Archaeal NSUN6 catalyzes m₅C72 modification on a wide-range of specific tRNAs

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ABSTRACT

Human NOL1/NOP2/Sun RNA methyltransferase family member 6 (hNSun6) generates 5-methylcytosine (m₅C) at C72 of four specific tRNAs, and its homologs are present only in higher eukaryotes and hyperthermophilic archaea. Archaeal NSun6 homologs possess conserved catalytic residues, but have distinct differences in their RNA recognition motifs from eukaryotic NSun6s. Until now, the biochemical properties and functions of archaeal NSun6 homologs were unknown. In archaeon Pyrococcus horikoshii OT3, the gene encoding the NSun6 homolog is PH1991. We demonstrated that the PH1991 protein could catalyze m₅C72 formation on some specific PhtRNAs in vitro and was thus named as PhNSun6. Remarkably, PhNSun6 has a much wider range of tRNA substrates than hNSun6, which was attributed to its tRNA substrate specificity. The mechanism was further elucidated using biochemical and crystallographic experiments. Structurally, the binding pocket for nucleotide 73 in PhNSun6 is specific to accommodate U73 or G73-containing PhtRNAs. Furthermore, PhNSun6 lacks the eukaryotic NSun6-specific Lys-rich loop, resulting in the non-recognition of D-stem region by PhNSun6. Functionally, the m₅C72 modification could slightly promote the thermal stability of PhtRNAs, but did not affect the amino acid accepting activity of PhtRNAs.

INTRODUCTION

As linker molecules between ribosomes and growing polypeptide chains, transfer RNAs (tRNAs) play a central role in protein synthesis. Recently, further functions of tRNAs have been identified in addition to translation (1–3). Multiple modifications are a characteristic property of tRNAs, and these modifications are crucial for tRNA stability, decoding accuracy, and cellular functions (4–8).

In contrast to tRNA modifications in prokaryotes and eukaryotes, study of archaeal tRNA modifications lags behind in aspects of genetic characterization and in vivo functions; however, archaeal tRNA modifications are indispensable (9,10). Archaeosine is exclusively found in most of known archaeal tRNAs at position G15 in the D-loop, ensuring the tRNA tertiary structure by tightening the tertiary base pair, G15:C48 (11,12). An agmatidine modification at the first anticodon position of C34 in archaeal tRNAIle(CAU) is essential for precise decoding (13,14). Archaea-specific isowyosine and 7-methylwyosine, which are guanosine-37 derivatives, demonstrate the complexity in archaeal wyosine derivatives synthesis (15–20).

5-methylcytosine (m₅C) is a ubiquitous modification in DNA and different cellular RNAs, which is formed by site-specific methyltransferases (MTases). The NSun family, which contains an S-adenosyl-methionine (SAM)-dependent NOL1/NOP2/Sun (NSun) domain, is the main RNA:m₅C MTase and comprises seven family members in eukaryotes. Among them, NSun2, NSun3 and NSun6 are primarily responsible for m₅C modifications on tRNAs (21–23). Genetic mutations and aberrant expression of several NSun family members are linked to human diseases and disorders, including intellectual disability (24,25), mitochondrial deficiency (26), Williams–Beuren syndrome (27), male infertility (28), and cancer development (29,30). In archaebacteria, m₅C modifications are widespread in cellular RNAs, especially in tRNAs. Positions C34, C38, C48, C49 and C50 of tRNAs are the known m₅C modification sites in archaea (31,32).

m₅C72 is a newly identified modification in the acceptor stem region of human cytoplasmic tRNAThr and tRNACys, established by human NSun6 (hNSun6) (23). Our previous study showed that wellfolded and sequence-specific tRNA substrates are strictly selected by hNSun6 (33). The struc-
ture of hNSun6 in complex with tRNA revealed that the MTase domain works together with pseudouridine synthase and archaeosine transglycosylase (PUA) domain to recognize regions in the acceptor and D-stem of tRNAs (34). Homologs of hNSun6 are present only in higher eukaryotes and hyperthermophilic archaea. In contrast to the well-studied identified genes that encode eukaryotic NSun6s, the corresponding genes in archaea are unknown.

In the genome of Pyrococcus horikoshii OT3 (P. horikoshii), five genes encode putative RNA m⁵C MTases: PH1337, PH1374, PH1078, PH1991, and PH0851. Among them, PH1991 shares the highest sequence similarity (23.9% identity) with hNSun6, suggesting that PH1991 is the hNSun6 homolog. Interestingly, compared with hNSun6, the amino acid sequence of PH1991 is shorter and the predicted RNA recognition residues, especially the predicted PUA domain (residues 86–164) and RNA recognition motifs (RRMs, residues 34–79 and 173–185), are extremely divergent from eukaryotic NSun6s (Supplementary Figure S1). Moreover, PH1991 lacks the eukaryotic NSun6-specific conserved Lys-rich loop in the PUA domain, which is essential for D-stem recognition by eukaryotic NSun6s. Until now, the properties and functions of PH1991 were uncharacterized. Thus, the present study aimed to determine whether PH1991 retains the m⁵C MTase catalytic activity; what are the RNA substrates of PH1991, considering the differences in the predicted RNA binding residues; and what is the biological function of PH1991.

Moreover, NSun6 homologs are conserved in other hyperthermophilic archaea, such as Pyrococcus kูลukañaï NCB100 (35), Pyrococcus abyssi GE5 (http://www.genoscope.cns.fr/Pab/), Thermococcus litoralis NS-C (36) and Thermococcus nautili 30-1 (37). These archaeal NSun6 homologs all share identity with PH1991 (Supplementary Figure S1). However, the biochemical properties and functions of PH1991 analogs are unknown.

In this study, we identified that PH1991 is a tRNA:m⁵C72 MTase NSun6 (PhNSun6). In contrast to hNSun6, which could only methylate four tRNAs with U73, PhNSun6 could catalyze m⁵C72 formation on eleven PhtRNAs harboring either U73 or G73. We further characterized the tRNA recognition elements for PhNSun6, which were quite different from those of hNSun6. To better understand the recognition mechanism of PhNSun6, we solved the crystal structures of PhNSun6 in the apo form and in complex with SAM, sinfungin (an analog of SAM, SFG), and S-adenosyl-homocysteine (demethylated SAM, SAH). Furthermore, we found that m⁵C72-modified PhtRNAs show slightly higher thermal stability than their unmodified counterparts, but this does not affect the amino acid accepting activity of PhtRNAs. Combined with previous work, we proposed an evolutionary model for eukaryotic and archaeal NSun6 and its tRNA recognition mechanism.

