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Dynamic modulation of Dnmt2-dependent tRNA methylation by the micronutrient queuine

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ABSTRACT

Dnmt2 enzymes are cytosine-5 methyltransferases that methylate C38 of several tRNAs. We report here that the activities of two Dnmt2 homologs, Pmt1 from *Schizosaccharomyces pombe* and DnmA from *Dictyostelium discoideum*, are strongly stimulated by prior queuosine (Q) modification of the substrate tRNA. *In vivo* tRNA methylation levels were stimulated by growth of cells in queuine-containing medium; *in vitro* Pmt1 activity was enhanced on Q-containing RNA; and queuine-stimulated *in vivo* methylation was abrogated by the absence of the enzyme that inserts queuine into tRNA, eukaryotic tRNA-guanine transglycosylase. Global analysis of tRNA methylation in *S. pombe* showed a striking selectivity of Pmt1 for $tRNA^{Asp}$ methylation, which distinguishes Pmt1 from other Dnmt2 homologs. The present analysis also revealed a novel Pmt1- and Q-independent tRNA methylation site in *S. pombe*, C34 of $tRNA^{Pro}$. Notably, queuine is a micronutrient that is scavenged by higher eukaryotes from the diet and gut microflora. This work therefore reveals an unanticipated route by which the environment can modulate tRNA modification in an organism.

INTRODUCTION

The post-transcriptional modification of nucleotides enables organisms to increase the functional repertoire of RNA as well as DNA molecules. A large variety of modifications occur on tRNA (1,2), which allows the dynamic fine-tuning of protein translation in order to adapt protein levels to changing environmental conditions (3). Modifications can affect the overall structure and stability of the tRNA or, when targeted to specific functional regions, can have a more direct effect on ribosome binding, codon misreading and frame shifting as well as aminoacylation (4). An important question is how external cues can affect tRNA modification patterns and thus regulate cellular protein translation.

Cytosine-5 methylation (m^5C) is a common modification in tRNAs of organisms from all domains of life (5). Methylation by the Dnmt2 family of methyltransferases leads to m^5C methylation at position C38 in the anticodon loop of certain tRNAs (6). Interestingly, Dnmt2 is phylogenetically related to DNA rather than RNA methyltransferases, and thus apparently has evolutionarily been functionalized to methylate tRNA rather than DNA (7). The most common substrate for Dnmt2 is $tRNA^{Asp}$, which is a Dnmt2 target in mammals, *Drosophila*, *Arabidopsis*, *Dictyostelium* and *Entamoeba histolytica* (8–12). Dnmt2 also methylates C38 in $tRNA^{Gly}$ and $tRNA^{Val}$ *in vivo* in *Drosophila* and mouse (10,13).

The fission yeast *Schizosaccharomyces pombe* contains a Dnmt2 homolog designated ***p**ombe* **m**ethyl**t**ransferase 1 (Pmt1), which we have shown to have *in vitro* methylation activity on $tRNA^{Asp}$ and, to a lesser extent, $tRNA^{Glu}$ (14). Notably, Pmt1 also provides *in vivo* tRNA methylation activity that is strongly controlled by nutritional cues, as $tRNA^{Asp}$ methylation is induced to 100% when cells are grown in the presence of peptone, and cultivation of *S. pombe* in minimal medium leads to approximately 23% of $tRNA^{Asp}$ methylation. There is selectivity to the induction, since methylation is not enhanced by stress conditions such as glucose starvation, heat stress or oxidative stress. The function of this modification in *S. pombe* is unclear, since cells lacking Pmt1 have no apparent phenotype and are insensitive to high temperature and oxidative stress (14).

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The function of Dnmt2-dependent tRNA methylation is also poorly understood in other organisms. *Drosophila* or mice lacking Dnmt2 are viable and fertile, but Dnmt2-deficient flies show an increased sensitivity to thermal and oxidative stress as well as viral infection, suggesting a role for Dnmt2 in stress tolerance (10,15). Furthermore, Dnmt2 in *Drosophila* controls silencing of retrotransposons and telomere integrity, and it is linked to the non-random segregation of sister chromatids during stem cell division, though the connection between tRNA methylation and these phenotypes remains unclear (16,17). In mice, Dnmt2 is involved in the inheritance of epialleles of the Kit gene, possibly through methylation of small RNAs (18). Importantly, the absence of Dnmt2 in mice is lethal in combination with the absence of the Trm4-family methyltransferase NSun2, which methylates C48, C49 and C50 of tRNA (13). Since all m^5C tRNA methylation is lacking in NSun2- and Dnmt2-deficient cells, this uncovers an essential role for C5 tRNA methylation in mammalian development.

While the methyl group in m^5C represents a rather simple chemical entity, other nucleotide modifications are more elaborate. One such example is queuosine (Q designates the nucleotide or nucleoside, the respective base is called queuine) (reviewed in (19)), which is a hyper-modified 7-deaza-guanosine that occurs at the wobble position (position 34) of tRNAs that contain the GUN anticodon ($tRNA^{Asn}$, $tRNA^{Asp}$, $tRNA^{His}$ and $tRNA^{Tyr}$) (2). Interestingly, the presence of queuosine in tRNA is widely distributed in the biosphere, but only eubacteria synthesize queuine *de novo* via a multi-step biosynthesis pathway that results in the presence of Q34 in tRNA (reviewed in (19)). In contrast, eukaryotes cannot synthesize queuine *de novo*, but rely on environmental sources and/or the gut microflora to obtain queuine, which is exchanged for the G34 guanine in $tRNA^{Asn}$, $tRNA^{Asp}$, $tRNA^{His}$ and $tRNA^{Tyr}$ (reviewed in (19)). The enzyme catalyzing this irreversible base exchange is eukaryotic tRNA-guanine transglycosylase (eTGT), a heterodimer consisting of a catalytic subunit (Qtrt1) and a regulatory subunit (Qtrtd1) (20,21). The absence of queuine has no obvious phenotype in gnotobiotic mice under laboratory conditions (22); however, subsequent research suggests that queuine limits tetrahydrobiopterin oxidation and may affect numerous physiological processes in eukaryotes (reviewed in (19)). Also, a recent study suggested that the bioavailability of queuine in drosophilids results in a shift of codon preference in their genome (23). Altogether, the biological relevance of queuine incorporation remains to be determined.

