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The RNA methyltransferase Dnmt2 methylates DNA in the structural context of a tRNA

Steffen Kaiser[a], Tomasz P. Jurkowski[b], Stefanie Kellner[a], Dirk Schneider[a], Albert Jeltsch[b], and Mark Helm [a]

[a]Institute of Pharmacy and Biochemistry, Johannes Gutenberg University Mainz, Mainz, Germany; [b]Institute of Biochemistry, Faculty of Chemistry, University Stuttgart, Stuttgart, Germany

ABSTRACT

The amino acid sequence of Dnmt2 is very similar to the catalytic domains of bacterial and eukaryotic DNA-(cytosine 5)-methyltransferases, but it efficiently catalyzes tRNA methylation, while its DNA methyltransferase activity is the subject of controversial reports with rates varying between zero and very weak. By using composite nucleic acid molecules as substrates, we surprisingly found that DNA fragments, when presented as covalent DNA-RNA hybrids in the structural context of a tRNA, can be more efficiently methylated than the corresponding natural tRNA substrate. Furthermore, by stepwise development of $tRNA^{Asp}$, we showed that this natural Dnmt2 substrate could be engineered to employ RNAs that act like guide RNAs *in vitro*. The 5′-half of $tRNA^{Asp}$ was able to efficiently guide methylation toward a single stranded tRNA fragment as would result from tRNA cleavage by tRNA specific nucleases. In a more artificial setting, a composite system of guide RNAs could ultimately be engineered to enable the enzyme to perform cytidine methylation on single stranded DNA *in vitro*.

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5-methylcytosine; DNA; Dnmt2; enzyme kinetics; modification pathway crosstalk; RNA methylation; RNA modification; tRNA

Introduction

A central question that may join the fields of DNA and RNA modification concerns the action of such rare enzymes that act on nucleobases in both types of nucleic acid.[1,2] Ever since the discovery of its robust tRNA methylation,[3] Dnmt2 has been a paradigm in this respect, because its nearest neighbors in evolution all are DNA-cytosine C5-methyltransferases (MTases) and methylate DNA.[4] While the deoxyribo-5-methylcytidine (5mC or dm^5C) residues formed by these related Dnmt enzymes are classical epigenetic marks, the role of Dnmt2 and its product, ribo-5-methylcytidine (rm^5C) at position 38 of different tRNAs, is ill understood at present, with different studies showing effects of this modification on tRNA fragmentation and stability,[5,6] tRNA charging[7] and fidelity in translation.[8] The search for a Dnmt2 substrate had been exclusively focused on DNA until the discovery of tRNA methylation activity of Dnmt2 in the seminal paper by Goll.[3] With the discovery of $tRNA^{Asp}$ as very active substrate the search for substrates took an unexpected turn, and reports on DNA methylation were now subject to highly controversial discussion.[3,9-13] Meanwhile, a recurrent set of tRNAs other than $tRNA^{Asp}$ have been identified as Dnmt2 substrates in various organisms. These include $tRNA^{Val}$ and $tRNA^{Gly}$ in mouse,[6] $tRNA^{Glu}$ in *Schizosaccharomyces pombe*,[14] $tRNA^{Gly}$ and $tRNA^{Glu}$ in *Dictyostelium discoideum*,[15] $tRNA^{Val}$, and $tRNA^{Gly}$ in *Drosophila melanogaster* [5,16] and $tRNA^{Gly}$ in *Arabidopsis thaliana*.[17] In *Geobacter sulfurreducens*, which so far represents the only prokaryote known to express a Dnmt2 protein, substrate specificity includes $tRNA^{Glu}$ but not $tRNA^{Asp}$, and the discrimination hinges upon the presence of certain sequence elements in the variable loop.[18]

Since the revelation of Dnmt2 as an RNA methyltransferase, the performance of methods for selective and sensitive detection of nucleic acid methylation has significantly improved.[19] One particularly important development was that bisulfite mapping of dm^5C in DNA, an established method that has seen dozens of improvements over the decades, was finally adapted to rm^5C detection in RNA.[16] This approach [17,20-24] and a related transcriptome-wide approach based on azacytidine [25] have produced numerous candidates for rm^5C in RNA, although most of these candidates were not attributed to Dnmt2 activity, but rather dependent on Trm4/NSUN2, a related *bona fide* RNA methyltransferases.[6] Although these approaches allegedly identified several RNAs as Dnmt2 substrates, to date there is no published biochemical data showing any *in vitro* activity of Dnmt2 on substrates other than tRNA.[4] This leaves open the possibility that additional factors influence or even determine substrate specificity of Dnmt2 *in vivo* or that search paradigms have not been correctly aimed.

Inspired by the ongoing discussion about whether Dnmt2 is capable of methylating DNA, or any other RNA in addition to tRNA, we have set out to determine if the biochemical potential for DNA methylation *in vitro* is present in the enzyme. We argued that composite nucleic acid molecules with varying contents of RNA versus DNA should allow gauging a preference of an enzyme for one or the other type of nucleic acid. We surprisingly found that Dnmt2 can efficiently methylate short DNA

CONTACT Mark Helm mhelm@uni-mainz.de Institute of Pharmacy and Biochemistry, Johannes Gutenberg University Mainz, Staudingerweg 5, 55128 Mainz, Germany.
Supplemental data for this article can be accessed on the publisher's website.

stretches when these are presented in the form of covalent chimeric molecules in the structural context of a tRNA. This activity is interpreted as a residual property originating from the evolutionary proximity of Dnmt2 to DNA methyltransferases.[4,26] Furthermore, starting from these structures, artificial guide RNAs can be developed, which allow targeting of Dnmt2 activity to the methylation of short single stranded RNA or DNA oligomers *in vitro*.

Results and discussion

Dnmt2, but not Trm4, methylates single deoxycytidines at the defined target site

Since linear double-strand DNA is a poor *in vitro* Dnmt2 substrate at best,[9] whereas $tRNA^{Asp}$ is efficiently methylated, we have constructed a series of $tRNA^{Asp}$ hybrids, in which RNA nucleotides were substituted with their corresponding deoxy-surrogates, starting with the single target nucleotide C38, which is methylated to rm^5C in native $tRNA^{Asp}$.[3] Several hybrid constructs of increasing DNA content were assembled from synthetic fragments by splint ligation [27] and their properties as methylation substrates of recombinant human Dnmt2 [28] were assessed in a tritium incorporation assay with ^{3}H-SAM.[29] As expected, the natural all-ribo $tRNA^{Asp}$ displayed robust methylation (Fig. 1).