**MATERIALS AND METHODS**

**Materials**

The m⁵C standard, dithiothreitol (DTT), guanosine monophosphate (GMP), sodium acetate (NaAc), Tris-base, SAH, benzonase, pyrophosphate, and phosphodiesterase I from Crotalus adamanteus venom were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). Bacterial alkaline phosphatase was obtained from Invitrogen (Shanghai, China). Tris–HCl, tryptone, yeast extract, isopropyl-D-thiogalactoside (IPTG), bovine serum albumin (BSA), MgCl₂, NaCl, sodium phosphate monobasic, sodium phosphate dibasic, Ethylene diamine tetraacetic acid (EDTA), ATP, CTP, GTP, and UTP were purchased from Sangon Biotech (Shanghai, China). DNA fragment rapid purification and plasmid extraction kits were purchased from Yuanpinghao Biotech (Tianjin, China). KOD-plus mutagenesis kit and KOD-plus-neo Kit were from TOYOBO (Osaka, Japan). Crystallization kits were from Hampton research (Aliso Viejo, CA, USA). T4 DNA ligase, the ribonuclease inhibitor, and all restriction endonucleases were obtained from Thermo Scientific (Waltham, MA, USA). [Methyl-³H] SAM (78.0 Ci/mmol) and [³H] L-serine were purchased from Perkin Elmer Inc. (Waltham, MA, USA). [¹⁴C] L-threonine was obtained from the Institut de Biologie Moléculaire et Cellulaire du CNRS (Strasbourg, France). The pET28a vector was from Merck–Millipore (Darmstadt, Germany). Escherichia coli Rosetta (DE3) cells were purchased from TIANGEN (Beijing, China). Nickel-nitrilotriacetate (Ni-NTA) Superflow resin was purchased from Qiagen, Inc. (Hilden, Germany). The Superdex™ 200 column (10/300 GL; column volume, 23.562 ml) was purchased from GE Healthcare (Fairfield, CT, USA). T7 RNA polymerase was purified from an overproduction strain in our laboratory (38).

**Preparation of tRNA**

The PhtRNAs genes were inserted into pTrc99b to construct pTrc99b-T7-PhtRNAs. Mutants of the PhtRNAThr(CGU) were chemically synthesized by BioSune and inserted into the pET28a vector using NdeI and NotI. Escherichia coli Rosetta (DE3) was transformed with the pET28a-PhNSun6 plasmid. Protein overproduction was induced by adding 0.2 mM IPTG into cells. After culturing for 12 h at 18°C, the cells were collected by centrifugation. PhNSun6 was purified by affinity
Crystallization was performed at 16°C by the hanging drop vapor diffusion method. For crystallization, PhNSun6 was concentrated to ~13.3 mg/ml. Protein solution (1 μl) was mixed with an equal volume of the reservoir solution, consisting of 0.2 M magnesium acetate tetrahydrate, 0.1 M sodium cacodylate trihydrate pH 6.5 and 20% (v/v) polyethylene glycol 8000. PhNSun6 and 1 mM SAM, or SFG, or SAH, were co-crystallized under the same conditions. The crystals were frozen in liquid nitrogen after transferring for a few seconds in the mother liquor, which contained 15% (v/v) glycerol as a cryoprotectant.

All crystal diffraction data sets were collected at the Shanghai Synchrotron Radiation Facility beamlines (SSRF, Shanghai, China) BL-19U1. The diffraction data were processed using the HKL3000 program package (43). Further data analysis was performed with the CCP4 suite (44). The structure of PhNSun6 in the apo form was initially solved by molecular replacement using PHASER (45) with hNSun6 (PDB ID: 5WWQ) structure as starting model. The model was further improved by manual adjustments using COOT (46). The structures of PhNSun6-SAM, PhNSun6-SFG, and PhNSun6-SAH were solved by molecular replacement using PHASER. All models were refined using REFINER program in the PHENIX suite (47). The quality of the final model was evaluated using MOLPROBITY (http://molprobity.biochem.duke.edu/). Figures were drawn using PyMOL (http://www.pymol.org/). A structure-based multiple amino acid sequence alignment of NSun6s from model organisms was generated using ESPript (48).

**Protein crystallization, structure determination, and refinement**

To measure the methyl transfer activity of PhNSun6 and its mutants, 5 μM of tRNAs were used as substrates. The reactions were performed at 65°C under the same conditions in a 25-μl reaction mixture containing 200 μM [3H]-SAM, 50 mM Tris–HCl (pH 7.5), 200 mM NaCl, 10 mM MgCl₂, 100 μg/ml BSA, and 5 mM DTT. Reactions were initiated by the addition of 100 nM PhNSun6. Aliquots (5 μl) of the reaction mixtures were removed at time intervals between 2 and 8 min, quenched on Whatman glass-fiber filter disks, and soaked in 5% trichloroacetic acid (TCA). Washing, the amount of radioactive [3H]-methyl-tRNA on each disc was measured in a Beckman Las6500 scintillation counting apparatus. The steady-state kinetics were measured under the same conditions with 50–300 nM PhNSun6 and 0.1–40 μM tRNA; the reaction time was 2 or 4 min. For mutants that exhibited extremely low activity, higher concentrations of enzyme and tRNA substrates were used. The data were fitted to Lineweaver-Burk plots, and the Kₘ, kₗ, and kₖcat/Kₗ values for each tRNA were calculated from the curve fitting.

To obtain m⁵C72_PhtRNAs, the reactions were performed at 65°C in a mixture comprising 2 μM PhNSun6, 200 μM SAM, 50 mM Tris–HCl (pH 7.5), 200 mM NaCl, 10 mM MgCl₂, 100 μg/ml BSA, and PhtRNAs. The wild-type (WT) PhtRNAs were prepared in the same mixture by adding the PhNSun6 restored buffer. The reaction was performed for 1 h and stopped using phenol/chloroform extraction. The PhtRNAs were precipitated using a 3-fold volume of ethanol.

**Aminoacylation assay**

The time course curve for aminoacylation by SerRS for PhtRNA⁶⁰⁰ was determined in a 25-μl mixture containing 60 mM Tris–HCl (pH 7.5), 10 mM MgCl₂, 5 mM DTT, 2.5 mM ATP, 100 μg/ml BSA, 40 μM [³H]serine, and 200 mM enzyme, with 5 μM of PhtRNA⁶⁰⁰ at 60°C. Aliquots (5 μl) of the reaction mixtures were removed at time intervals between 1 and 4 min, quenched on Whatman glass-fiber filter disks, and soaked in 5% TCA. The time course curve for aminoacylation by ThrRS for PhtRNA³⁸⁸ was determined at 37°C, as described previously (41).