In this study, we investigated the stimulation of Dnmt2 activity by nutritional supplementation with queuine. Significantly, we found that growth of *S. pombe* cells in the presence of queuine strongly stimulated *in vivo* Pmt1-dependent methylation of C38 in $tRNA^{Asp}$, thus showing an unanticipated dependence of methylation on queuosine. Importantly, C38 methylation *in vivo* required the incorporation of queuine into tRNA by eTGTs. Furthermore, the dependence of Dnmt2 on queuine incorporation was evolutionarily conserved between *S. pombe* and *Dictyostelium discoideum*. A genome-wide tRNA methylation analysis by high-throughput bisulfite sequencing revealed a striking selectivity of Pmt1- and queuine-dependent tRNA methylation in *S. pombe*, because $tRNA^{Asp}$ was the only Pmt1 tRNA target identified. During this analysis, we identified a novel Pmt1- and queuine-independent m^5C site in *S. pombe*, C34 of $tRNA^{Pro}$. Our data show a remarkable regulation of tRNA methylation by a micronutrient, which reveals an unanticipated route by which the gut microbiome and the environment could influence protein translation in higher eukaryotes.

MATERIALS AND METHODS

Strains and growth conditions

The *S. pombe* and *D. discoideum* strains used in this study are shown in Supplementary Table S2. *S. pombe* strains were cultured in standard full medium (YES) (5 g/l yeast extract, 30 g/l glucose, 250 mg/l adenine, 250 mg/l histidine, 250 mg/l leucine, 250 mg/l uracil, 250 mg/l lysine). Gene knockouts in *S. pombe* and *D. discoideum* were obtained by homologous recombination, and correct knockout was verified by polymerase chain reaction (PCR) analysis (24–26). Primer sequences are listed in Supplementary Tables S3 and S4. All *Dictyostelium* strains used in this study are derived from strain Ax2–214. Cells were cultured in HL5 medium (Formedium) containing ampicillin (50 μg/ml), amphotericin-B (0.25 μg/ml), penicillin/ streptomycin (100 μg/ml) and chloramphenicol (34 μg/ml). Where required, antibiotics for selection (blasticidin S and/or G418 sulfate, 10 μg/ml each) were added. For RNA preparation, cells were grown shaking under continuous light at 22°C up to a cell density of 2×10^6 cells/ml. Queuine isolated from bovine amniotic fluid was added to *S. pombe* cultures in YES medium at 0.03 μM. *D. discoideum* cultures were grown for at least four days with 0.1 μM queuine, kindly provided by Klaus Reuter, Marburg (27).

In vitro RNA methylation assays

The *in vitro* methylation of total RNA from *S. pombe* or *D. discoideum* with recombinant Pmt1 or DnmA was performed as described earlier (11,14). Briefly, 2 μg of total RNA were incubated with 3 μM enzyme for 90 min at 22°C in 20 μl of methylation buffer (5 mM Tris–HCl pH 7.5, 5 mM NaCl, 0.5 mM $MgCl_2$, 0.1 mM dithiotreitol (DTT)) containing 1.25 nM [methyl-^{3}H]-AdoMet (Hartmann Analytic). For methylation assays with isolated $tRNA^{Asp}$, this tRNA was obtained from total RNA of *pmt1Δ* (AEP8) grown in YES with or without 0.03 μM queuine by hybridization with a biotinylated DNA oligonucleotide (see Supplementary Data for details). The time course of methylation of isolated $tRNA^{Asp}$ from *S. pombe* was performed using 0.5 μM $tRNA^{Asp}$ and 1 μM recombinant Pmt1. Reactions were separated on 7 M urea 12% denaturing polyacrylamide gels and quantification of methylation product formation was done by autoradiography followed by densitometric analysis of films using Image Lab Software (Bio-Rad).

Bisulfite sequencing of individual tRNAs

Bisulfite sequencing of tRNAs was performed as described previously (11,14,28). Briefly, PCR amplicons of tRNAs

were generated from bisulfite-treated total RNA of *S. pombe* or *D. discoideum* by reverse transcription using a tRNA 3′-specific stem-loop primer, followed by amplification with primers binding only to the deaminated sequences at the 5' end. Subsequently, standard cloning of the PCR amplicons and sequencing of several independent subclones was performed. Primer sequences are listed in Supplementary Tables S3 and S4. For high-throughput RNA bisulfite sequencing of individual tRNAs from *S. pombe*, PCR products obtained after bisulfite treatment of total RNA, reverse transcription and PCR amplification were subjected to Illumina sequencing (28). Library preparation was performed by ligation of Illumina TrueSeq LT indexes to PCR products using T4 ligase (NEB). Post-ligation amplification was carried out with Herculase II Fusion DNA Polymerase (Agilent) for 8–12 cycles. A total of 150 bp paired-end sequencing was done using the Illumina MiSeq v3 platform. Reads were processed using in-house R scripting and the Bioconductor package ShortRead (29). Processing included trimming of PCR primers, selection of high quality reads and sorting of the reads based on the sequence in the degenerate region of the RT-primer. Bisulfite conversion in 1024 unique reads per sample were plotted.

Genome-wide tRNA methylome analysis

tRNAs from *S. pombe* were obtained by separating total RNA on a denaturing polyacrylamide gel and size-selection for tRNAs. Extracted RNAs were subjected to bisulfite conversion using the EZ RNA Methylation kit (Zymo). 3′ dephosphorylation and 5′ phosphorylation were performed using T4 polynucleotide kinase (TaKaRa). Library preparation for deep sequencing was done using the NEBNext Small RNA Library Prep Set for Illumina (New England Biolabs). A total of 200 ng of tRNAs per library were used as starting material and ligations done with undiluted adaptors. Adaptor-ligated cDNA was amplified with 12 cycles of PCR reaction and purified using the QIAQuick PCR Purification kit (Qiagen). Libraries were size selected with a 6% Novex tris-borate-EDTA (TBE) polyacrylamide gel (Life Technologies), extracted and ethanol precipitated according to NEBNext instruction manual and resuspended in EB buffer (Qiagen). The libraries were multiplexed in equimolar ratios and sequenced on one lane of the Illumina HiSeq 2000 platform using paired-end 100 bp sequencing. The sequencing data was quality trimmed to a minimum aggregate score of 30 using the fastq quality trimmer integrated in Galaxy and adapter-trimmed using cutadapt version 1.8.1 (30), then aligned to all known genomic tRNA sequences which were obtained from the genomic tRNA database (31), using bsmap version 2.74 (32), allowing for 5% mismatch rate for each read and reporting all repeat hits. Custom Python scripts were then used to calculate bisulfite conversion rates for each cytosine position in the reference and combined with R scripting to generate heatmap plots.

