Gly}$(CCC)	GCAUUGGUGGUUCAGUGGUAGAAUUCUCGCCUCCCA CGCGGGAGACCCGGGUUCAAUUCCCGGCCAAUGCAC CA
$tRNA^{His}$(GUG)	GCCGUGAUCGUAUAGUGGUUAGUACUCUGCGUUGUG GCCGCAGCAACCUCGGUUCGAAUCCGAGUCACGGCA CCA
$tRNA^{Leu}$(AAG)	GGUAGCGUGGCCGAGCGGUCUAAGGCGCUGGAUUAA GGCUCCAGUCUCUUCGGGGGCGUGGGUUCGAAUCCC ACCGCUGCCA
$tRNA^{Leu}$(UAG)	GGUAGCGUGGCCGAGCGGUCUAAGGCGCUGGAUUUA GGCUCCAGUCUCUUCGGAGGCGUGGGUUCGAAUCCC

	ACCGCUGCCACCA
$tRNA^{Val}$(AAC)	GUUUCCGUAGUGUAGUGGUUAUCACGUUCGCCUAAC ACGCGAAAGGUCCCCGGUUCGAAACCGGGCGGAAAC ACCA
$tRNA^{Val}$(CAC)	GUUUCCGUAGUGUAGUGGUUAUCACGUUCGCCUCAC ACGCGAAAGGUCCCCGGUUCGAAACCGGGCGGAAAC ACCA
$tRNA^{Val}$(UAC)	GGUUCCAUAGUGUAGUGGUUAUCACGUCUGCUUUAC ACGCAGAAGGUCCUGGGUUCGAGCCCCAGUGGAACC ACCA

The 38^{th} nucleotides of these tRNAs are marked as red.

Supplementary Table 3. The biotinylated DNA probes for fishing tRNAs.

tRNAs	probes
$tRNA^{Asp}$(GUC)	5'biotin-TGGCTCCCCGTCGGGGAATCGAACCCCGGT
$tRNA^{Gly}$(GCC)	5'biotin-CGAGAATTCTACCACTGAACCACCAATGC