**Mass spectrometry analysis of m⁵C modification**

One microgram of tRNA was hydrolyzed using benzonase, phosphodiesterase I, and bacterial alkaline phosphatase overnight at 37°C in a 20-μl solution including 20 mM Tris– HCl (pH 8.0), 2 mM MgCl₂, and 20 mM NaCl. The solution was then diluted with H₂O 100 times and 10 μl was applied to Ultra-Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS). The nucleosides were separated using UPLC on a C18 column (Agilent Zorbax Eclipse Plus C18, 2.1 × 50 mm, 1.8-Micron) and then detected using a triple-quadrupole mass spectrometer (Agilent 6400 QQQ) in the positive ion multiple reaction-monitoring (MRM) mode. Mass transition from m/z 258 to 126 (m⁵C) was monitored and recorded.

**tRNA methyl transfer assay**

To measure the methyl transfer activity of PhNSun6 and its mutants, 5 μM of tRNAs were used as substrates. The reactions were performed at 65°C under the same conditions in a 25-μl reaction mixture containing 200 μM [³H]-SAM,
melting temperature curve was measured at 260 nm using a heating rate of 1°C/min from 25 to 95°C via an Agilent Cary 100 spectrophotometer. The $T_m$ value was determined from the first derivative of the melting curve.

**RESULTS**

**PH1991 is the *P. horikoshii* tRNA:m^5C72 MTase NSun6**

PH1991 is the homolog of eukaryotic NSun6 in *P. horikoshii*. To validate whether PH1991 has the tRNA:m^5C72 MTase catalytic activity, we synthesized the gene that encodes PH1991 and prepared possible tRNA substrates: PhtRNA^Thr*(CGU), PhtRNA^Thr*(GGU), PhtRNA^Thr*(UGU) and PhtRNA^Cys*(GCA), corresponding to the tRNA substrates of hNSun6, using T7 transcription.

Subsequently, we purified PH1991 and subjected it to SDS-PAGE analysis (Figure 1A). To assay the MTase activity of PH1991 for PhtRNA^Thr*(CGU), we tested the methyl transfer activity at 37, 55 and 65°C. The highest methyl transfer activity of PH1991 was at 65°C (Supplementary Figure S2). We found that PH1991 was able to catalyze the methylation of three PhtRNA^Thr isoacceptors and PhtRNA^Cys*(GCA) at 65°C, similar to hNSun6 at 37°C (Figure 1B). The PhtRNA^Thr*(CGU) methylated by PH1991 were analyzed using UPLC-MS and the modification was confirmed to be m^5C (Figure 1C), as it was in the methylated PhtRNA^Thr*(GGU), PhtRNA^Thr*(UGU), and PhtRNA^Cys*(GCA) (Supplementary Figure S3). To confirm that C72 was the target site, three mutants, PhtRNA^Thr*(CGU)-C2G:G71C, -C2G:G71U, and -C72U, were constructed. None of these mutants could be methylated by PHNSun6 (Figure 1D). We measured the steady state kinetic parameters of PHNSun6 in the presence of four PhtRNA substrates (Supplementary Table S1). The $K_m$ values for the four PhtRNAs were approximately 0.5 μM; however, the $k_{cat}$ values showed differences. The $k_{cat}$ values of PHNSun6 for three PhtRNA^Thr isoacceptors were approximately 4.30 min⁻¹, which was higher than that for PhtRNA^Cys*(3.04 min⁻¹). Thus, we identified that PH1991 is the *P. horikoshii* tRNA:m^5C72 MTase NSun6 and named it PHNSun6.

**PHNSun6 shows a different recognition mechanism for the acceptor stem of tRNAs compared with that of hNSun6**

Our previous study on hNSun6 showed that the CCA terminus, the target site C72, the discriminator base U73, and base pairs 2:71 and 3:70 in the acceptor stem are the tRNA recognition elements (33). We then determined whether these recognition elements in the acceptor stem were conserved in tRNA recognition by PHNSun6. We assayed the methylation of a PhtRNA^Thr*(CGU) mutant with a deleted CCA terminus and several mutations in U73 and C2:G71 (Figure 2A).

PHNSun6 could not methylate the PhtRNA^Thr*(CGU) mutant lacking the CCA terminus (Figure 2B), implying that the common CCA terminus of tRNA was still essential for the methylation reaction catalyzed by PHNSun6.

To determine whether U73 was necessary for methylation by PHNSun6, we replaced U73 with other three bases and obtained the PhtRNA^Thr*(CGU)-U73A, -U73C and -U73G mutants (Figure 2A). Remarkably, PHNSun6 could methylate PhtRNA^Thr*(CGU)-U73G, but not PhtRNA^Thr*(CGU)-U73A or -U73C (Figure 2C). Steady-state kinetic assays showed that the $K_m$ value of PHNSun6 for PhtRNA^Thr*(CGU)-U73G was 0.59 μM, which was slightly higher than that for PhtRNA^Thr*(CGU) (0.43 μM). Whereas the $k_{cat}$ value of PHNSun6 for PhtRNA^Thr*(CGU)-U73G was 2.11 min⁻¹, which was almost a half that determined for PhtRNA^Thr*(CGU) (4.30 min⁻¹) (Supplementary Table S2). These results showed that the efficiency of PhtRNA^Thr-mediated methylation of C72 of PhtRNA^Thr*(CGU)-U73G is lower than that for PhtRNA^Thr*(CGU). However, unlike hNSun6, which only recognizes U73, PHNSun6 can recognize both U73 and G73.

According to the tRNA database (31,49), the second base pair in the acceptor stem is C2:G71 or G2:C71 in most PhtRNAs, and in few PhtRNAs is G2:U71; the third base pair exists as C3:G70, G3:C70, A3:U70 and G3:U70. In the four above-mentioned PhtRNAs, the second and third base pairs are C2:G71 and C3:G70, respectively. To determine whether PHNSun6 recognized the second base pair, two mutants, PhtRNA^Thr*(CGU)-C2G:G71C and -C2G:G71U, were constructed and their methylation by PHNSun6 was assessed (Figure 2A). The results showed that PHNSun6 could not methylate these two mutants (Figure 2D; Supplementary Table S2), indicating clearly that C2:G71 is a determinant base pair of tRNA recognition by PHNSun6.