However, we surprisingly found that a chimeric tRNA with a single deoxyribose substitution at the target site C38 ($tRNA^{Asp}$dC38) was an even better substrate for hDnmt2 (Fig. 1a, b, Fig. S1). As a negative control, a dm^5C38 hybrid containing synthetic dm^5C at position 38 did not show any methyl group acceptor properties in the tritium incorporation assay (Fig. 1b, blue trace). To confirm the structure of the methylated nucleotide, both methylated substrates were re-isolated from the reaction mixture, hydrolyzed to nucleosides, and analyzed by LC-MS/MS. Using fragmentation patterns from synthetic rm^5C and dm^5C (Fig. 2a), we established a multiple reaction monitoring (MRM) fragmentation method with a limit of detection in the single digit femtomol range.[30] While no methylated deoxycytidine was present in the substrates before the reaction, analysis of the material after the reaction revealed the presence of a new peak in the hybrid $tRNA^{Asp}$dC38 that corresponds to dm^5C in both retention time and fragmentation pattern (Fig. 2b). Both RNAs contained trace amounts of rm^5C, that was already present in the synthetic oligonucleotides used for ligation of the full length substrates,[30] but a strong increase of rm^5C in the all-ribo substrate was detected upon incubation with Dnmt2. These results clearly show methylation of dC38, and prove that Dnmt2 can accept and modify deoxynucleotides in its active site. To analyze, whether this is a particular feature of Dnmt2, or a generic features of m^5C: MTases, we performed analogous experiments with yeast Trm4, an enzyme known to methylate cytidines in the variable loop of yeast tRNAs.[31] While recombinant Trm4 showed robust tritium incorporation into an all-ribo tRNA (Fig. S2), substitution of the known target sites rC48 and rC49 with dC led to complete ablation of tritium incorporation, suggesting strongly that Trm4, in contrast to Dnmt2, is not capable of DNA methylation.

Dnmt2 methylates deoxycytidines faster than ribocytidines at its target site

An in-depth analysis of the methylation reaction showed that it followed Michaelis Menten kinetics (Fig. S3), yielding similar apparent K_m values for all-ribo $tRNA^{Asp}$ and hybrid $tRNA^{Asp}$dC38 (4.2 μM vs. 4.0 μM), while the k_{cat} value of the hybrid $tRNA^{Asp}$dC38 was increased by a factor of 2.3. This indicates similar binding properties combined with an accelerated turnover as a consequence of the lack of the 2'-hydroxylgroup at position 38. In the absence of a crystal structure of Dnmt2 [29] complexed to its tRNA substrate, it is unclear if the 2'-hydroxylgroup is interacting directly with the enzyme,[32] as an influence on base-flipping is possible as well.[33]

Yet more surprising observations were made in comparing hybrid tRNA with further increasing DNA content:

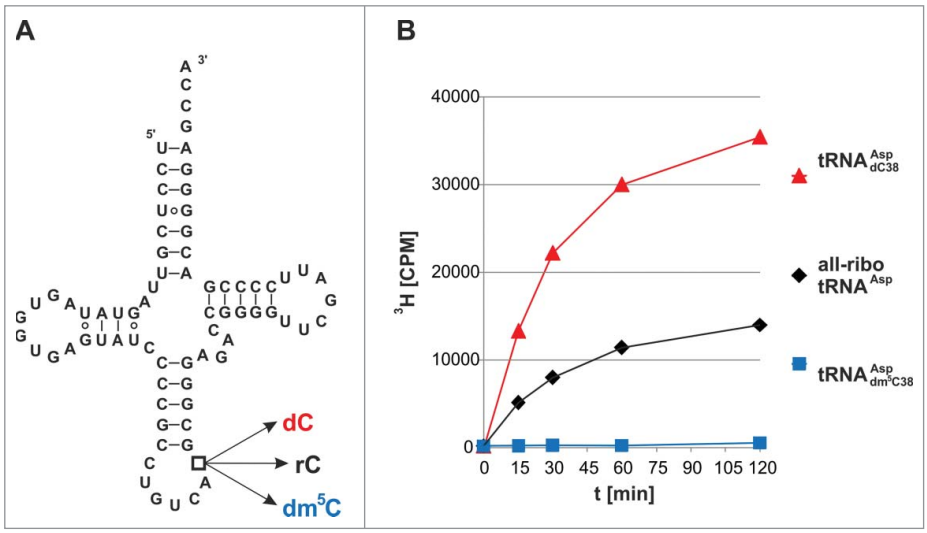

Figure 1. Tritium incorporation assay suggests that Dnmt2 methylates a tRNA containing a deoxycytidine at position 38. (A) Cloverleaf structure of unmodified human cytosolic tRNAAsp. Position 38 has been engineered to contain rC, dC, or dm5C in three hybrid tRNAs of corresponding name. (B) *In vitro* methylation measured by tritium incorporation from 3H-SAM. Standard deviations of three replicates are given in Fig. S1.

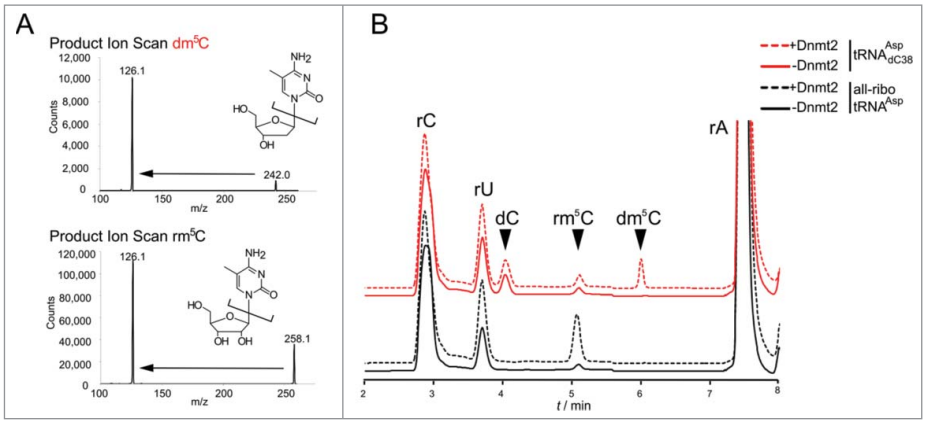

Figure 2. LC-MS analysis confirms that Dnmt2 methylates a tRNA containing a deoxycytidine at position 38. (A) Fragmentation patterns and mass transitions used for scanning of rm^5C and dm^5C in LC-MS. (B) LC-MS analysis of all-ribo $tRNA^{Asp}$ and hybrid $tRNA^{Asp}$dC38 before and after in vitro methylation by human Dnmt2.

introduction of a 3 nucleotide DNA stretch spanning from d37 to d39 did also improve methylation in comparison to all-ribo $tRNA^{Asp}$ (Upper curves in Fig. 3).

Dnmt2 methylates DNA presented as covalent hybrid with RNA in the structural context of a tRNA

In chimeras with longer deoxynucleotide stretches, substrate quality decreased with the DNA content, but a hybrid $tRNA^{Asp}$d36-40, containing a stretch of 5 deoxynucleotides between positions 36 and 40, was still a substrate comparable to the all-ribo $tRNA^{Asp}$. Chimeric $tRNA^{Asp}$d34-43 (10 deoxynucleotides) was the first to show clearly decreased, albeit still readily detectable activity (Fig. 3).