See Supplementary Data for additional materials and methods.

RESULTS

In vivo methylation of tRNA^{Asp} by Dnmt2 was induced by the presence of queuine

Methylation of carbon 5 in cytosine 38 of tRNA^{Asp} in *S. pombe* is dependent on nutritional signals in that the cultivation of *S. pombe* in the presence of peptone strongly induces the methylation of C38 by Pmt1 (14). Peptone, a complex mixture of nutrients generated by proteolytic digestion of animal protein, is a source of the micronutrient queuine (which in peptone occurs as a nucleoside or nucleotide (33), but is salvaged by *S. pombe* to queuine (34)). Queuine is absent in standard *S. pombe* growth medium. Therefore, we tested the possibility that queuine induces Pmt1-dependent tRNA methylation. In a first approach, RNA was extracted from wild-type (wt) and *pmt1Δ* cells grown in full medium either in the presence or the absence of queuine, and recombinant Pmt1 was subsequently tested for its ability to methylate these samples *in vitro*. If prior *in vivo* methylation is high, subsequent *in vitro* methylation is expected to be low, whereas low *in vivo* methylation will result in high *in vitro* remethylation (14). RNA from *pmt1Δ* cells showed high *in vitro* remethylation by recombinant Pmt1, regardless of the prior presence of queuine in the growth medium. Importantly, while RNA from wt cells grown without queuine was efficiently remethylated, *in vitro* remethylation was absent in RNA from wt cells grown in the presence of queuine (Figure 1A). This finding suggested that the presence of queuine in the growth medium resulted in complete methylation of Pmt1 targets *in vivo*.

To support this suggestion, the methylation state of tRNA^{Asp}, a known target of Pmt1, was analyzed by RNA bisulfite sequencing. Briefly, PCR amplicons of tRNA^{Asp} were generated from bisulfite-treated total RNA, and the sequence of independent subclones was determined. Using this method, cells grown in full medium without queuine showed less than 6% tRNA^{Asp} methylation at C38 (zero out of 16 subclones), whereas the presence of queuine in the medium led to 100% methylation at this residue (Figure 1B). Thus, Pmt1-dependent tRNA^{Asp} methylation was strongly induced *in vivo* by the presence of queuine. One possible explanation for the above results is that the expression of the *pmt1*$^{+}$ gene might become induced by queuine. However, we observed no significant expression differences of *pmt1*$^{+}$ between cells grown with and without queuine (Supplementary Figure S1), and Pmt1 protein levels were unaffected by the presence of peptone (14), arguing for a more direct stimulation of Pmt1 by queuine.

The methyltransferase activity of Dnmt2 on C38 of tRNA^{Asp} is largely conserved across species (35). We therefore tested whether another Dnmt2 homolog, DnmA from *D. discoideum*, was also stimulated by the presence of queuine. For this purpose, RNA extracted from wt, dnmA- cells and a dnmA overexpression strain (dnmA GFP) was analyzed in *in vitro* remethylation assays. As expected, *in vivo* methylation was absent in the dnmA- mutant and high in the dnmA-GFP overexpressing strain, as indicated by the strongly reduced *in vitro* remethylation signal upon DnmA overexpression (Figure 1A). Furthermore, wt cells showed low *in vivo* methylation in both rich and minimal medium.