Similarly, we performed C3G:G70C, C3A:G70U and C3G:G70U base pair replacement in PhtRNA^Thr*(CGU). Unexpectedly, PHNSun6 could methylate all these mutants (Supplementary Figure S4 and Table S2), implying that the C3:G70 base pair is not a discriminator for tRNA methylation by PHNSun6.

Collectively, we identified four elements in the acceptor stems of tRNAs that are essential for substrate recognition by PHNSun6: The target site C72, the CCA terminus, U or G73, and the second base pair C2:G71.

**The D-stem is not recognized by PHNSun6**

Besides elements in the acceptor stem, hNSun6 also interacts with the D-stem of tRNAs (33,34). Base pairs 11:24 and 12:23 of the D-stem are recognized via the eukaryotic NSun6-specific Lys-rich loop (residues 157-174 KCKKGAK163 in hNSun6, Supplementary Figure S1). However, archaean NSun6 homologs lack the corresponding eukaryotic NSun6-specific Lys-rich loop, indicating that archaean NSun6 homologs presumably possess a distinct recognition mechanism in D-stem of tRNA substrates. Therefore, we constructed a series of PhtRNA^Thr*(CGU) mutants by replacing C11:G24 or C12:G23 with A:U, U:A, G:C, G:U and U:G, respectively (Figure 3A). Interestingly, these mutants could all be methylated by PHNSun6 (Figure 3B, C), and the $K_m$ and $k_{cat}$ values of PHNSun6 for these mutants were similar to that for PhtRNA^Thr*(CGU) (Supplementary Table S2).

These results suggested that C11:G24 and C12:G23 base pairs in the D-stem are not involved in RNA recognition by PHNSun6.
PhNSun6 has a wider specificity of tRNA substrates than hNSun6

Based on the above results, PhNSun6 requires fewer identity elements for tRNA substrates than hNSun6. To examine whether PhNSun6 has other tRNA substrates besides the above four PhtRNAs (three PhtRNAThr isoacceptors and PhtRNAArg(GCA)), we determined the methylation activity of PhNSun6 for all the PhtRNAs that harbor the recognition elements of PhNSun6. These PhtRNAs are PhtRNASer(UGA), -CGA, -GGA and -GCU, PhtRNAAsn(GUu), PhtRNAAsp(GUC) and PhtRNAArg(GCG). In addition, PhtRNAPro(GAA) with A73 and G2:C71, which does not match the recognition requirements for PhNSun6, was chosen as a negative control (Figure 4A). Intriguingly, PhNSun6 could catalyze methylation of the above seven tRNAs but not the negative control PhtRNAPro(GAA) (Figure 4B; Supplementary Table S3).

Thus, the results showed that PhNSun6 could efficiently catalyze m^5C72 formation on eleven PhtRNAs, which suggested that PhNSun6 has a wider range of tRNA substrates than hNSun6.
Crystal structures of PhNSun6

To fully decipher the RNA recognition mechanism of PhNSun6, we obtained the crystal structures of PhNSun6 with and without its cofactors, including apo-PhNSun6, and PhNSun6 complexed with SAM, SFG and SAH, which diffracted to 2.6, 2.5, 2.5 and 2.2 Å, respectively (Table 1). PhNSun6, with 12 α-helices and 19 β-strands, is divided into two distinct domains with different sizes: the MTase domain, including an RRM motif (residues 34–79 and residues 173–185) and a Rossmann-fold catalytic core (residues 185–389), and the PUA domain (residues 86–164). The PUA domain is inserted into the MTase domain via two linkers: Residues 79–86 and residues 164–173 (Figure 5A). A non-conserved extension motif (residues 1–34) was observed at the N-terminus.

Cofactors bind at the Rossmann-fold catalytic core of PhNSun6. When comparing the structures of PhNSun6 in the apo form and in the form bound with cofactors, the overall structures do not show distinct changes, except that helix α9 moves a slightly toward the cofactors (Supplementary Figure S5). Residues Met208, Ala209, Pro279, Phe308, Asp260, Asp233, Arg238, Ala211, Asp277, Lys215, Tyr304, Gly213 and Gly214 form the binding pocket for the cofactor SAH, as shown in Figure 5B. These residues are mostly conserved or semi-conserved in eukaryotic and archaeal NSun6s, except for the archaeal-specific residue Tyr304, which recognizes N6 and N7 of the adenine ring of SAH. In the PhNSun6-SAM and PhNSun6-SFG complexes, similar binding models of the cofactors to that of SAH were observed, but are not discussed here.

Although PhNSun6 contains a different RNA recognition motif compared with that in hNSun6, the main structure of PhNSun6 adopts a similar topology to hNSun6 and the two active Cys residues of PhNSun6 are conserved. The two conserved Cys residues point from opposite sides of the cofactor, which is the same as that in hNSun6 (Figure 5C).

Some structural differences were observed between PhNSun6 and hNSun6. The first difference is that hNSun6 contains two unique insertions (hNSun6 specific insertion 1 and 2) in the surface of MTase catalytic core (Supplementary Figure S6). Second, for the PUA domain, most
Figure 3. D-stem region is not recognized by \textit{Ph}NSun6. (A) The secondary structure of Ph\textsubscript{tRNA}\textsubscript{Thr} (CGU) summarizing the mutations in C11:G24 and C12:G23 base pairs. The capacity of Ph\textsubscript{tRNA}\textsubscript{Thr} (CGU) with various mutations in C11:G24 (B) and C12:G23 (C) to be methylated by Ph\textsubscript{NSun6}.

