

# Molecular basis for human mitochondrial tRNA m<sup>3</sup>C modification by alternatively spliced METTL8

Meng-Han Huang<sup>1</sup>, Gui-Xin Peng<sup>1,3</sup>, Xue-Ling Mao<sup>1</sup>, Jin-Tao Wang<sup>1</sup>, Jing-Bo Zhou<sup>1</sup>, Jian-Hui Zhang<sup>1</sup>, Meirong Chen<sup>2</sup>, En-Duo Wang<sup>1,3,\*</sup> and Xiao-Long Zhou<sup>1,\*</sup>

<sup>1</sup>State Key Laboratory of Molecular Biology, CAS Center for Excellence in Molecular Cell Science, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, University of Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai 200031, China, <sup>2</sup>School of Pharmacy, China Pharmaceutical University, 639 Longmian Avenue, Nanjing 211198, Jiangsu, China and <sup>3</sup>School of Life Science and Technology, ShanghaiTech University, 393 Middle Hua Xia Road, Shanghai 201210, China

Received January 06, 2022; Revised March 06, 2022; Editorial Decision March 08, 2022; Accepted March 10, 2022

## ABSTRACT

**METTL8 has recently been identified as the methyltransferase catalyzing 3-methylcytidine biogenesis at position 32 (m<sup>3</sup>C32) of mitochondrial tRNAs. METTL8 also potentially participates in mRNA methylation and R-loop biogenesis. How METTL8 plays multiple roles in distinct cell compartments and catalyzes mitochondrial tRNA m<sup>3</sup>C formation remain unclear. Here, we discovered that alternative mRNA splicing generated several isoforms of METTL8. One isoform (METTL8-Iso1) was targeted to mitochondria via an N-terminal pre-sequence, while another one (METTL8-Iso4) mainly localized to the nucleolus. METTL8-Iso1-mediated m<sup>3</sup>C32 modification of human mitochondrial tRNA<sup>Thr</sup> (hmtRNA<sup>Thr</sup>) was not reliant on t<sup>6</sup>A modification at A37 (t<sup>6</sup>A37), while that of hmtRNA<sup>Ser</sup>(UCN) critically depended on i<sup>6</sup>A modification at A37 (i<sup>6</sup>A37). We clarified the hmtRNA<sup>Thr</sup> substrate recognition mechanism, which was obviously different from that of hmtRNA<sup>Ser</sup>(UCN), in terms of requiring a G35 determinant. Moreover, SARS2 (mitochondrial seryl-tRNA synthetase) interacted with METTL8-Iso1 in an RNA-independent manner and modestly accelerated m<sup>3</sup>C modification activity. We further elucidated how nonsubstrate tRNAs in human mitochondria were efficiently discriminated by METTL8-Iso1. In summary, our results established the expression pattern of METTL8, clarified the molecular basis for m<sup>3</sup>C32 modification by METTL8-Iso1 and provided the rationale for the involvement of METTL8 in tRNA modification, mRNA methylation or R-loop biogenesis.**

## INTRODUCTION

Transfer RNA (tRNA) is one of the most abundant RNA species in the cell (1,2). It functions as an adaptor during the translation of the genetic code at the ribosome. tRNAs exhibit a cloverleaf secondary structure with several conserved domains, including amino acid accepting stem, D-stem and loop, anticodon stem and loop, variable stem or loop and TψC stem and loop.

After transcription by RNA polymerase, tRNA precursors usually undergo posttranscriptional processing, including numerous base or sugar modifications, by various tRNA modifying enzymes (2,3). In fact, tRNAs harbor the most extensive posttranscriptional modification among all cellular RNA species (1). Specifically, bases in the anticodon loop are the most heavily modified. These modifications play various critical roles in maintaining tRNA structure and stability, limiting or expanding codon-anticodon base pair capacity, restricting the occurrence of frameshifting and promoting tRNA aminoacylation (2–4).

3-Methylcytidine modification at position 32 (m<sup>3</sup>C32) is present only in eukaryotic cytoplasmic tRNA<sup>Thr</sup>, tRNA<sup>Ser</sup>, tRNA<sup>Arg</sup> (only in mammals) and mitochondrial tRNA<sup>Thr</sup> and tRNA<sup>Ser</sup> species (5–9). The biological function of tRNA m<sup>3</sup>C32 modification in mRNA translation has not been fully established. Considering its location in the anticodon loop, we suggest that it likely participates in regulating loop conformation, codon-anticodon base pairing and/or the biogenesis of tRNA-derived small regulatory RNAs. Loss of cytoplasmic tRNA<sup>Ser</sup> m<sup>3</sup>C formation was found to influence pluripotency and cancer cell growth (10).