Exploring the limits of DNA substitution in $tRNA^{Asp}$

The above data clearly show methylation of DNA, provided it is presented in a structure that can be assumed to resemble the canonical tRNA substrate structure to a reasonable degree. The data also implies a drop in methylation efficiency with increasing DNA content. These findings provoke numerous questions as to where and how much DNA could be incorporated into the tRNA substrate, and if, ultimately, this approach would reveal an all-DNA substrate.

In the investigation of further DNA-tRNA chimeras of increasing DNA content, the substrate quality was assessed in an endpoint assay after 120 minutes, foregoing a time-consuming determination of Michaelis-Menten parameters. Results, as given in Table 1a, were normalized to the all-ribo tRNA as a reference. Based on this quantitative data, a qualitative ranking in descending order of substrate quality was visualized in Fig. 4 to facilitate the following discussion. When the DNA stretch starting at position 34, was further extended toward, and into, the 5'-side of the T-stem, methylation efficiency remained at a moderate level of ~25-40% of the all-ribo tRNA (Fig. 4e,f,g). To assess the general requirement for the 3D structure of a tRNA, an anticodon stem loop construct (ASL) of $tRNA^{Asp}$ was tested, but found to not be a substrate. Because this strongly suggested that typical elements of tRNA architecture were required for substrate recognition and methylation by Dnmt2, the possibility of extending the DNA stretch into the D- and T-stems was tested by chimeras featuring either an all-deoxy-T-domain or an all-deoxy-D-domain. However, the fact that replacement of either the

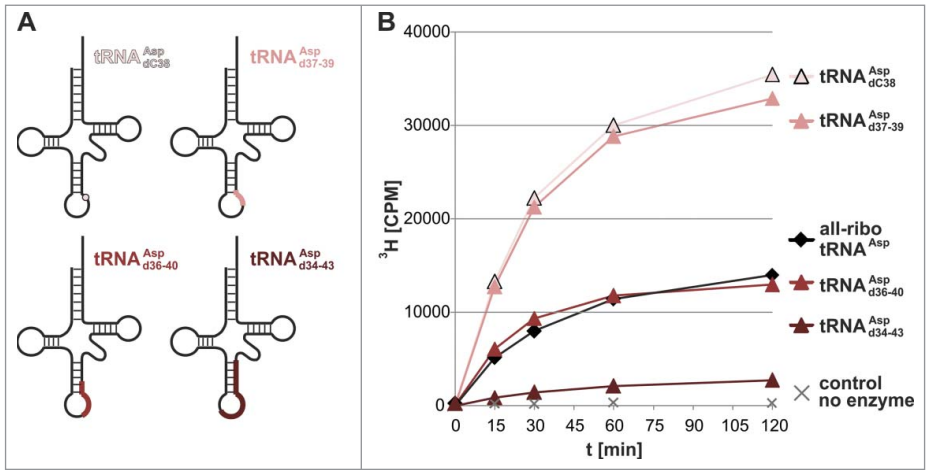

Figure 3. Dnmt2 methylates hybrid tRNAs containing up to 10 deoxynucleotides. (A) Positions of DNA substitutions in the tRNA cloverleaf in hybrid tRNAs. (B) Methylation of hybrid tRNAs of increasing DNA content. Average values and standard deviations are given in Fig. S1.

Table 1. tRNA-derivatives constructed by ligation and/or hybridization. Numbering in column 2 corresponds to that used in Figs. 4 and 5.

Name	tRNA derivative	Relative efficiency / %	N =	SD / %
(a)	See Fig. 4 for graphical representation			
all-ribo tRNA^{Asp}	4c, 5b	100		
tRNA^{Asp}dC38	4a	177	3	74
tRNA^{Asp}d37-39	4b	180	3	56
tRNA^{Asp}d36-40	4d	103	3	12
tRNA^{Asp}d34-51	4e	38	3	4
tRNA^{Asp}d34-46	4f	32	3	3
tRNA^{Asp}d34-43	4g	25	3	6
(b)	See Fig. 5 for graphical representation			
*nicked tRNA^{Asp} dC*dC38*	5a	145	3	20
*nicked tRNA^{Asp}dC*dC38-43*	5c	72	3	7
nicked tRNA^{Asp} d27-33	5d	71	3	3
nicked tRNA^{Asp}dC38	5e	43	3	5
nicked tRNA^{Asp}I	5f	43	3	3
nicked tRNA^{Asp}dX-43-3'HX	5g	22	3	3
nicked tRNA^{Asp}dX-53-5'HX	5h	13	3	1
nicked all-ribo tRNA^{Asp}	5i	11	3	0
nicked tRNA^{Asp}dX-53	5j	9	3	1
	See Fig. 6			
RgD	6A	2	3	0,2

entire D-loop or the entire T-loop led to undetectable levels of methylation, ablated the idea that an all-DNA substrate might be derived from the tRNA structure in its full length.

In vitro *development of an RNA-guided system for DNA methylation (RgD)*

Having delineated the limits of the DNA content in a covalent DNA-RNA chimera, we turned to the task, of developing a non-covalent RNA-DNA hybrid that might support DNA methyltransferase activity to Dnmt2. Using the previous chimera as a starting point, we tested the effects on methylation of several manipulations, designed to lead to guide-RNAs for Dnmt2 methylation of a ssDNA fragment. These manipulations are graphically summarized in the inset in Fig. 5, and comprise (i) the introduction of a nick in the anticodon at a previous junction of RNA and DNA domains, *e.g.* at position 34 of constructs 4e-4g (constructs 5e, 5i). Furthermore, (ii) the DNA content would be enlarged similarly to what was discussed above, including also an oligo-dC tail adjacent to the target C38 site to offer multiple potential methylation sites (5a, 5c, 5f-h, 5j). The generated bipartite system would then (iii) be stabilized by extending basepairs in the newly opened anticodon (construct 5g), and (iv) near the 3'-CCA end of the tRNA (construct 5h). The results in Table 1b and the activity scale in Fig. 5 show, that a nick between positions 33 and 34 as in substrates 5i and 5e, which results in an open anticodon loop, is still tolerated by the enzyme, although substrate activity of construct 5i is clearly reduced in comparison to the intact all-ribo tRNA (4c,5b). This result, although obtained rather in passing, is of biological significance. It shows that a hybrid of 2 tRNA-fragments (tRFs), which is essentially a nicked tRNA as would result from specific cleavage by a tRNase such as angiogenin,[5] was still methylated. Therefore, the 5'-tRF half could be viewed as a "guide RNA" that steers the enzyme to an RNA target, in this case the 3'-tRF. We took the biochemical proof of concept of a tRF as guide RNA for methylation as an important prerequisite to engineer an artificial system for ssDNA methylation. To this