Table 1. Data collection and refinement statistics

| Measurement                              | Ph\textsubscript{NSun6-Apo} | Ph\textsubscript{NSun6-SAH} | Ph\textsubscript{NSun6-SAM} | Ph\textsubscript{NSun6-SFG} |
|------------------------------------------|------------------------------|-----------------------------|----------------------------|------------------------------|
| Data collection                          | P1                           | P1                          | P1                         | P1                           |
| Space group                              |                              |                             |                            |                              |
| Cell dimensions                          |                              |                             |                            |                              |
| \(a, b, c\) (Å)                          | 52.19, 52.60, 83.968         | 52.98, 52.17, 83.804        | 52.11, 52.98, 83.801       | 52.47, 52.94, 84.18          |
| \(\alpha, \beta, \gamma\) (°)           | 81.78, 81.78, 67.93          | 80.63, 80.58, 67.90         | 80.80, 80.75, 67.84        | 81.49, 81.38, 67.45          |
| Resolution (Å)                           | 50–2.60 (2.64–2.60)          | 50–2.18 (2.22–2.18)         | 50–2.50 (2.54–2.50)        | 50–2.50 (2.54–2.50)          |
| \(R_{sym}\)                              | 11.5 (39.2)                  | 13.3 (42.1)                 | 10.9 (33.4)                | 11.2 (32.7)                  |
| Completeness (%)                         | 91.4 (67.0)                  | 93.4 (73.8)                 | 91.9 (70.7)                | 92.2 (67.1)                  |
| Redundancy                               | 6.7 (5.4)                    | 6.5 (4.6)                   | 6.6 (4.9)                  | 6.0 (4.4)                    |
| Refinement                               |                              |                             |                            |                              |
| Resolution (Å)                           | 48.1–2.60                    | 41.1–2.18                   | 48.8–2.50                 | 41.4–2.50                    |
| No. reflections (work/free)              | 22105/1064                   | 39519/1997                  | 25054/1259                | 25193/1285                   |
| \(R_{work}/R_{free}\)                   | 0.177/0.236                  | 0.189/0.236                 | 0.179/0.236                | 0.182/0.229                  |
| No. atoms                                |                              |                             |                            |                              |
| Protein                                  | 6156 (2 chains)              | 6156 (2 chains)             | 6156 (2 chains)            | 6156 (2 chains)              |
| Ligand                                   | 52 (2xSAH)                   | 54 (2xSAM)                  | 54 (2xSFG)                | 54 (2xSFG)                   |
| \(B\)-factors                            |                              |                             |                            |                              |
| Protein                                  | 37.9                         | 31.6                        | 36.8                      | 36.8                         |
| Ligand                                   | 30.4                         | 39.3                        | 41.1                      |                              |
| Bond lengths (Å)                         | 0.005                        | 0.004                       | 0.005                     | 0.005                        |
| Bond angles (°)                          | 0.889                        | 0.901                       | 0.925                     | 0.925                        |

\*Values in parentheses are for the highest-resolution shell.
Figure 4. *PhNSun6* has wide-range of tRNA substrates. The secondary structures (A) and the methylation capacity by *PhNSun6* (B) of PthtRNA^{Ser}(UGA), -(CGA), -(GGA) and -(GCU), PthtRNA^{Asp}(GUU), PthtRNA^{Asp}(GUC), PthtRNA^{Asp}(GCG) and PthRNA^{Phe}(GAA).

The CCA terminus of tRNA is essential for *PhNSun6* recognition. According to the *PhNSun6*/*tRNA* model, all the three nucleotides of the CCA terminus interact extensively with residues from the PUA domain and linkers (Figure 6B, C). The binding mode between the CCA terminus and *PhNSun6* is very similar to that observed from the hNSun6/*tRNA* complex. However, as mentioned above, the PUA domain, especially those residues on the protein surface, are quite divergent between *PhNSun6* and hNSun6. To test this superposition model, we mutated putative CCA terminus-interacting residues (Asn98, Glu174 and Tyr112) of *PhNSun6* to Ala. All the three mutants of the eukaryotic NSun6-specific Lys-rich loop (Figure 6A), which is consistent with D-stem of tRNA not being recognized by *PhNSun6*.
Figure 5. Overall structure of PhNSun6. (A) Ribbon diagram showing the overall structure of PhNSun6 in Apo form and in complex with cofactors. The N-terminal extension (green, residues 1–34), linkers (cyan, Linker 1: residues 79–86, and Linker 2: residues 164–173), PUA domain (magenta, residues 86–164) and the MTase domain (residues 34–79 and 173–389) including an RRM motif (dark blue, residues 34–79 and residues 173–185) and a catalytic core (gray, residues 185–389) are highlighted. (B) SAH binding details in the PhNSun6-SAH complex. SAH is represented in sticks with green color for carbon atoms, and the hydrogen bonds are indicated as black dotted lines. (C) The position of two conserved Cys residues relative to SAH (green, stick representation).

showed reduced methyltransferase activities compared with WT protein. The $K_m$ values of PhNSun6-N98A and -E174A for PhtRNA^{Thr}(CGU) were $\sim2$-fold higher than that of PhNSun6, while their $k_{cat}$ values remain similar (Figure 6D; Supplementary Table S4); the $K_m$ value of PhNSun6-Y112A for PhtRNA^{Thr}(CGU) was 5.3-fold higher than that of PhNSun6 while its $k_{cat}$ value decreased (Figure 6D; Supplementary Table S4). These results suggest that all the three residues of PhNSun6 contribute to tRNA binding. Thus, we conjectured that the PUA domain has retained the CCA binding capability during evolution.

The hypothetical binding pocket of PhNSun6 for the base at the 73rd nucleotide of the PhtRNA substrate comprises Ser190, Gln107, and Tyr41 (Figure 6E), which are absolutely conserved in archaeal NSun6 homologs. Intriguingly, the archaeal-specific hypothetical binding pocket for N73 seems propitious to accommodate either U or G, mainly thanks to the orientation of helix $\alpha_4$, which enlarges the size of the pocket in PhNSun6. In contrast, the corresponding helix $\alpha_5$ in hNSun6 limits the binding pocket to U73 only (Figure 6F). In the PhNSun6/tRNA model, the O4 of U73 or O6 of G73 could form a hydrogen bond with the main chain amide group of Tyr41. However, when U73/G73 is substituted by C73 or A73, the N4 of C73 or N6 of A73 could not form this hydrogen bond, thus tRNAs with C73 or A73 will be excluded by PhNSun6. The archaeal-specific residues in the hypothetical binding pocket for N73 might explain why PhNSun6 could accommodate U or G, but not C or A, at site 73 of tRNA substrates. To test our hypothesis, we made mutations on Ser190 and Gln107 of PhNSun6. The OH group from the side chain of Ser190 forms a hydrogen bond to the phosphate group of N73. When Ser190 was mutated to Ala, PhNSun6-S190A had little methylation activity compared to PhNSun6 (Figure 6G). On the other hand, in the hypothetical binding model, side chain of Gln107 is close to the uracil ring of U73. To modify the size and the charge of the binding pocket for the base ring of N73, Gln107 was replaced by Trp with large side chain or by Arg with positive charge respectively. Both PhNSun6-Q107W and -Q107R mutants lost methylation activity completely (Figure 6G). These results suggest that residues in the proposed N73 binding pocket are important for methylation of PhNSun6.
The m$^5$C72 modification of tRNA does not affect the aminoacylation activity of aminoacyl-tRNA synthetase (aaRS)