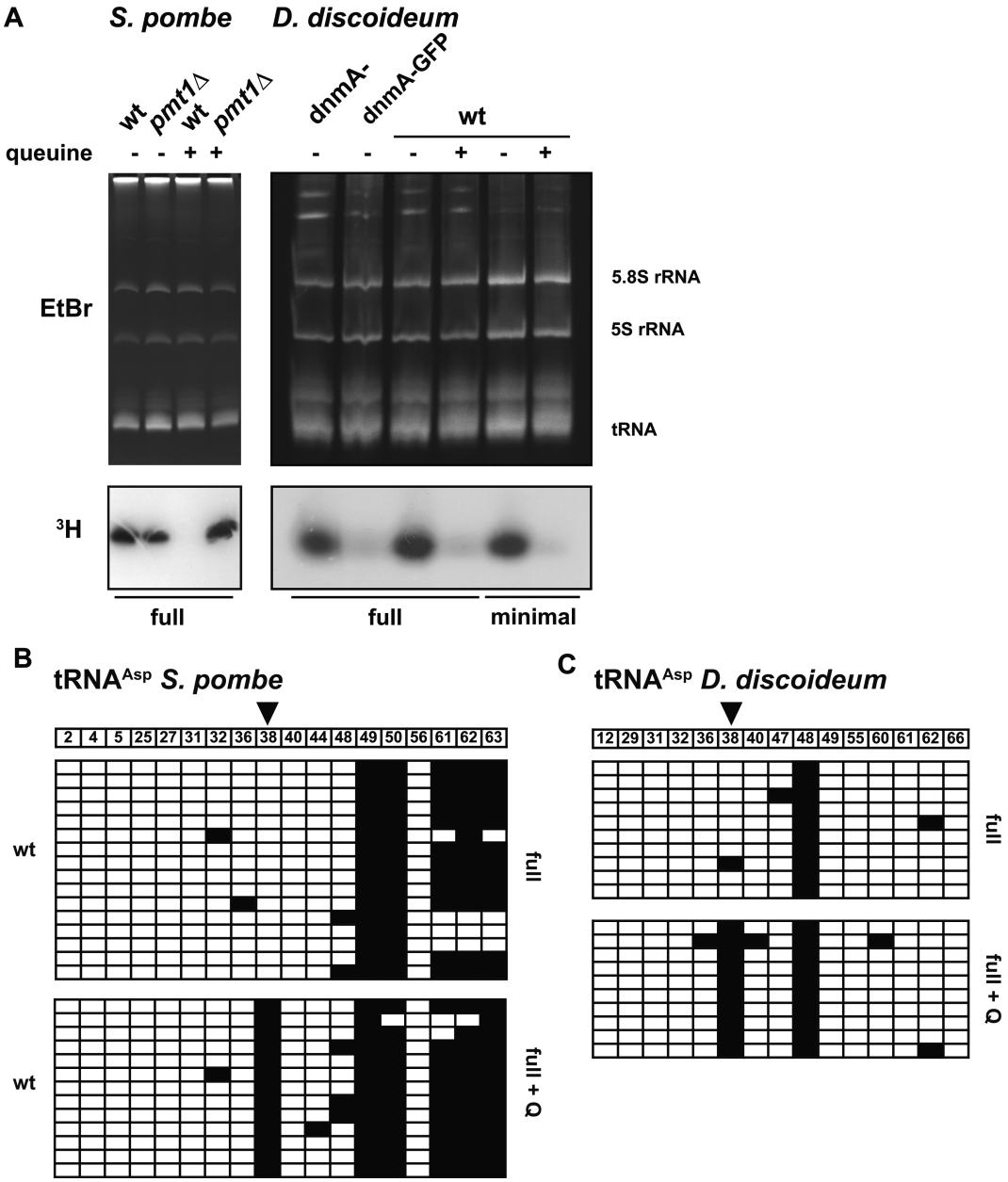

Figure 1. *In vivo* methylation of tRNA^{Asp} by the Dnmt2 homologs Pmt1 (*Schizosaccharomyces pombe*) and DnmA (*Dictyostelium discoideum*) was stimulated by growth of cells in the presence of queuine. (**A**) *In vitro* methylation of total RNA from *S. pombe* (left) and *D. discoideum* strains (right) that were either wild-type (wt), lacked Dnmt2 (*pmt1Δ*, *dnmA*-) or overexpressed DnmA (dnmA-GFP) and were grown in the indicated medium (*S.pombe*: full—YES; *D. discoideum*: full—HL5, minimal—FM) with or without queuine (0.03 μM in *S. pombe*; 0.1 μM in *D. discoideum*). Methylation was performed by recombinant Pmt1 (left) or DnmA (right). Top, ethidium bromide-stained gel (EtBr; size of ribosomal and tRNAs indicated), bottom, autoradiogram of methylated RNA (^{3}H). (**B**) *In vivo* RNA bisulfite sequencing of tRNA^{Asp} from total RNA of wild-type *S. pombe* grown with or without queuine (Q) as described in (A). The cytosine residues present in tRNA^{Asp} are indicated in the top row. Each subsequent row represents an independent clone that was sequenced. Black boxes indicate methylated cytosines, white boxes indicate unmethylated cytosines, the arrow indicates the methylation position for Dnmt2 enzymes. Data for wt are taken from (14). (**C**) *In vivo* RNA bisulfite sequencing of tRNA^{Asp} from total RNA of *D. discoideum*, representation as in (B).

However, the addition of queuine to either medium resulted in significantly increased *in vivo* methylation as indicated by the reduced signal upon *in vitro* remethylation, thus suggesting that DnmA activity, like its homolog from *S. pombe*, was increased by the presence of queuine.

We next tested *in vivo* methylation of *D. discoideum* tRNA^{Asp} by RNA bisulfite sequencing. Importantly, the addition of queuine to the growth medium increased C38 methylation from 10 to 100%, while other cytosines remained unaffected (Figure 1C). As in *S. pombe*, this effect was not attributed to a difference in the expression of the *dnmA* gene (Supplementary Figure S1). The low level of C38 methylation without supplemental queuine may be caused by a residual level of queuine in the full medium, or by a basal level of DnmA activity on queuine-free tRNA.

Taken together, these results showed that growing cells in the presence of queuine resulted in a substantial induction of the *in vivo* activity of two Dnmt2 enzymes towards $tRNA^{Asp}$, thus revealing an unanticipated dependence of C38 methylation on the presence of queuine that is conserved between *S. pombe* and *D. discoideum*. This dependence was not due to transcriptional regulation of the respective genes by queuine, suggesting a more direct effect of queuine on the activity of Pmt1 and DnmA, possibly by its incorporation into the tRNA substrate itself.

Queuine incorporation into RNA stimulated Pmt1 activity *in vivo* and *in vitro*

To further examine the influence of queuine on the methylation of $tRNA^{Asp}$, we next asked whether queuine was incorporated into *S. pombe* RNA *in vivo*. For this purpose, total RNA of wt and *pmt1Δ* cells grown in medium with or without queuine was analyzed by LC-MS/MS. Importantly, queuosine was present in RNA from both wt and *pmt1Δ* cells when grown in medium containing queuine, while it was not detected in RNA from cells grown in its absence (Figure 2A). Thus, queuine had been incorporated into *S. pombe* RNA, and its incorporation was not dependent on Pmt1. The observation of queuosine in total RNA was in good agreement with previous observation of queuosine in *S. pombe* $tRNA^{Asp}$ (34).

We next asked whether queuine incorporation into (t)RNA resulted in a difference in the kinetics of *in vitro* methylation by Pmt1. To this end, RNA that was unmethylated at Pmt1 target sites was obtained from *pmt1Δ* cells grown with or without queuine and subjected to a time-course of *in vitro* remethylation with recombinant Pmt1. Significantly, Pmt1 activity was increased on Q34-RNA compared to G34-RNA (Figure 2B). Furthermore, Pmt1 activity was also observed on $tRNA^{Asp}$ purified *ex vivo* from *pmt1Δ* cells and its activity was increased on Q34-$tRNA^{Asp}$ (Figure 2B). This showed that the presence of queuosine in RNA stimulated not only the *in vivo*, but also the *in vitro* activity of Pmt1. It should be noted, however, that there was substantial *in vitro* activity on unmodified tRNA, suggesting an additional layer of Pmt1 regulation *in vivo* (for instance by tRNA folding or occlusion of the methylation site by proteins bound to the tRNA).

Stimulation of $tRNA^{Asp}$ methylation *in vivo* by Dnmt2 was dependent on eukaryotic type tRNA-guanine transglycosylases

The incorporation of queuine at position 34 of eukaryotic tRNAs containing a GUN anticodon is catalyzed by eukaryotic type tRNA-guanine transglycosylases (eTGTs), a complex consisting of Qtrt1 and Qtrtd1 (21). The absence of Qtrt1 has been shown to abrogate the presence of queuosine in tRNA in *S. pombe* (34). Therefore, to further test whether the incorporation of queuine into tRNA was a prerequisite for *in vivo* stimulation of C38 methylation by Pmt1, the $tRNA^{Asp}$ of *qtrt1Δ*, *qtrtd1Δ* and a *qtrt1Δ qtrtd1Δ* double mutant were analyzed by high-throughput RNA bisulfite sequencing. While the C38 methylation level in wt cells grown with queuine was 96%, the level in the absence of queuine amounted to 18% using this method (Figure 2C), a level slightly higher than that found by conventional bisulfite sequencing (Figure 1B). Importantly, the mutants displayed methylation levels at $tRNA^{Asp}$ C38 ranging from 11 and 12% in *qtrt1Δ* and *qtrtd1Δ* to 17% in the double mutant (Figure 2C), which was similar to the level in wt cells grown without queuine. Thus, the inability to incorporate queuine into tRNA reduced C38 methylation to levels found in the absence of queuine, indicating that the presence of queuine alone was insufficient to stimulate Pmt1 and that queuine incorporation into the tRNA was likely required to stimulate Pmt1 activity *in vivo*.