With respect to the biogenesis of tRNA m<sup>3</sup>C32 in budding yeast, Trm140 is responsible for m<sup>3</sup>C32 modification of both tRNA<sup>Thr</sup> and tRNA<sup>Ser</sup> (5,6); however, two enzymes, Trm140 and Trm141, catalyze tRNA<sup>Thr</sup> and tRNA<sup>Ser</sup> m<sup>3</sup>C32 modification, respectively, in fission yeast

\*To whom correspondence should be addressed. Tel: +86 21 5492 1247; Fax: +86 21 5492 1011; Email: xlzhou@sibcb.ac.cn  
Correspondence may also be addressed to Prof. En-Duo Wang. Tel: +86 21 5492 1241; Fax: +86 21 5492 1011; Email: edwang@sibcb.ac.cn

(11). The rationale for the evolution of the additional gene (Trm141) in fission yeast is unclear. *In vivo* genetic data have shown that *N*<sup>6</sup>-threonylcarbamoyladenine (t<sup>6</sup>A) or *N*<sup>6</sup>-isopentenyladenine (i<sup>6</sup>A) modification at position 37 (t<sup>6</sup>A37 or i<sup>6</sup>A37) of yeast tRNA<sup>Thr</sup> or tRNA<sup>Ser</sup> functions as a positive determinant in catalyzing m<sup>3</sup>C32 biogenesis by Trm140 and Trm141 (11,12). In mice, Mettl2 has been identified as the m<sup>3</sup>C32 methyltransferase for tRNA<sup>Thr</sup> and tRNA<sup>Arg</sup>; Mettl6 has been identified as the methyltransferase for tRNA<sup>Ser</sup>, based on the observation that m<sup>3</sup>C32 formation on these specific tRNAs was impaired after deleting the corresponding genes. Interestingly, it has been shown that mRNA also undergoes m<sup>3</sup>C modification, which is mediated by Mettl8. Moreover, Mettl8 knockout has little effect on the m<sup>3</sup>C32 abundance of tRNAs, leading to the hypothesis that Mettl8 is not a tRNA but rather a mRNA m<sup>3</sup>C methyltransferase (13). A recent study reported that METTL8 is a potential regulator of R-loop stability in the ribosomal RNA (rRNA) gene locus (14). Indeed, another recent study reported that m<sup>3</sup>C abundance in mRNAs is lower than that in tRNAs (15). In human cells, two Mettl2 homologs, METTL2A and METTL2B, have further evolved, with amino acid differences at only six positions, for unclear reasons (16). Recently, we purified human METTL2A, METTL2B and METTL6 and established the m<sup>3</sup>C32 modification activities of all these enzymes. We found that t<sup>6</sup>A37 in human cytoplasmic tRNA<sup>Thr</sup> is a critical determinant of m<sup>3</sup>C32 biogenesis by METTL2A/2B; however, t<sup>6</sup>A37 in human cytoplasmic tRNA<sup>Ser</sup>(GCU) plays little role in modification by METTL6, in assistance with cytoplasmic seryl-tRNA synthetase (SerRS, encoded by *SARS1*) (16). Very recently, we further determined the crystal structure of METTL6, defining its active site and substrate binding patterns (17).

Among the 22 human mitochondrial tRNAs (hmtRNAs), only tRNA<sup>Thr</sup> (hmtRNA<sup>Thr</sup>) and tRNA<sup>Ser</sup>(UCN) (hmtRNA<sup>Ser</sup>(UCN)) contain m<sup>3</sup>C32 modification (9). Meanwhile, hmtRNA<sup>Thr</sup> and hmtRNA<sup>Ser</sup>(UCN) harbor a t<sup>6</sup>A37 modification, catalyzed by YRDC and OSGEPL1 (18,19), and a i<sup>6</sup>A37 modification mediated by TRIT1 (20,21), respectively. Of the four known m<sup>3</sup>C modifying enzymes (METTL2A, METTL2B, METTL6 and METTL8), METTL2A/2B and METTL6 are distributed in the cytoplasm but not the mitochondria, suggesting that they do not function as mitochondrial m<sup>3</sup>C32-modifying enzymes (16). Therefore, the enzyme responsible for mammalian tRNA<sup>Thr</sup> and tRNA<sup>Ser</sup>(UCN) m<sup>3</sup>C32 biogenesis has long been a mystery. However, a recent mitochondrial proteome screening found that METTL8 is located in the mitochondrial fraction, eliciting the question of whether METTL8 is the missing mitochondrial tRNA m<sup>3</sup>C32 methyltransferase (22). Indeed, METTL8 has very recently been proven to be the mitochondrial tRNA m<sup>3</sup>C32 methyltransferase (23).