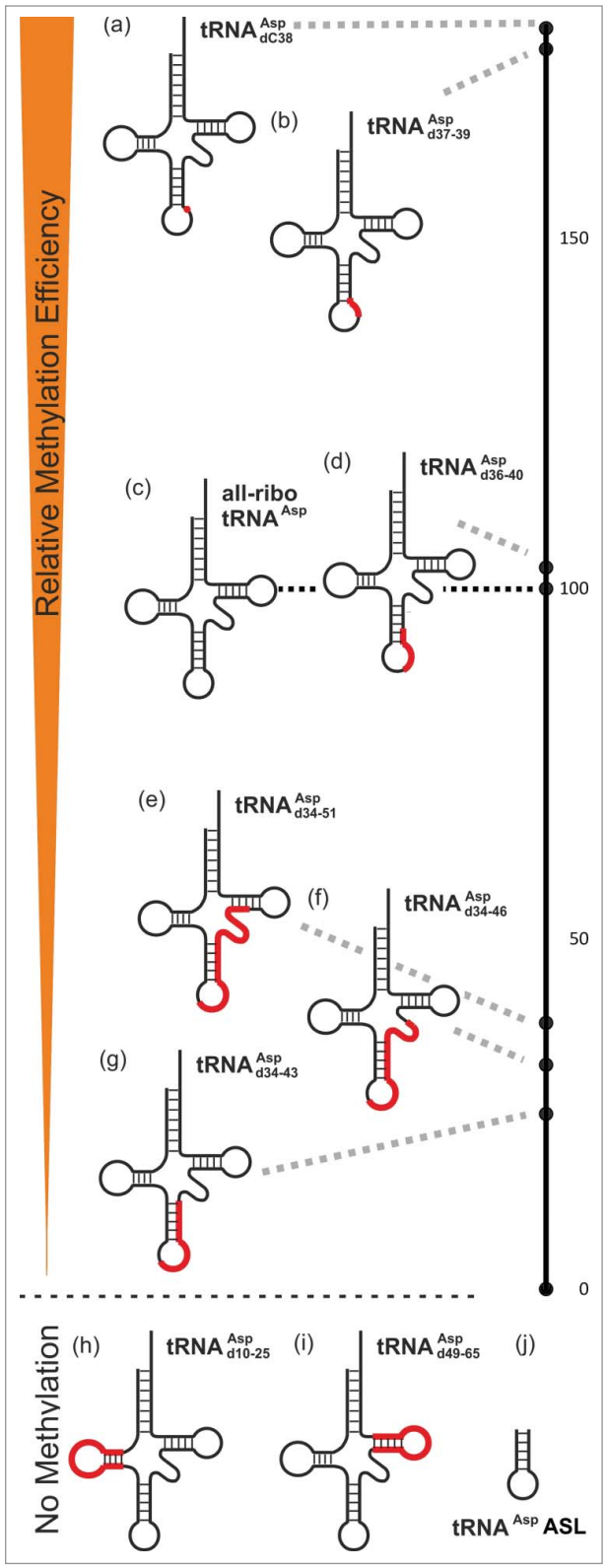

Figure 4. Relative methylation efficiency of covalent chimeric tRNAs^{Asp} compared to the all-ribo tRNA^{Asp} (entry 4c in Table 1), which is set to 100 on the black scale bar on the right.

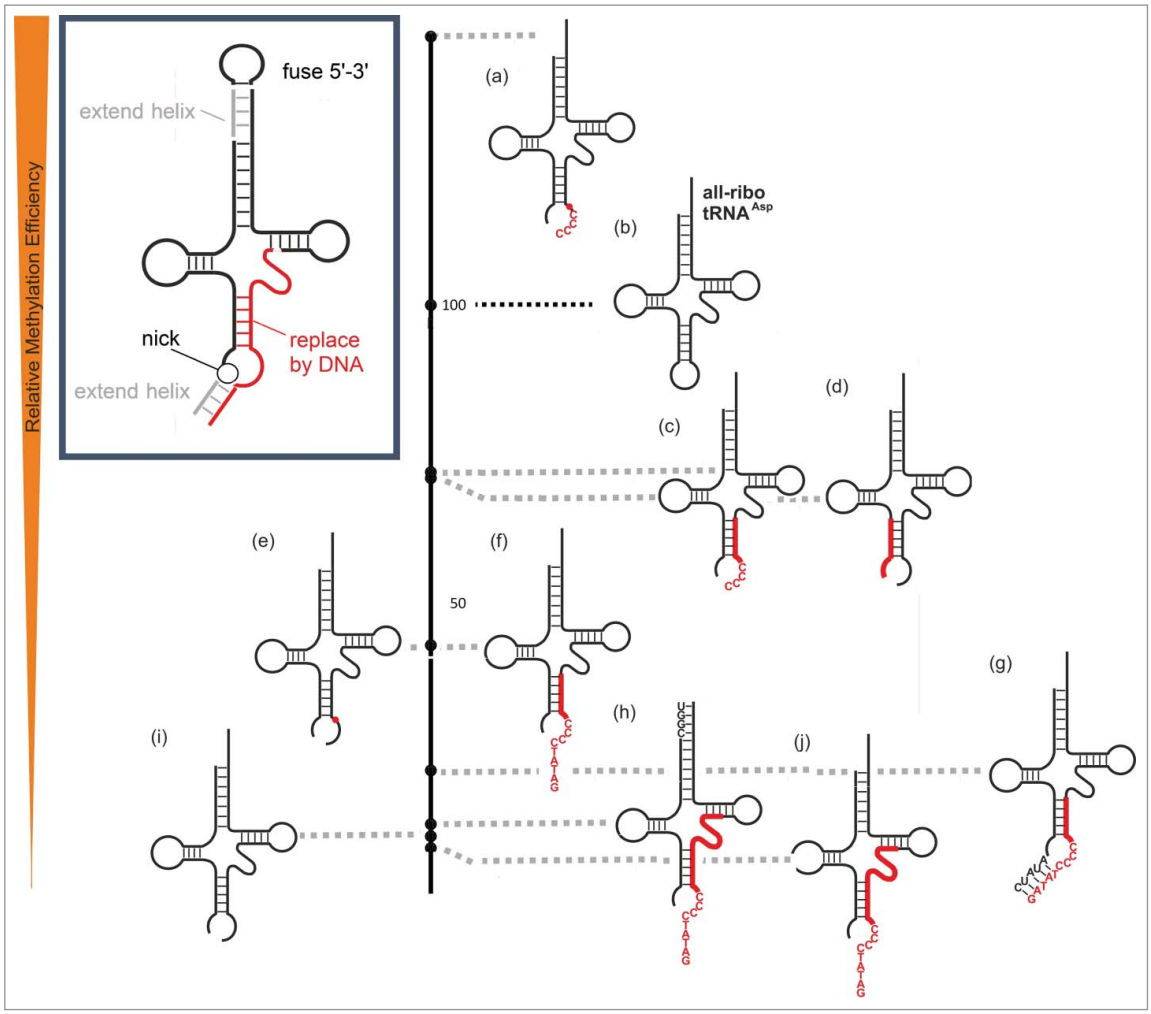

Figure 5. Relative methylation efficiency of 2-component hybrid-chimeric tRNAs^{Asp} compared to the all-ribo tRNA^{Asp} (entry 4c/5b in Table 1) which is set to 100 on the black scale bar in the middle. Deviations from the native tRNA^{Asp} sequence are presented in capital letters, DNA is highlighted in red.