To further test the generality of this observation, two independent *D. discoideum qtrt1*- mutants were examined in *in vitro* remethylation assays for an effect in DnmA-dependent methylation. Importantly, the absence of Qtrt1 decreased queuine-dependent *in vivo* methylation, as indicated by high levels of *in vitro* remethylation, suggesting that *in vivo* methylation was low in the mutants and that the added queuine did not stimulate *in vivo* methylation when cells lacked eTGT (Figure 2D). As observed above, the rich medium (HL5) contains peptone and therefore provides the cells with low levels of queuine, which causes some *in vivo* methylation in wt cells, but this was further reduced in the *qtrt1* mutants, supporting the interpretation that queuine incorporation into RNA stimulated DnmA activity *in vivo*. In summary, these results showed that the mere presence of queuine in the medium was not sufficient to stimulate Dnmt2 methylation activity *in vivo* and suggested that it had to be incorporated into the RNA substrate of Pmt1 and DnmA in order to increase their activity *in vivo*.

Pmt1 was selective for $tRNA^{Asp}$ methylation

In addition to $tRNA^{Asp}$, Dnmt2 homologs also methylate other tRNA targets at C38, namely (depending on the organism) the tRNAs for glutamate, glycine and valine (10,11,13). In *S. pombe*, $tRNA^{Glu}$ also is methylated by Pmt1, but only to a level of 25% upon *pmt1*$^{+}$ overexpression and is not affected by the presence of peptone (14). *S. pombe* $tRNA^{His}$ (which is potentially Q34) and $tRNA^{Val}$ also carry a C38, but their *in vivo* methylation is not known, because they so far have been recalcitrant to conventional RNA bisulfite sequencing (14). Also, some *S. pombe* isoforms of $tRNA^{Leu}$ and $tRNA^{Arg}$ have a C38.

We therefore asked whether queuine affected additional *in vivo* tRNA targets of Pmt1. For this purpose, we determined the complete tRNA methylome of *S. pombe* by combined RNA bisulfite treatment and next-generation sequencing of tRNAs. The retrieved reads were mapped to the known *S. pombe* tRNAs (31) and analyzed for cytosine methylation. For $tRNA^{Asp}$, this method showed a level of 14% C38 methylation in wt cells grown in queuine-free medium, whereas this was increased to 97% by addition of queuine, and *pmt1Δ* cells showed a background level of 2% C38 methylation independently of queuine (Figure 3A, Supplementary Figure S2, Supplementary Table S1). However, investigation of C38-containing tRNAs for histidine, glutamate and valine showed no methylation at this position (Figure 3A), indicating that these residues were not substrates for Pmt1. This was surprising for $tRNA^{His}$, because