Considering that the m$^5$C72 modification is close to the CCA terminus, we investigated whether the m$^5$C72 modification affected the amino acid accepting activity of tRNA by the corresponding aaRS. We tested seven (PhttRNA$^{\text{Ser}}$(CGA), -(GGA), -(UGA) and -(GCU), PhttRNA$^{\text{Thr}}$(CGU), -(GGU) and -(UGU)) of the eleven PhttRNAs. The aminoacylation activity of aaRS for the m$^5$C72-modified PhttRNAs showed no obvious difference compared that for their unmodified counterparts (Supplementary Figure S7). These results suggest that the m$^5$C72 modification does not affect the amino acid accepting activity of the tested PhttRNAs.

The m$^5$C72 modification slightly increases the thermal stability of PhttRNAs

Archaea usually exist in extreme environments, such as hot springs and salt lakes. *P. horikoshii* is a hyperthermophilic anaerobic archaeon, growing optimally at 98$^\circ$C (50). Critically, according to statistical analyses, archaeal NSun6 homologs only exist in hyperthermophilic archaea.

In hyperthermophilic organisms, tRNAs have to tolerate high temperature, resulting in the development of certain strategies to protect tRNAs from degradation and denaturation, and to increase the stability of tRNA tertiary structure, such as high GC-content and tRNA modifications (16,18,51). Notably, loss of some tRNA modifications in hyperthermophilic tRNAs affects their thermal stability.
For example, in Thermus thermophilus, lack of the m^5G46 modification causes decreased T_m values of class I tRNAs and degradation of tRNA^{Ph} and tRNA^{Ac}, resulting in reduced protein synthesis at high temperatures (32).

To determine whether there is a correlation between the m^C72 modification and the thermal stability of tRNAs, we measured the T_m of the eleven m^C72-modified Ph tRNAs and their unmodified counterparts. The data were shown in Table 2. Except for Ph tRNA^{Glu} (GGC), the T_m values for the other ten Ph tRNAs with the m^C72 modifications all increased compared with those for their unmodified counterparts. The delta T_m values are between ~1 and 2°C in most cases. Only Ph tRNA^{Glu} showed a 3°C difference between methylated and unmethylated tRNAs. The results suggested that m^C72 contributes thermal stability towards tRNA, although the effect from this single modification is small.

**Archaeal or eukaryotic NSun6 distinguish their cognate tRNA substrates**

Based on our work and our previous work on eukaryotic NSun6 (33,34), a remarkable aspect of NSun6 is the difference in substrate specificity between archaeal and eukaryotic homologs (23,33). This raises an interesting question of whether archaeal or eukaryotic NSun6 could discriminate each other’s tRNA substrates.

First, we verified whether the tRNA substrates of hNSun6 could be methylated by PhNSun6. Considering the stability of hctRNAs, we tested the methyl transferase activity of PhNSun6 to hctRNA^{Thr}(AGU), hctRNA^{Thr}(UGU), and hctRNA^{Cys}(GCA) at 65 or 55°C. The results showed that the tRNA substrates of hNSun6 could not be methylated by PhNSun6 at 65°C (Figure 7A) or 55°C (Figure 7B). Considering that C2:G71 is the determinant element for PhNSun6 recognition, we speculated that the tRNA substrates of hNSun6 were not distinguished by PhNSun6 because these native tRNAs harbor a C2:G71 base pair.

We also tested the methylation of the eleven PhNSun6 tRNA substrates by hNSun6. The results showed that none of the eleven tRNA substrates of PhNSun6 could be catalyzed by hNSun6 (Figure 7C). For hNSun6 recognition, tRNAs with U73 are strictly recognized. The seven Ph tRNAs with G73: Ph tRNA^{Ser}(UGA), -(CGA), -(GGA) and -(GCU), Ph tRNA^{Asp}(GUU), Ph tRNA^{Glu}(GCU) and Ph tRNA^{Asp}(GCG) should not be methylated by hNSun6.

### Table 2. Melting temperatures (T_m) of Ph tRNAs or m^C72-modified Ph tRNAs

| tRNAs          | T_m (°C) Ph tRNAs | T_m (°C) m^C72-modified Ph tRNAs | ΔT_m(°C) |
|----------------|-------------------|----------------------------------|----------|
| Ph tRNA^{Thr}(CGU) | 65.3±0.4          | 67.3±0.4                         | 2.0      |
| (GGU)          | 61.3±0.4          | 62.3±0.4                         | 1.0      |
| (UGU)          | 69.3±0.5          | 71.3±0.5                         | 2.0      |
| Ph tRNA^{Cys}(GCA) | 61.2±0.5          | 62.8±0.5                         | 1.6      |
| (CGA)          | 65.8±0.4          | 66.8±0.4                         | 1.0      |
| (GGA)          | 67.5±0.4          | 69.5±0.6                         | 2.0      |
| (GCU)          | 65.2±0.5          | 66.8±0.5                         | 1.6      |
| Ph tRNA^{Asp}(GUU) | 71.6±0.5          | 73.0±0.6                         | 1.4      |
| Ph tRNA^{Glu}(GUC) | 73.2±0.9          | 76.2±0.5                         | 3.0      |
| Ph tRNA^{Glu}(GCG) | 68.0±0.1          | 68.7±0.5                         | 0.7      |

Each of melting temperature was calculated from the average of three independent measurements and standard deviation values were also shown.

**DISCUSSION**

**tRNA recognition mechanism by archaeal NSun6s**

In this study, we identified that PH1991 is the P. horikoshii tRNA: m^C72 MTase PhNSun6, PhNSun6. The tRNA substrates recognized by PhNSun6 were based on (i) the 3’CCA terminus, (ii) the target site C72, (iii) U73 or G73, and (iv) the discriminatory base pair C2:G71 at the acceptor stem. The CCA terminus and the target site C72 are common recognition elements for PhNSun6 and hNSun6. However, they exist in almost all tRNAs, which are not enough to distinguish substrate tRNAs of NSun6. U73/G73 and C2:G71 function as discriminatory elements to identify PhNSun6 substrate tRNAs from nonsubstrate tRNAs.