Despite being revealed as a multifunctional protein, however, the localization of METTL8 in mitochondria as a tRNA m<sup>3</sup>C32 modifying enzyme (23) seemingly contradicts the findings that Mettl8 potentially mediates mRNA m<sup>3</sup>C formation in mouse cells (13) and regulates R-loop biogenesis and stability in human cells (14), which require the pres-

ence of METTL8 in the cytoplasm and/or nucleolar fraction. To more clearly understand these seemingly inconsistent findings about the multiple roles of METTL8, we initially analyzed all potential mRNA and protein isoforms in detail and identified several protein variants that were generated via alternative splicing of METTL8 mRNA. The longest variant, METTL8-Iso1, harbors an N-terminal mitochondrial targeting sequence (MTS), which facilitates its import into mitochondria. We further precisely established the mature form of METTL8-Iso1 and reconstituted both hmtRNA<sup>Thr</sup> and hmtRNA<sup>Ser</sup>(UCN) m<sup>3</sup>C32 modification activity of METTL8-Iso1. Our results showed that i<sup>6</sup>A37 of hmtRNA<sup>Ser</sup>(UCN) but not t<sup>6</sup>A37 of hmtRNA<sup>Thr</sup> constituted the key determinant in modification and further established a tRNA recognition mechanism by METTL8-Iso1 using hmtRNA<sup>Thr</sup> transcript. METTL8-Iso1 interacted with SARS2 (mitochondrial SerRS) but exhibited SARS2-independent activity.

In summary, our results significantly deepen our understanding of METTL8 gene expression and the mitochondrial tRNA m<sup>3</sup>C32 modification mechanism of METTL8-Iso1. In combination with our recent elucidation of the cytoplasmic tRNA m<sup>3</sup>C32 biogenesis mechanism involving METTL2A and METTL6 (16), these results collectively provide valuable insights into both the similarity and divergence between cytoplasmic and mitochondrial tRNA m<sup>3</sup>C32 modifications.

## MATERIALS AND METHODS

### Materials

S-Adenosylmethionine (SAM) was obtained from New England Biolabs (Ipswich, MA, USA). Protease inhibitor cocktail, horseradish peroxidase (HRP)-conjugated secondary antibodies, protein standards for gel filtration chromatography, anti-FLAG (F7425), anti-Myc (M4439) and anti-GAPDH (G8795) antibodies were purchased from Sigma (St. Louis, MO, USA). Anti-GFP (4B10) antibody was obtained from Cell Signaling Technology (Boston, MA, USA). Dynabeads Protein G, MitoTracker, RNase I (RNase) and restriction enzymes were obtained from Thermo Scientific (Waltham, MA, USA). Lipofectamine 8000 transfection reagent and 4',6-diamidino-2-phenylindole (DAPI) were obtained from Beyotime Biotech (Shanghai, China). Polyvinylidene fluoride (PVDF) membranes were purchased from Merck (Darmstadt, Germany). Primers were synthesized by Biosune (Shanghai, China), and DNA sequencing was performed by Tsingke (Shanghai, China). PrimeScript RT Master Mix was obtained from Takara Bio (Kusatsu, Japan). Dimethylallyl diphosphate (DMAPP) was obtained from APEX BIO Technology (Houston, TX, USA). The BCA Protein Assay Kit was obtained from TIANGEN Biotech (Beijing, China). [<sup>3</sup>H] SAM and [<sup>3</sup>H] DMAPP were obtained from Perkin Elmer, Inc. (Waltham, MA, USA). t<sup>6</sup>A (sc-286478) and m<sup>3</sup>C (HY-111645) standard samples for mass spectrometry were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and MedChemExpress (Monmouth Junction, NJ, USA), respectively.