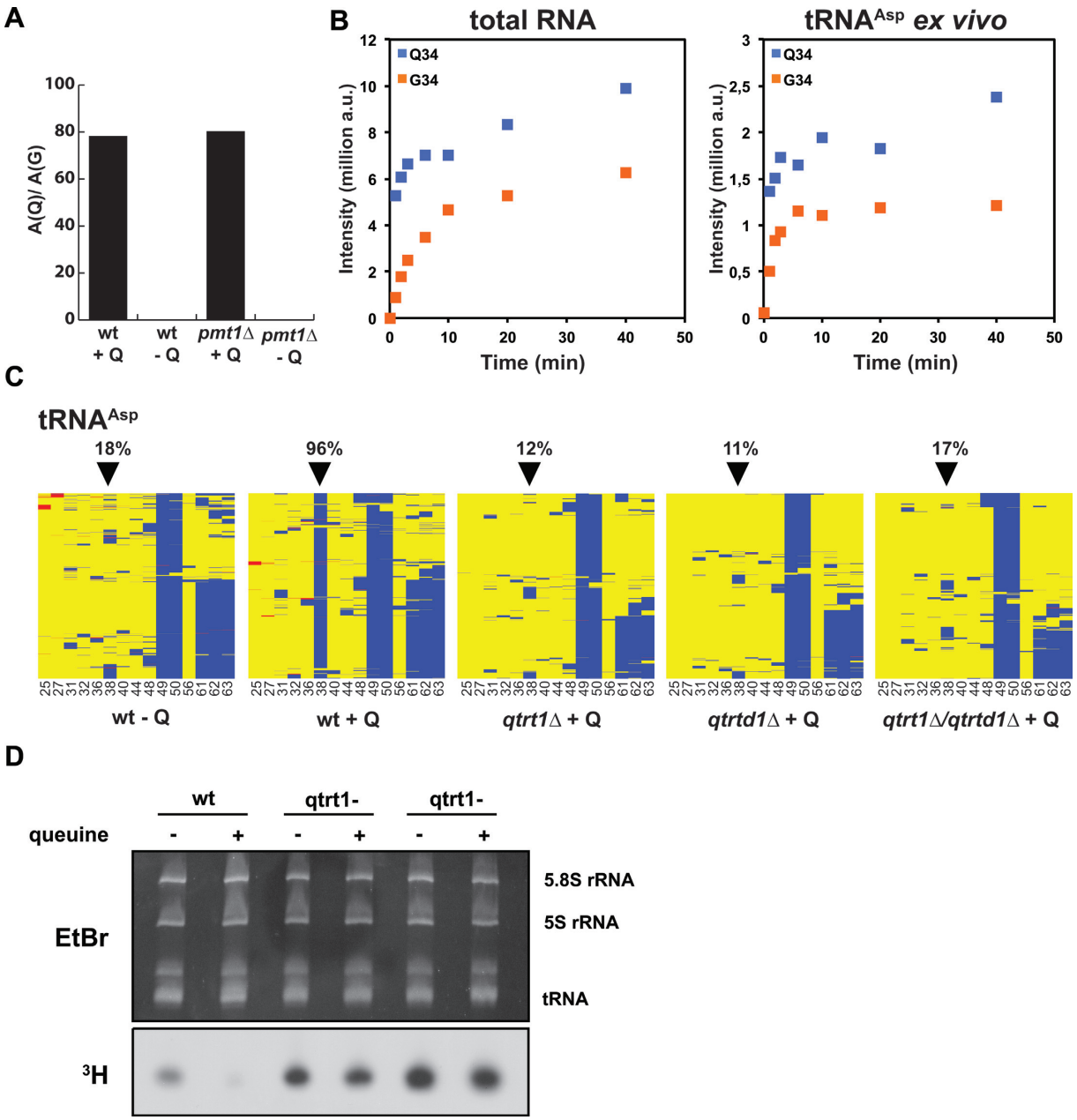

Figure 2. Queuine incorporation into tRNA *in vivo* stimulated Dnmt2 enzymes *in vivo* and *in vitro*. (**A**) Quantitation of queuine levels in total RNA from *Schizosaccharomyces pombe* (wt and *pmt1Δ*) grown with or without queuine, as determined by LC-MS/MS. Extracted peak areas for queuine were normalized to the UV peak area of guanosine. (**B**) Pmt1 activity *in vitro* was stimulated on tRNA containing queuine (Q34) as compared to Q-free tRNA (G34). A time course of methylation by Pmt1 (1 μM) is shown. The left panel shows the time course of incorporation of ^{3}H-labeled methyl groups into total RNA isolated from *pmt1Δ* cells grown with (Q34) or without queuine (G34). Methylation was quantitated by densitometric analysis of autoradiograms. Right panel, *in vitro* methylation of tRNA^{Asp} isolated *ex vivo* from *pmt1Δ* with or without queuine. Representation and data analysis as in the left panel. Time courses of methylation were reproduced with an independent batch with comparable results. (**C**) *In vivo* stimulation of Pmt1 required incorporation of queuine into tRNA by the eukaryotic tRNA-guanine transglycosylase (eTGT). High-throughput bisulfite sequencing of tRNA^{Asp} from strains that were wild-type or lacked Qtrt1, Qtrtd1 or both grown in full medium (YES) with or without queuine as indicated was performed. Results from 1024 independent sequences are shown. Yellow, unmethylated cytosine; blue, methylated cytosine; red, mismatch. Arrows indicate the C38 position. Methylation levels are given in %. (**D**) DnmA activity *in vivo* on RNA required the presence of eTGT in *Dictyostelium discoideum*. Total RNA from the wild-type and two independent *qtrt1-* mutant strains grown with or without queuine was methylated *in vitro* by recombinant DnmA. Presentation as in Figure 1A.

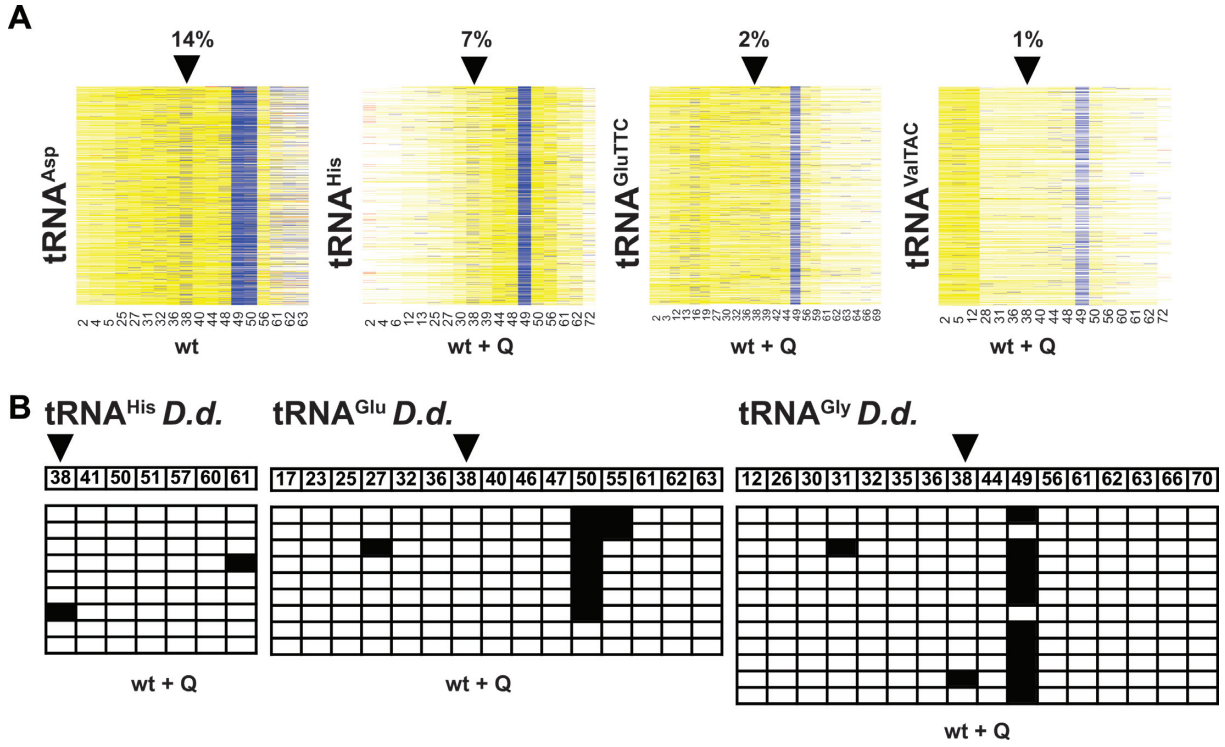

Figure 3. *In vivo* methylation of C38-containing tRNAs other than tRNA^{Asp} was not stimulated by queuine. (**A**) Cytosine methylation levels of tRNA^{Asp}, tRNA^{His}, tRNA^{Glu} (TTC) and tRNA^{Val} (TAC) in *Schizosaccharomyces pombe* as determined by whole tRNA bisulfite sequencing are shown. Wild-type cells were grown with or without queuine as indicated. Yellow, unmethylated cytosine; blue, methylated cytosine; red, mismatch. The arrow indicates the C38 position, values for methylation levels are indicated above. For tRNA^{His}, tRNA^{Glu} and tRNA^{Val}, this corresponds to background values that were also obtained in the absence of queuine or Pmt1 (see Supplementary Table S1). (**B**) Methylation of mitochondrial tRNA^{His}, tRNA^{Glu} and tRNA^{Gly} was not stimulated by growth in queuine in *Dictyostelium discoideum*. Analysis as in Figure 1C.

it contains Q34 and C38. The other tRNAs have no Q34, and this finding therefore corroborated the notion that the presence of queuosine in the tRNA, rather than queuine in the medium, is necessary in order to stimulate Pmt1 activity. A similar result was observed in *D. discoideum*, where neither tRNA^{His} nor tRNA^{Glu} and tRNA^{Gly} showed DnmA-dependent C38 methylation as determined by conventional bisulfite sequencing (Figure 3B, Supplementary Figure S3).