end DNA residues were successively introduced into the target strand, and trends already observed were indeed recapitulated. For example, an exchange of rC38 against dC38 improved methylation (5i vs. 5e), or substitution of the 3'-side of the anticodon stem against DNA entailed moderate losses in methylation (5c, 5f-5h, 5j) as was previously the case with covalent chimeras (4e, 4f, and 4g).

An extension of the DNA fragment on its 5'-end was tolerated, albeit with some loss of activity (compare 5c vs. 5f). In comparison to the covalent chimeras, the newly implemented exchange of the Asp anticodon sequence against an oligo-C stretch was also tolerated by the enzyme (5 a,c,f,g,h,j). Subsequent extension of the acceptor stem helix led to moderate improvement (5j vs. 5h), while the artificial introduction of an extra helix in the anticodon domain led to a drop in methylation (5f vs. 5g) pointing to a dangling DNA single strand as the better substrate. Indeed, a construct featuring a 5'-tRNA-half hybridized to an RNA-DNA chimera with an oligo-C segment was a good substrate (5a) and its DNA content could be extended to the 3' (5c) and the 5' (5f) and still retained appreciable activity. An extension of the 5'-tRNA-half, designed to form an additional helix, lowered activity (compare 5f vs. 5g), pointing to a dangling DNA single strand as the better substrate.

The next step was aimed at replacing the covalent RNA-DNA chimera with 2 separate oligonucleotides, one DNA and one RNA. The underlying rationale was that this would lead to a ternary system of 2 RNA strands, which would be assembled into a methylation competent complex upon hybridization with the substrate DNA strand, effectively acting as artificial guide RNAs *in vitro*. In case of the construct (5h), the hitherto covalent link between the DNA and the RNA part was simply omitted by using 2 separate oligomers, in essence replacing that covalent link with a nick between positions 51 and 52. This particular construct (Fig. 6A) was chosen for 2 reasons, namely (i) to have a maximum length of the DNA for hybridization onto/into the tRNA scaffold, and (ii) because of the stretch of 4 consecutive G residues at positions 51-54 in the T-stem, which was liable to induce potential misfolding by hybridization to C residues 26-29 if the nick was placed elsewhere. The corresponding 3 oligonucleotides needed to assemble the ternary tRNA structural mimic were submitted to a hybridization protocol, but a subsequent test for tritium incorporation showed methylation activity that was only ~2 fold above the background signal. We therefore changed the assay conditions to include higher enzyme and SAM concentrations, and sampled at 15 and 120 minutes of incubation. Under these enhanced conditions however, a clear signal was measured, exceeding background by a

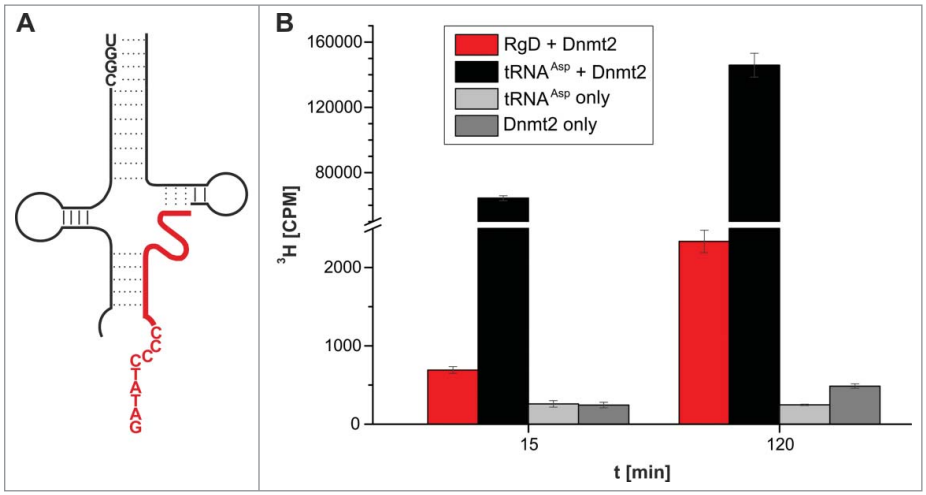

Figure 6. Methylation of an RNA-guided DNA oligonucleotide by Dnmt2. (A) Structure of the hybridized construct. DNA shown in red. (B) Average values and standard deviations of 3 tritium incorporation assays are shown.

factor of 2.8 after 15 minutes, and by a factor of 9.3 after 120 minutes in 3 replicates (Fig. 6B). Omission of the RNA fragments resulted in signal intensity of background level (not shown), confirming that the methylation depended on the presence of the 2 "guide" RNAs.

To verify, that the methyl group of ^{3}H-SAM was indeed incorporated into the DNA strand our customary LC-MS assay turned out to be inadequate, since the synthetic oligomers used to assemble the various substrates were discovered to already contain substantial amounts of non-radioactive m^{5}C in both RNA and DNA,[30] which increased the background to a level (compare Fig. 2) that precluded adequate quantification. Of note, the relative content of tritium in ^{3}H-SAM is so low, that detection of ^{3}H-by LC-MS would require quantities incompatible with biochemical handling and radiation safety. Instead, we resorted to thin-layer chromatography (TLC) for detection of tritium-containing dm^{5}C. The reaction mixture was precipitated, digested to mononucleosides, and separated by TLC in 2 dimensions after addition of authentic standards.[34] The corresponding spots were visualized on the TLC by UV light, scratched from the plate, and the tritium quantified by scintillation counting. As shown in Fig. 7, recovered radioactivity resided in the dm^{5}C spot, showing that no formation of rm^{5}C was catalyzed in the RNA part of the substrate complex.

Discussion

As an opening remark, 3 classes of new Dnmt2 substrates have been identified purely *in vitro*, and we are well aware that evidence for their biological significance is scarce in the absence of *in vivo* data. However, one of the new substrate classes has a known correspondent in tRNA biology, namely the all-RNA hybrid composed of 2 RNA strands in Fig. 5j. Such tRNAs with a nicked anticodon may result from incorrect tRNA splicing, the action of ribotoxins or other endonuclease enzymes like angiogenin, and the latter actually cleaves the Dnmt2 substrate tRNA^{Asp}.[5] Our results imply, that tRNAs may still be methylated after cleavage, but also that the 5'-tRNA half can, in principle act as a the equivalent of a guide RNA in trans, directing the methylation of Dnmt2 onto a single stranded short RNA such as a tRF. Given that tRFs were found associated to RISC,[36] modifications can be expected to affect RNAi, and might constitute a pathway through which Dnmt2 influences RNAi.