In the superimposition model of PhNSun6/tRNA, the binding pocket of site 73 is capable of accommodating both U and G, which was in line with our methylation experiment in which all Ph tRNAs with U73 or G73 could be substrates of PhNSun6. In the model, the base moiety of G71 was not in contact with PhNSun6. However, based on our results, C2:G71 functions as a discriminatory base pair that can select substrate tRNAs from other Ph tRNAs. The exact recognition mechanism of PhNSun6 for C2:G71 requires further mechanistic and structural studies.

Based on the PhNSun6/tRNA superimposition model, PhNSun6 does not interact with the D-stem region of the tRNA, because of the lack of the eukaryotic NSun6-specific Lys-rich loop, which is consistent with biochemical data on the effect of the D-stem on activity of PhNSun6. Thus, our biochemical and crystallographic data suggest that PhNSun6 ignores the sequential specificity of the D-stem
of PhtRNA substrates. Considering that archaeal NSun6 homologs share high identity with each other, we speculated that the tRNA substrate specificity of PhNSun6 is conserved in other archaeal NSun6 homologs.

A proposed evolutionary model for NSun6

Based on the results of this study, together with previous findings on hNSun6, we proposed an evolutionary model for NSun6 (Figure 8). Originally, the RRM and PUA domain were recruited and inserted into an ancient mC catalytic core to bind RNA substrates, resulting in the formation of NSun6’s last universal common ancestor (LUCA). Specifically, for the recognition by NSun6’s LUCA of tRNA substrates, the PUA domain recognizes the common CCA terminus; the RRM recognizes site 73; and the m5C catalytic core recognizes C72 and base pairs near site 72. Later in evolution, NSun6 divided into two branches: Archaeal NSun6 and eukaryotic NSun6. The divergence of the RRM and PUA domain during evolution, which increased the precision and accuracy of RNA substrate discrimination, resulted in the formation of NSun6’s last universal common ancestor (LUCA). Specifically, for the recognition by NSun6’s LUCA of tRNA substrates, the PUA domain recognizes the common CCA terminus; the RRM recognizes site 73; and the m5C catalytic core recognizes C72 and base pairs near site 72. Later in evolution, NSun6 divided into two branches: Archaeal NSun6 and eukaryotic NSun6. The divergence of the RRM and PUA domain during evolution, which increased the precision and accuracy of RNA substrate discrimination, resulted in the formation of NSun6’s last universal common ancestor (LUCA).

Biological functions of m5C72 modification

The presence of m5C modifications in RNA has been observed across the three domains of life. However, the tRNA:m5C modification is present only in archaea and eukaryotes (31,32,53). The m5C modification could increase hydrophobicity and reinforce stacking interactions, probably leading to tRNA stabilization (54). In Saccharomyces cerevisiae, the m5C40 modification of tRNA-Phe stabilizes the tRNA anticodon stem (55); lack of m5C48 and m5C49, together with m7G46, cause the rapid decay of certain tRNAs (56).

In archaea, NSun6s are present only in hyperthermophilic species (57). In terms of tRNA canonical functions, m5C72 modification does not affect the amino acid accepting activity of tRNAs, but slightly increases the thermal stability of PhtRNAs. Acceptor stem of tRNA is rarely modified, and m5C72 is one of the few modifications in this region. It remains possible that m5C72 has other biological functions in vivo, which awaits further studies on both archaeal and eukaryotic NSun6s.

DATA AVAILABILITY

Protein Data Bank: atomic coordinates and structure factors for apo PhNSun6 have been deposited with accession code 5ZVD; for the PhNSun6-SAM, PhNSun6-SFG, and PhNSun6-SAH complex under accession codes 5ZVG, 5ZVH and 5ZVE, respectively.
Figure 8. A proposed evolutionary model for NSun6. Originally, ancient m^4C catalytic core recruited and inserted RRM motif and PUA domain, resulting in NSun6’s LUCA. Later in evolution, NSun6 divided into two branches: archaeal NSun6 and eukaryotic NSun6. Due to the divergent RRM motif and PUA domain during evolution, archaeal and eukaryotic NSun6 exhibit different discrimination mechanisms for the cognate tRNA substrates. For RNA substrates recognition, eukaryotic NSun6 recognizes the acceptor stem and D-stem region, while archaeal NSun6 only interacts with the acceptor stem region. The tRNA elements that are recognized by archaeal or eukaryotic NSun6 are summarized here and highlighted in sticks.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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REFERENCES
1. Gustilo, E.M., Vendeix, F.A. and Agris, P.F. (2008) tRNA’s modifications bring order to gene expression. *Curr. Opin. Microbiol.*, 11, 134–140.
2. El Yacoubi, B., Bailly, M. and de Crécy-Lagard, V. (2012) Biosynthesis and function of posttranscriptional modifications of transfer RNAs. *Annu. Rev. Genet.*, 46, 69–95.
3. Schimmel, P. (2017) The emerging complexity of the tRNA world: mammalian tRNAs beyond protein synthesis. *Nat. Rev. Mol. Cell Biol.*, 19, 45–58.
4. Motorin, Y. and Helm, M. (2010) tRNA stabilization by modified nucleotides. *Biochemistry*, 49, 4934–4944.
5. Kirchner, S. and Ignatova, Z. (2015) Emerging roles of tRNA in adaptive translation, signalling dynamics and disease. *Nat. Rev. Genet.*, 16, 98–112.
6. Popis, M.C., Blanco, S. and Frye, M. (2016) Posttranscriptional methylation of transfer and ribosomal RNA in stress response pathways, cell differentiation, and cancer. *Curr. Opin. Oncol.*, 28, 65–71.
7. Bednárová, A., Hanna, M., Durham, I., VanCleave, T., England, A., Chaudhuri, A. and Krishnan, N. (2017) Lost in translation: defects in transfer RNA modifications and neurological disorders. *Front. Mol. Neurosci.*, 10, 135.
8. Orioli, A. (2017) tRNA biology in the omics era: stress signalling dynamics and cancer progression. *Bioessays*, 39, 1600158.
11. Gregson, J.M., Crain, P.F., Edmonds, C.G., Gupta, R., Hashizume, T., RajBhandary, U.L. (2010) Agmatidine, a modified cytidine in the anticondol of archaeal tRNA. *Nucleic Acids Res.*, **38**, 683–693.