We subsequently expanded the analysis to the cytosine residues in all *S. pombe* tRNAs. Strikingly, while known as well as new methylation sites were detected (Figures 3A and 4A, see below), the only site that was strongly dependent on both Pmt1 and queuine was C38 of tRNA^{Asp} (Figure 4A, Supplementary Table S1). One additional site, C61 of tRNA^{Arg} (TCG), appeared to have a weak decrease of methylation in the absence of Pmt1, but the number of reads for this site was extremely low (Supplementary Table S1), such that we do not interpret this as a true Pmt1 dependency. Thus, this analysis revealed a remarkable level of selectivity of Pmt1 for C38 of tRNA^{Asp} and the requirement for queuine incorporation for Pmt1 activity. Pmt1 therefore is more selective than its homologs in mouse and flies.

Notably, methylation at C11 of tRNA^{Ser} (GCT) showed an inverse correlation to the presence of Pmt1 and queuine in that its level increased from 23 to 37% in the absence of Pmt1 (Figure 4B, statistical significance at 95% confidence interval, Supplementary Figure S4). Perhaps the loss of tRNA^{Asp} C38 methylation causes a compensatory methylation of tRNA^{Ser} by another methyltransferase (36), the significance of which remains to be determined. Also, there appeared to be an increase in methylation of C62 of tRNA^{Arg}(TCG), but, as described above for C61 of the same tRNA, coverage was very low, such that we do not interpret this as a true increase in methylation.

Detection of novel candidate methylation sites in tRNAs of *S. pombe*: C61–C63 of tRNA^{Gly} and C34 of tRNA^{Pro}

Our determination of genome-wide tRNA methylation levels by bisulfite sequencing allowed us to ask whether methylation sites were present in *S. pombe* that so far have not been reported. Indeed, we identified additional Pmt1- and Q-independent candidate methylation sites in tRNA^{Gly} (GCC) at C61–C63 (19, 27 and 27%, respectively). We furthermore found evidence for strong C34 methylation in tRNA^{Pro} (CGG) (95%; independent of Pmt1 and Q), which, according to relevant databases (37,38), would represent a novel modification site. Methylation of C34 so far has been thought to be specific for tRNA^{Leu} (39). This residue lies at the wobble position of the anticodon, and its methylation has been shown to affect the efficiency of translation of codon-biased tRNAs in response to oxidative stress (3,5). Our identification of C34 methylation in tRNA^{Pro} suggests that this tRNA may likewise contribute to translational stress response.