Two other classes of newly defined Dnmt2 substrates are of interest in our quest to understand the origin and mechanism of Dnmt2 catalytic action. The definition of a number of covalent RNA-DNA chimeras with variegated substrate properties illustrates, that Dnmt2 still bears strong traits of a DNA methyltransferase. These findings are strongly supporting the result

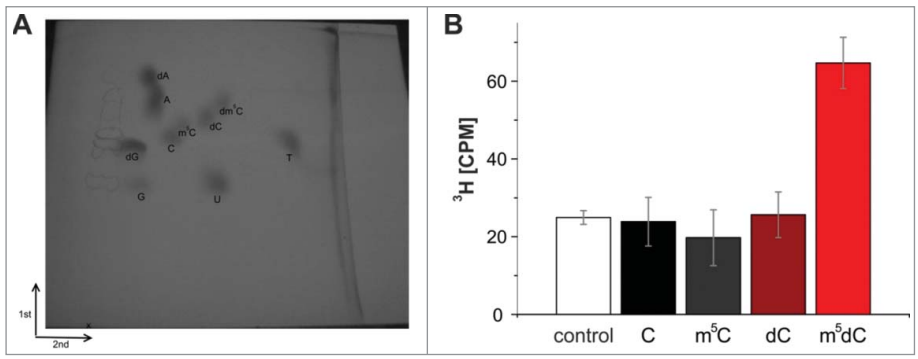

Figure 7. (A) Two-dimensional thin-layer chromatography of nucleosides on a 10 cm x 10 cm cellulose TLC plate. The starting point is marked by an X. (B) Methylation of an RNA-guided DNA oligonucleotide by Dnmt2. The oligonucleotides were hydrolyzed to nucleosides after the tritium incorporation assay, separated by 2D thin-layer chromatography and analyzed with the Cherenkov counter. Tritium could only be detected in m^{5}dC. Average values and standard deviations of 3 experiments are shown. Control corresponds to background signal of the TLC plate. Note that an identical Fig. S4 with enhanced contrast can be found in the supplement.

of multiple sequence alignments indicating that Dnmt2 has evolved from a DNA methyltransferase precursor and the DNA methylation activity might be considered an evolutionary "relict" in terms of biochemical catalysis.[26]

Although our unexpected results cannot prove that any DNA methylation by Dnmt2 does indeed occur *in vivo*, the fact that constructs featuring small DNA stretches near the target site (substrates 3a, 3b) actually *improve* methylation over the *bona fide* natural substrate $tRNA^{Asp}$, is indeed very suggestive. Based on our data one may speculate that different full or partially single stranded all-DNA structures which present a target deoxycytidine in a loop region, might function as Dnmt2 substrates.

Should Dnmt2 indeed never methylate DNA *in vivo*, then our finding might be construed to signify that DNA at the target site of a methyltransferase is more easily amenable to catalysis than RNA in general, *e.g.,* because it facilitates base flipping.[37] However, this is not the case for the TRM4 enzyme, which does not accept a deoxyribonucleotide at its target position C49 at all (Fig S1), thus supporting once more that the DNA methylation activity of Dnmt2 is likely related to its evolutionary ancestry among DNA methyltransferases.[26]

The substrates 3a-4i all contain DNA covalently linked to RNA, and these chimeras are derived from a tRNA scaffold whose structure depends, among other features, on the presence of A-form helices and a number of hydrogen bridges mediated by 2'-OH groups.[32] Both these features are gradually diminished upon increasing the DNA content and the resulting chimeras can reasonably be expected to gradually lose their structural resemblance to a proper tRNA, a view which receives support from the concomitantly fading methylation activity.

Our construction of an artificial guide RNA system that can efficiently direct methylation activity onto a non-tRNA oligoribonucleotide, and, less efficiently, onto a single stranded DNA strand, brings new concepts to the currently very lively field of nucleic acid modification. While guide RNAs are well established parts of several RNP complexes, *e.g.* effecting pseudouridinylation,[38,39] 2'O-methylation,[40,41] or U-insertion,[42,43] the fact that tRNA derived fragments may potentially turn any given modification enzyme into an RNA-guided modification RNP complex, can reasonably be expected to give impulse to the field. Of interest might be our attempts to simplify the tripartite DNA methylation system to a dual one by fusing the 2 guide RNA fragments. By applying again the splint ligation approach we obtain ~60% ligation yield, independently of where within the tRNA structure the junction is situated.[44] However, a series of resulting constructs featuring the former CCA-3'-end now forming a loop between the former 5' and 3'-ends (Fig. 5 inset) were, surprisingly, completely inactive for reasons yet to be evaluated, but likely linked to particular structural features of this loop (data not shown). This structural impact is likely to somehow affect the local structure at the remote modification site, because the known data on the enzyme suggest that its interaction with the substrate tRNA is restricted to the anticodon stem loop structure.[4,45]

As a concluding remark, we are aware that the low efficiency of our artificial DNA methylation system certainly quenches hope that this very construct might exhibit any activity *in vivo*. However, the principle possibility that Dnmt2 might do so in a more appropriate structural context should certainly be considered. Since such context would be expected to contain both RNA and DNA, possibly covalently linked, a future search might be directed toward replication forks, transcription sites, DNA repair events, or retroviral replication. However, even viral, bacterial and eukaryotic RNA contain a certain amount of DNA.[46]

Material and methods

Splinted ligation of hybrid tRNAs

Splinted ligation was performed as described previously, by annealing 2 synthetic fragments (IBA Göttingen, Germany, see Table 2) of RNA or DNA:RNA hybrids, corresponding in sequence to $tRNA^{Asp}$ from human (or mouse, as the sequences are identical) onto a 52 nt long complementary oligodeoxynucleotide.[47] Appropriate fragments (4 nmol) were 5'-phosphorylated by incubating in KL buffer supplemented with 5 mM ATP, 5 mM DTT and 0.75 u/μl T4 polynucleotide kinase (PNK, Fermentas, Germany) in a final volume of 150 μl in the thermomixer at 37°C for 1 h. To the phosphorylation reaction mixture an equimolar amount of the 5'-fragment and the DNA splint were added, as well as KL buffer, ATP (5 mM) and DTT (5 mM) leading to a final volume of 500 μl and a 8 μM concentration of each fragment. The RNA fragments were hybridized to the DNA splint by heating to 75°C in the thermomixer for 4 min and letting the reaction mixture cool down to room temperature for 15 min. Then T4 DNA ligase (1.5 u/μl; Fermentas) and T4 RNA ligase 2 (22 ng/μl) were added and the ligation was performed in the thermomixer at 16°C over night. Template DNA in constructs $tRNA^{Asp}$dC38 and all-ribo $tRNA^{Asp}$ was removed by addition of 1.5 u/μL DNase I (Fermentas), followed by 1 h of incubation at 37°C. The DNase digestion was omitted for hybrids containing DNA stretches of 3 nucleotides in length or more. All tRNA and tRNA hybrids were purified from ligation mixtures by denaturing PAGE, excised and eluted from the gel, and precipitated with ethanol. Concentrations were calculated from absorption at 254 nm, as determined on a Nanodrop ND-1000 spectrometer.