### Plasmid construction and gene expression

Genes encoding different METTL8 (UniProt No. Q9H825) isoforms were amplified from cDNA obtained by reverse transcription of total RNA from human embryonic kidney 293T (HEK293T) cells and inserted between the XhoI and EcoRI sites of pEGFP-N2 with a C-terminal EGFP tag. The initiating Met codon (ATG) of EGFP was mutated to the Ile codon (ATC) to prevent the potential generation of free EGFP. The gene encoding METTL8-Iso1 was also inserted between the NotI and XhoI sites of pCMV-3Tag-3A with C-terminal 3 × FLAG tags. Mature METTL8-Iso1 and METTL8-Iso4 were inserted between the NcoI and XhoI sites of pET28a with a C-terminal His<sub>6</sub>-tag for expression and purification in an *E. coli* expression system. A plasmid expressing mature SARS2 (Thr<sup>35</sup>-Ser<sup>518</sup>) was constructed and expressed as described in a previous report (19). Full-length SARS2 was inserted between the EcoRI and NotI sites of pcDNA3.1 with a C-terminal Myc-tag. The gene encoding *Escherichia coli* MiaA (*EcMiaA*) (UniProt No. P16384) was amplified by PCR from the *E. coli* genome and inserted between the NcoI and XhoI sites of pET28a with a C-terminal His<sub>6</sub>-tag. The plasmid expressing *Saccharomyces cerevisiae* Sua5 (*ScSua5*) (UniProt No. P32579) was constructed and expressed as reported previously (19,24). All primers used in gene cloning are listed in Supplementary Table 1.

For gene expression in *E. coli*, constructs were transformed into *E. coli* BL21 (DE3) cells. METTL8-Iso1, METTL8-Iso4 and *EcMiaA* gene expression was induced with a final concentration of 200 μM isopropyl β-D-1-thiogalactopyranoside (IPTG), and *S. cerevisiae* KEOPS (*ScKEOPS*) expression from pJ241-*ScKEOPS* (25) was induced with a final concentration of 500 μM IPTG when the initial cell culture reached an absorbance at 600 nm (*A*<sub>600</sub>) of 0.6–0.8. Transformants were cultured overnight at 18°C. Proteins were purified by Ni<sup>2+</sup>-NTA Superflow resin according to the manufacturer's protocol. The purified METTL8 proteins were concentrated and further purified on a HiTrap Q FF column and dialyzed against storage buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM DDT). The purified *EcMiaA* proteins were concentrated and dialyzed against storage buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM DDT). The purified *ScKEOPS* was concentrated and further purified via ion exchange chromatography (Mono Q column) and then dialyzed against storage buffer (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM β-ME). All purified proteins were mixed with an equal volume of glycerol and then stored at –20°C. The protein concentrations were determined by using a BCA Protein Assay Kit according to the manufacturer's instructions.

### tRNA gene cloning and transcription

Plasmids for *in vitro* transcription of hmtRNA<sup>Thr</sup>, hmtRNA<sup>Lys</sup>-Ki and hmtRNA<sup>Trp</sup> were constructed as previously reported (19,24,26). hmtRNA<sup>Ser</sup>(UCN), hmtRNA<sup>Tyr</sup>, hmtRNA<sup>Leu</sup>(UUR), hmtRNA<sup>Phe</sup> and hmtRNA<sup>Met</sup> were engineered into the pTrc99b plasmid. A hammerhead ribozyme was added between the T7

promoter and hmtRNA<sup>Phe</sup> gene to improve transcription efficiency (27). Note that a mutant of hmtRNA<sup>Lys</sup>, hmtRNA<sup>Lys</sup>-Ki (U50:A64) (with A50:U64 in wild-type hmtRNA<sup>Lys</sup> replaced with U50:A64), which has been shown to substitute well for wild-type hmtRNA<sup>Lys</sup> (28), was transcribed and used in this study to avoid misfolding. tRNA gene mutagenesis was performed according to the protocol provided with the KOD-plus mutagenesis kit. All tRNAs were obtained by *in vitro* tRNA transcription, which was performed as previously described (19,29). The primers used for template preparation are listed in Supplementary Table 1.

### Determination of *in vitro* m<sup>3</sup>C and i<sup>6</sup>A modification

The m<sup>3</sup>C modification reaction was performed at 37 °C in a 40 μl reaction mixture containing 50 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM spermidine, 100 μM [<sup>3</sup>H] SAM, 5 μM hmtRNAs (or variants) and 1 μM mature METTL8-Iso1 (or METTL8-Iso1 and SARS2 in different molar ratios). The i<sup>6</sup>A modification reaction was performed at 37 °C in a 10 μl reaction mixture containing 50 mM Tris-HCl (pH 7.5), 3.5 mM MgCl<sub>2</sub>, 1 mg/ml BSA, 5 mM β-ME, 30 μM [<sup>3</sup>H] DMAPP, 5–15 μM hmtRNAs (or variants) and 1 μM *EcMiaA*. Aliquots of the reaction solution were added to filter pads at various time points (for m<sup>3</sup>C modification) or at 15 min (for i<sup>6</sup>A modification) and quenched with cold 5% TCA. The pads were washed three times for 15 min each with cold 5% TCA and then three times for 10 min each with 100% ethanol. Finally, the pads were dried under a heat lamp, and the radioactivity of the precipitates was quantified using a scintillation counter (Beckman Coulter, Atlanta, GA, USA).