14. Mandal, D., Köhrer, C., Su, D., Russell, S.P., Krivos, K., McCloskey, J.A., Graham, D.E., Zhou, S.L., Crain, P.F., Ibba, M., Konisky, J., Söll, D. and Olsen, G.J. (2001) Post-transcriptional modification in archaeal tRNAs: identities and phylogenetic relations of nucleotides from mesophilic and hyperthermophilic *Methanococcales*. *Nucleic Acids Res.*, **29**, 4699–4706.

18. Zhao, S.L., Sitaramiah, D., Noon, K.R., Guymon, R., Crain, P.F., McCloskey, J.A., Thomm, M., Lim, J. and Cavichioli, R. (2003) Influence of temperature on modification of archaeal tRNAs: identities and phylogenetic relations of nucleotides from archaeal tRNA. *Nucleic Acids Res.*, **31**, 32–89.

22. Nakano, S., Suzuki, T., Kawarada, L., Iwata, H., Asano, K. and Suzuki, T. (2016) NSUN3 methylase initiates 5-formylcytidine biogenesis in human mitochondrial tRNA^Met*. *Bioorg. Chem.*, **60**, 546–551.

28. Khosronezhad, N., Colagar, A.H. and Jorsarayi, S.G.A. (2015) T26248G-transversion mutation in exon 7 of the putative methyltransferase Nsun7 gene causes a change in protein folding associated with reduced sperm motility in asthenospermic men. *Reprod. Fertil. Dev.*, **27**, 471–480.

30. Frye, M. and Watt, F.M. (2006) The RNA methyltransferase Msu (NSun2) mediates Myc-induced proliferation and is upregulated in tumors. *Cancer Biol. Ther.*, **16**, 971–981.

32. Edelheit, S., Schwartz, S., Mumbach, M.R., Wurtzel, O. and Sorek, R. (2013) Transcriptome-wide mapping of 5-methylcytidine RNA modifications in bacteria, archaea, and yeast reveals m^5^C within archaebacterial m^5^C methyltransferase NSun6. *Nucleic Acids Res.*, **41**, D303–D307.

34. Liu, R.J., Long, T., Li, J., Zhou, M.Y., Zhou, X.L., Liu, R.J. and Wang, E.D. (2016) Sequence-specific and shape-selective RNA recognition by the human 5-methylcytosine methyltransferase NSun6. *Nucleic Acids Res.*, **45**, 6684–6697.

36. Omer, P.M., Callac, N., Oger-Desfieux, C., Hughes, S., Gillet, B., Jebar, M. and Godfruy, A. (2017) Complete genome sequence of the hyperthermophilic piezophilic archaeon *Pyrococcus kawakamii NCB100* isolated from the Rebecca’s roost hydrothermal vent in the guaymas basin. *Genome Announc.*, **5**, e01667-16.

40. Kibbe, W.A. (2007) OligoCalc: an online oligonucleotide properties calculator. *Nucleic Acids Res.*, **35**, W43–W49.

41. Chen, Y., Ruan, Z.R., Wang, Y., Huang, Q., Xue, M.Q., Zhou, X.L. and Wang, E.D. (2018) A threonyl-tRNA synthetase-like protein has tRNA aminoacylation and editing activities. *Nucleic Acids Res.*, **46**, 3643–3656.

44. Bailey, S. (1994) The CCP4 suite—programs for protein crystallography. *Acta Crystallogr. D.*, **50**, 760–763.

45. McCoy, A.J., Grosse-Kunstleve, R.W., Adams, P.D., Winn, M.D., Stachiw, E.S., Echini, M., Headd, J.J., Edington, D. and McKee, F.H. (2007) Phaser crystallographic software. *J. Appl. Crystallogr.*, **40**, 658–674.

46. Otwinowski, Z. and Minor, W. (1997) Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.*, **283**, 307–326.

47. Otwinowski, Z. and Minor, W. (1997) Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.*, **283**, 307–326.
automated crystallographic structure determination. Acta Crystallogr. D, 58, 1948–1954.
48. Robert,X. and Gouet,P. (2014) Deciphering key features in protein structures with the new ENDscript server. Nucleic Acids Res., 42, W320–W324.
49. Chan,P.P. and Lowe,T.M. (2016) GtRNAdb 2.0: an expanded database of transfer RNA genes identified in complete and draft genomes. Nucleic Acids Res., 44, D184–D189.
50. González,J.M., Masuchi,Y., Robb,F.T., Ammerman,J.W., Maeder,D.L., Yanagibayashi,M., Tamaoka,J and Kato,C. (1998) Pyrococcus horikoshii sp. nov., a hyperthermophilic archaeon isolated from a hydrothermal vent at the Okinawa Trough. Extremophiles, 2, 123–130.
51. Kowalak,J.A., Dalluge,J.J., McCloskey,J.A. and Stetter,K.O. (1994) The role of posttranscriptional modification in stabilization of transfer RNA from hyperthermophiles. Biochemistry, 33, 7869–7876.
52. Tomikawa,C., Yokogawa,T., Kanai,T. and Hori,H. (2010) N⁷-methylguanine at position 46 (m⁷G46) in tRNA from Thermus thermophilus is required for cell viability at high temperatures through a tRNA modification network. Nucleic Acids Res., 38, 942–957.
53. Squires,J.E., Patel,H.R., Nousch,M., Sibbritt,T., Humphreys,D.T., Parker,B.J., Suter,C.M. and Preiss,T. (2012) Widespread occurrence of 5-methylcytosine in human coding and non-coding RNA. Nucleic Acids Res., 40, 5023–5033.
54. Squires,J.E. and Preiss,T. (2010) Function and detection of 5-methylcytosine in eukaryotic RNA. Epigenomics, 2, 709–715.
55. Chen,Y., Sierpowska-Gracz,H., Guenther,R., Everett,K. and Agris,P.F. (1993) 5-methylcytidine is required for cooperative binding of Mg²⁺ and a conformational transition at the anticodon stem-loop of yeast phenylalanine tRNA. Biochemistry, 32, 10249–10253.
56. Alexandrov,A., Chernyakov,I., Gu,W., Hiley,S.L., Hughes,T.R., Grayhack,E.J. and Phizicky,E.M. (2006) Rapid tRNA decay can result from lack of nonessential modifications. Mol. Cell, 21, 87–96.
57. Apweiler,R., Bairoch,A., Wu,C.H., Barker,W.C., Boeckmann,B., Ferro,S., Gasteiger,E., Huang,H., Lopez,R., Magrane,M. et al. (2004) UniProt: the universal protein knowledgebase. Nucleic Acids Res., 32, D115–D119.