Protein preparation

Cloning, expression and purification by IMAC of the human DNMT2-His6 fusion protein was performed as described before.[9,28] Expression was done in *E. coli* (DE3) Rosetta2 pLysS cells. The protein expression was induced at $OD_{(600\ nm)} = 0.6$ with 1 mM IPTG and the cells were harvested 3 h after induction.

Tritium incorporation assay of in vitro methylation

In vitro methylation was done essentially as described.[27] The *in vitro* methylation assay measures the transfer of a tritiated methyl group from its donor [^{3}H]-S-adenosyL-methionine (^{3}H-SAM , 1 μCi/μL, 80 Ci/ mmol, from Hartmann Analytics, Braunschweig, Germany) onto the target tRNA, which is catalyzed by m^5C-methyltransferases. The tRNA is precipitated on small filters and the radioactive signal counted in a scintillation

Table 2. Oligonucleotides used in this study. Ribonucleotides are indicated by plain letters, deoxyribonucleotides are indicated as dC, dT, dA, dG, or dm^5C.

Construct name		5′ fragment		3′ fragment
all-ribo $tRNA^{Asp}$	MH 565	UCCUCGUUAGUAUAGUG GUGAGUAUCCCCGCCU	MH 566	GUCACGCGGGAGACCGGGGUU CGAUUCCCCGACGGGGAGCCA
$tRNA^{Asp}_{dC38}$	MH 565	UCCUCGUUAGUAUAGUGG UGAGUAUCCCCGCCU	MH 571	GUCAdCGCGGGAGACCGGGGU UCGAUUCCCCGACGGGGAGCCA
$tRNA^{Asp}_{d37\text{-}39}$	MH 565	UCCUCGUUAGUAUAGUGGU GAGUAUCCCCGCCU	MH 584	GUCdAdCdGCGGGAGACCGGGGU UCGAUUCCCCGACGGGGAGCCA
$tRNA^{Asp}_{d36\text{-}40}$	MH 565	UCCUCGUUAGUAUAGUGGU GAGUAUCCCCGCCU	MH 585	GUdCdAdCdGdCGGGAGACCGGG GUUCGAUUCCCCGACGGGGAGCCA
$tRNA^{Asp}_{d34\text{-}43}$	MH 565	UCCUCGUUAGUAUAGUGG UGAGUAUCCCCGCCU	MH 587	dGdTdCdAdCdGdCdGdGdGAGACC GGGGUUCGAUUCCCCGACGGGGAGCCA
$tRNA^{Asp}_{d34\text{-}46}$	MH 565	UCCUCGUUAGUAUAGUGGU GAGUAUCCCCGCCU	MH 623	dGdTdCdAdCdGdCdGdGd GdAdGdACCGGGGUUCGAU UCCCCGACGGGGAGCCA
$tRNA^{Asp}_{d34\text{-}51}$	MH 565	UCCUCGUUAGUAUAGUG GUGAGUAUCCCCGCCU	MH 642	dGdTdCdAdCdGdCdGdGdGd AdGdAdCdCdGdGGGUUCGAUUC CCCGACGGGGAGCCA
$tRNA^{Asp}_{d27\text{-}43}$	MH 586	UCCUCGUUAGUAUAGUGGUGA GUAUCdCdCdCdGdCdCdT	MH 587	dGdTdCdAdCdGdCdGdGdGAGACC GGGGUUCGAUUCCCCGACG GGGAGCCA
$tRNA^{Asp}_{d27\text{-}33}$	MH 586	UCCUCGUUAGUAUAGUGGUGA GUAUCdCdCdCdGdCdCdT	MH 566	GUCACGCGGGAGACCGGGGUU CGAUUCCCCGACGGGGAGCCA
$tRNA^{Asp}_{d48\text{-}49}$	MH 565	UCCUCGUUAGUAUAGUGG UGAGUAUCCCCGCCU	MH 621	GUCACGCGGGAGAdCdCGGGGUUC GAUUCCCCGACGGGGAGCCA
$tRNA^{Asp}_{dm5C38}$	MH 565	UCCUCGUUAGUAUAGUGGU GAGUAUCCCCGCCU	MH 569	GUCAdm^{5}CGCGGGAGACCGGGGUUC GAUUCCCCGACGGGGAGCCA
Splint	MH 570	dGdGdAdAdTdCdGdAdAdCdCd CdCdGdGdTdCdTdCdCdCdGd CdGdTdGdAdCdAdGdGdCdG dGd GdGdAdTdAdCdTdCdAd CdCdAdCdTdAdTdAdC		
nicked tRNAAsp dC*dC38	MH 565	UCCUCGUUAGUAUAGUGG UGAGUAUCCCCGCCU	MH 619	dCdCdCdCdCGCGGGA GACCGGGGUUCGAUUCCCCG ACGGGGAGCCA
nicked tRNAAspdC*dC38-43	MH 565	UCCUCGUUAGUAUAGUGG UGAGUAUCCCCGCCU	MH 638	dCdCdCdCdCdGdCdGdGd GAGACCGGGGUUCGAUUCCC CGACGGGGAGCCA
nicked tRNAAspdX-43	MH 565	UCCUCGUUAGUAUAGUGG UGAGUAUCCCCGCCU	MH 643	dGdAdTdAdTdCdCdCdCdCd GdCdGdGdGAGACCGGGGU UCGAUUCCCCGACGGGGAGCCA
nicked tRNAAspdX-43-3′HX	MH 646	UCCUCGUUAGUAUAGUGG UGAGUAUCCCCGCCUAUAUC	MH 643	dGdAdTdAdTdCdCdCdCdGd CdGdGdGAGACCGGGGUUCG AUUCCCCGACGGGGAGCCA
nicked tRNAAspdC38	MH 565	UCCUCGUUAGUAUAGUG GUGAGUAUCCCCGCCU	MH 571	GUCAdCGCGGGAGACCGGGGU UCGAUUCCCCGACGGGGAGCCA
nicked tRNAAsp d27-33	MH 586	UCCUCGUUAGUAUAGUGGU GAGUAUCdCdCdCdGdCdCdT	MH 566	GUCACGCGGGAGACCGGGGU UCGAUUCCCCGACGGGGAGCCA
nicked tRNAAspdX-53	MH 565	UCCUCGUUAGUAUAGUG GUGAGUAUCCCCGCCU	MH 647	dGdAdTdAdTdCdCdCdCd CdGdCdGdGdGdAdGdAdCdCd GdGGGUUCGAUUCCCCGACG GGGAGCCA
nicked all-ribo tRNAAsp	MH 565	UCCUCGUUAGUAUAGUG GUGAGUAUCCCCGCCU	MH 566	GUCACGCGGGAGACCGGGGU UCGAUUCCCCGACGGGGAGCCA
nicked tRNAAspdX-53-5′HX	MH 648	UGGCUCCUCGUUAGUAUAG UGGUGAGUAUCCCCGCCU	MH 647	dGdAdTdAdTdCdCdCdCdCd GdCdGdGdGdAdGdAdCdCd GdGGGUUCGAUUCCCCGACG GGGAGCCA
RgD	MH 648	UGGCUCCUCGUUAGUAUA GUGGUGAGUAUCCCCGCCU	MH 649	GGUUCGAUUCCC CGACGGGGAGCCA
	MH 650 (middle)	dGdAdTdAdTdCdCdCdCdCdGd CdGdGdGdAdGdAdCdCdGdG		