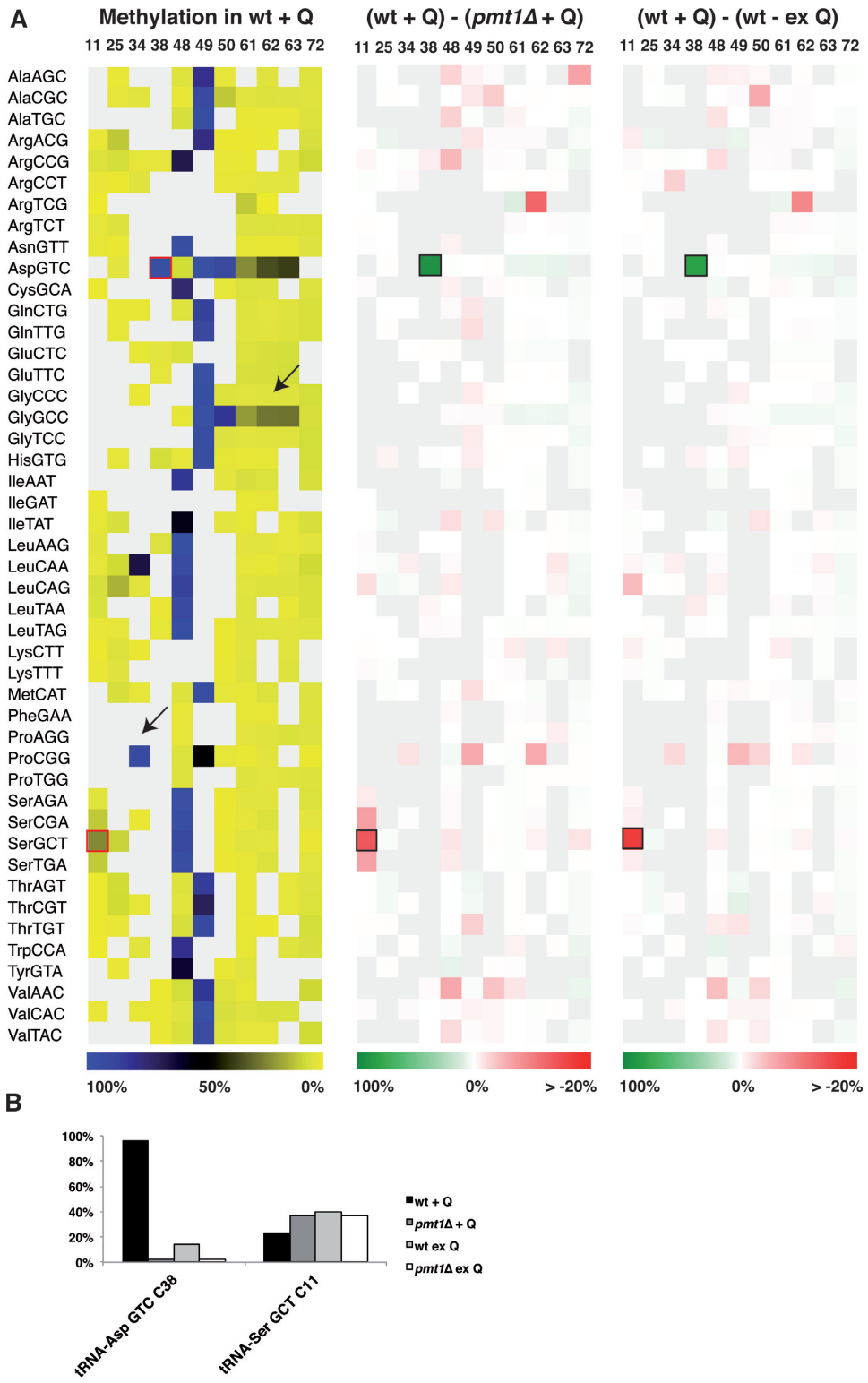

Figure 4. Genome-wide high-throughput tRNA bisulfite sequencing in *Schizosaccharomyces pombe* reveals novel candidate tRNA methylation sites and strong selectivity of Pmt1 for $tRNA^{Asp}$. (**A**) Left, cytosine methylation levels are shown for individual tRNA species in wt cells grown with queuine. The top row indicates the position of the cytosine using standard tRNA numbering. Blue, strong methylation; yellow, no methylation. C38 of $tRNA^{Asp}$ and C11 of $tRNA^{Ser}$ (GCT) are boxed in red/ black for clarity. Novel candidate methylation sites (C61–C63 of $tRNA^{Gly}$ and C34 of $tRNA^{Pro}$) are highlighted with an arrow. Middle, difference in tRNA methylation between wt and *pmt1Δ* cells grown in the presence of queuine. Green colors indicate a strong loss of methylation in *pmt1Δ* compared to wt. Red colors indicate an increase in methylation in *pmt1Δ* compared to wt. Right, difference in tRNA methylation between wt cells cultivated with and without queuine (ex Q, cells were grown in YES). Data is represented as in the middle panel. (**B**) Methylation levels of the indicated tRNAs in wt and *pmt1Δ* cells grown with or without queuine. $tRNA^{Ser}$ (GCT) methylation was mildly increased in the absence of Pmt1 and queuine.

Investigation of the overall tRNA methylation pattern furthermore revealed that C11 and C25 appeared slightly methylated (11–23%) in five tRNAs ($tRNA^{Ser}$ (CGA), $tRNA^{Ser}$ (GCT), $tRNA^{Ser}$ (TGA), $tRNA^{Arg}$ (ACG) and $tRNA^{Leu}$ (CAG)). Also, position C48, C49 or C50 were methylated in the majority of tRNAs in *S. pombe* (46 methylated, 10 unmethylated). In $tRNA^{Asp}$, we previously showed C49 and C50 methylation (in addition to C61–C63) to depend on the two Trm4 homologs (14). It will be important to validate the newly detected sites with independent approaches and to determine which of these sites depend on the Trm4 homologs.

DISCUSSION

External stimuli such as oxidative stress can lead to alterations in cellular tRNA modifications, which subsequently cause changes in protein translation that modulate the cellular response to the stimulus (39). Here, we have identified a novel pathway by which the environment can regulate cellular tRNA modifications. We found that the micronutrient queuine, which is incorporated into tRNAs at the wobble position of the anticodon, may directly regulate the enzymatic activity of the tRNA methyltransferase Dnmt2 in *S. pombe* and *D. discoideum*. Since queuine in higher eukaryotes is scavenged from the diet as well as from gut microbes, this provides an unanticipated mechanism for the microbiome and the environment to exert a direct effect on protein translation in its host by regulating a tRNA-modifying enzyme. This is one of only few examples of a sequential order of two separate tRNA modifications (40–43). Since our analysis only included tRNA methylation, but not other modifications, it is possible that Q34 and C38 methylation, regulate one or several of the over seventy other tRNA modifications, such that a cascade of tRNA modifications is initiated by queuosine and Dnmt2 that co-ordinately regulates tRNA function.

How do Q34 and C38 methylation of tRNA affect translation? The most obvious explanation is an effect of queuosine on the codon-anticodon decoding due to its presence in the anticodon (19). For $tRNA^{Asp}$ (GUC), a modeling study suggested that the unmodified tRNA strongly preferred the cognate GAC codon for Asp over GAU, whereas a Q34-containing tRNA showed no bias for either codon (44), which is notable in light of the fact that *S. pombe* and *D. discoideum* contain only tRNAs with the GUC anticodon, but an average ratio of GAC to GAU of 0.4 and 0.05, respectively, in the genome (31,45,46). Thus, queuosine modification of $tRNA^{Asp}$ may serve to enhance decoding of the non-cognate GAU Asp codon. For Dnmt2-dependent tRNA methylation, some of its biological effects may be attributed to its prevention of stress-induced tRNA cleavage (10,15). However, Dnmt2 recently has also been shown to directly affect translation (47) in that it suppresses mistranslation and thus, like queuosine, contributes to the discrimination of near-cognate tRNAs (48). We thus speculate that the combined actions of queuosine modification in the anticodon and C38 methylation of tRNA in the anticodon loop influence mRNA translation by globally affecting decoding and mistranslation. While the impact of either modification at a single codon or mRNA may be modest, the cumulative effect of both modifications across the entire proteome likely causes more pronounced changes. On an evolutionary scale, such effects on the speed and decoding of codons can result in a shift in codon preference within a genome, as has been demonstrated for queuosine modification in the drosophilid lineage (23).

Another possibility for the biological effect of combined queuosine and Dnmt2 tRNA modification is enhanced protection of the anticodon loop from endonucleolytic cleavage. C38 m^5C has been shown to inhibit cleavage in the tRNA loop by RNA endonucleases (10). This likewise may be the case for Q34 modification (49) and Q34 in $tRNA^{Asp}$ in *Dictyostelium* has been reported to increase its stability (50). In this way, both modifications may co-ordinately regulate cellular tRNA decay and thus availability for translation, much like what has been described for other tRNA methylation sites in *S. cerevisiae* (termed the rapid tRNA degradation pathway) (51).

Interestingly, we observed an unexpected selectivity of Pmt1 and queuosine in *S. pombe* for methylation of $tRNA^{Asp}$, but no other tRNA target, which distinguishes Pmt1 from its homologs in other organisms that also methylate non-Q-tRNAs. Thus, it will be interesting to see whether Dnmt2 homologs apart from DnmA also are susceptible to queuine incorporation. In such organisms, the Q34 sensitivity of Dnmt2 potentially affords a mechanism for the environment to provide an additional layer of translational control over aspartate codons as opposed other codons. In addition, since queuosine has also been identified in non-tRNA species (19), it will be of interest to see whether queuosine and Dnmt2-dependent methylation are present in any non-tRNA targets.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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