counter. The standard reaction volume was 40 μl. Unless stated otherwise, 120 pmol tRNA were diluted in water and heated to 65°C for 2 min. tRNA-MT-assay buffer and DTT were added immediately to final concentrations of 100 mM Tris-HCl pH 8.0; 100 mM NH_4OAc; 0.1 mM EDTA, 10 mM $MgCl_2$ and 10 mM DTT. ^{3}H-SAM-Stock solution (10x; containing cold and ^{3}H-SAM) was added to a final concentration of 0.9 μM SAM and 1 μCi per sample. The enzyme was added to final concentrations of 1 μM and mixed well by pipetting. At various time points 8 μL aliquots were spotted onto small Whatman filters and precipitated in 5 % ice-cold TCA, followed by 2 washes at room temperature for 20 min and 10 min, respectively in 5 % TCA. Then the filters were swirled in EtOH. After drying, the Whatman filters were transferred into scintillation vials and 3 ml

of Ultima Gold MV liquid scintillation cocktail (PerkinElmer, Waltham, USA) were added into each vial. The incorporated tritium was measured by liquid scintillation counting with a Wallac 1409 liquid scintillation counter (PerkinElmer, Waltham, USA). Measuring duration was 60 sec per sample. 1 μL of the 10x SAM-stock solution was spotted in duplicate as a standard for specific activity. Initial reaction rates were extracted from methylation kinetics (Fig. S2) by linear regression after background correction. From the initial rates, K_m and V_{max} values were obtained by a non-linear 2 component least square fit, and k_{cat} was calculated from V_{max}.

HPLC -MS analysis

0.5 μg of either Dnmt2 treated or untreated tRNA was dissolved in 20 mM NH_4OAc pH 5.3 and digested to nucleosides as described before.[48] Additionally the commercial oligomer MH569 (Table 1) containing deoxy-5-methyl-cytidine was digested and used as a reference sample for MS fragmentation experiments. For ribo-5-methyl-cytidine a commercial nucleoside was used (Sigma-Aldrich, Missouri, US).

The digested RNA was analyzed on an Agilent 1260 series equipped with a diode array detector (DAD) and Triple Quadrupol mass spectrometer Agilent 6460. A Synergy Fusion RP column (4 μm particle size, 80 Å pore size, 250 mm length, 2 mm inner diameter) from Phenomenex (Aschaffenburg, Germany) was used at 35 °C. The solvents consisted of 5 mM ammonium acetate buffer adjusted to pH 5.3 using acetic acid (solvent A) and pure acetonitrile (solvent B). The elution started with 100% solvent A followed by a linear gradient to 20% solvent B at 10 min. Initial conditions were regenerated by rinsing with 100% solvent A for 7 minutes. The flow rate was 0.5 mL/min.

The effluent from the column was first measured photometrical at 254 nm by the DAD followed by the mass spectrometer equipped with an electrospray ion source (Agilent Jet Stream). ESI parameters were as follows: gas temperature 300°C, Gas flow 5 L/min, Nebulizer pressure 35 psi, Sheath gas temperature 350°C, Sheath gas flow 12 L/min, capillary voltage 3500 V. The MS was operated in positive ion mode monitoring multiple fragmentation reactions (MRM mode) at previously optimized conditions. The transitions and retention times used for identification of nucleosides can be found in Table 3.

Thin layer chromatography

For the RgD Experiments the concentration of nucleic acid and enzyme was doubled and the ratio of hot to cold SAM was increased (2.3 μCi per sample). The total reaction volume was 50 μl. At two time points after reaction start (15 and 120 minutes), aliquots were spotted on a whatman filter paper and measured with the Cherenkov counter. The remaining portion of the sample was subjected to a nucleic acid precipitation with $LiClO_4$ in acetone to purify the nucleic acids. The precipitation with perchlorate and acetone was chosen over standard EtOH precipitation, as it is more suited to precipitate small oligonucleotides. The pellet was then resuspended in H_2O and further purified with a MicroSpin G50 size-exclusion column (GE Healthcare, Solingen, Germany). The purified oligonucleotides were enzymatically hydrolyzed to nucleosides with nuclease P1, snake venom phosphodiesterase and shrimp alkaline phosphatase. After the addition of commercially available nucleosides as standards (all Sigma-Aldrich, Missouri, US, except dm^5C, Berry & Associates, Dexter, USA), the samples were concentrated and spotted on a 10 cm × 10 cm cellulose TLC plate (Merck ref#1.05577.000, Darmstadt, Germany) and subjected to 2-dimensional thin-layer chromatography. For the first dimension the solvent was isobutyric acid : concentrated ammonia : H_2O (50 : 1.1 : 28.9 [v : v : v]) and for the second dimension isopropanol : concentrated HCl : H_2O (68 : 18 : 14 [v : v : v]). After each run the TLC plate was dried for several hours in a fume hood. The nucleoside spots were visualized with a UV-lamp (256 nm), marked with a pencil, and the cellulose corresponding to a spot was scraped off with a scalpel and transferred to a reaction tube. The nucleosides were extracted with water and subjected to liquid scintillation counting.

Table 3. Mass transitions for MRM and retention times in LC-MS analysis.

Nucleoside	Precursor ion [m/z]	Product ion [m/z]	Retention time [min]
ribocytidine (C)	244	112	2.8
ribouridine	245.2	113	3.7
deoxycytidine (dC)	228	112	4.0
ribomethylcytidine (rm^5C)	258.1	126.1	5.1
Deoxymethylcytidine (dm^5C)	242	126.1	6.0
adenosine	268	136	7.5

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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ORCID

Mark Helm http://orcid.org/0000-0002-0154-0928

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