### Reverse transcription and identification of METTL8 mRNAs

Total RNA was extracted from HEK293T cells using TRIzol reagent. cDNAs were reverse-transcribed using PrimeScript RT Master Mix according to the manufacturer's protocol. After cDNA synthesis, PCR was performed with different primers using 2 × Taq Master Mix according to the manufacturer's protocol. PCR products were separated via agarose gel electrophoresis, the relevant bands were excised, and the DNA was extracted with a HiPure Gel Pure Micro Kit and sequenced by Tsingke. The DNA sequencing results were then compared with various exon sequences to define alternative splicing patterns. The locations of the primers and exons are described in the Results section, and the primer sequences are listed in Supplementary Table 1.

### Cell culture, transfection and coimmunoprecipitation (Co-IP)

HEK293T cells were cultured in DMEM supplemented with 10% FBS in a 37°C incubator with 5% CO<sub>2</sub> at a confluence of 70% before transfection using Lipofectamine 8000 transfection reagent according to the manufacturer's protocol. For co-IP, at 24 h after transfection, cells were harvested, washed with ice-cold PBS three times, and lysed for 30 min at 4 °C with rotation in 1 ml of ice-cold lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% NP-40 and 0.25% sodium deoxycholate) supplemented



with a protease inhibitor cocktail. The supernatant was collected by centrifugation at 12,000 g for 20 min. Whole cell lysates were supplemented with anti-FLAG antibodies, incubated overnight with rotation, and then treated with Dynabeads protein G for an additional 4 h. The recovered immune complexes were washed three times with ice-cold PBS containing 0.05% Tween-20 (PBST). Protein was eluted from the beads in 2 × protein loading buffer comprising 100 mM Tris-HCl (pH 7.0), 4% SDS, 0.2% bromophenol blue, 20% glycerol and 200 mM DTT.

### Fluorescence assays

After cell culture and transfection, the cells were incubated with MitoTracker at a 1:20,000 dilution for 30 min in a 37 °C incubator. After washing 3 times, the cells were fixed in 4% paraformaldehyde for 30 min, washed with PBS 3 times, and then permeabilized in 0.2% Triton X-100 and 1% BSA for 5 min on ice. After washing with PBS 3 times again, the fixed and permeabilized cells were blocked in PBS containing 4% BSA, washed with PBS 3 times, and then immunolabeled with the nuclear counterstain DAPI in PBS at a 1:1000 dilution for 5 min at RT. Fluorescent images were taken using a Leica TCS SP8 STED confocal microscope and analyzed.

### Preparation of t<sup>6</sup>A- and i<sup>6</sup>A-modified tRNAs

For preparation of t<sup>6</sup>A-modified hmtRNA<sup>Thr</sup>, the t<sup>6</sup>A modification reaction was performed at 37 °C for 1 h in a 200 μl reaction mixture containing 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 15 mM MgCl<sub>2</sub>, 4 mM ATP, 5 mM DTT, 5 mM MnCl<sub>2</sub>, 50 mM NaHCO<sub>3</sub>, 1 mM L-Thr, 10 μM hmtRNA<sup>Thr</sup> and 5 μM yeast Sua5 (30) and KEOPS complex (31). For the preparation of i<sup>6</sup>A-modified hmtRNAs, an i<sup>6</sup>A modification reaction was performed at 37 °C for 30 min in a 200 μl reaction mixture containing 50 mM Tris-HCl (pH 7.5), 3.5 mM MgCl<sub>2</sub>, 1 mg/ml BSA, 5 mM β-ME, 0.5 mM DMAPP, 20 μM hmtRNAs and 5 μM *EcMiaA* (32). The t<sup>6</sup>A- or i<sup>6</sup>A-modified tRNA was purified by phenol/chloroform and precipitated by EtOH with NaAc overnight at -20 °C. The modified tRNA concentration was determined by denaturing urea-PAGE based on linear curves from tRNA transcript samples with known concentrations.

### Ultra-performance liquid chromatography-mass spectrometry/mass spectrometry (UPLC-MS/MS) analysis

For UPLC-MS/MS, 500 ng of unmodified or m<sup>3</sup>C-, t<sup>6</sup>A- or i<sup>6</sup>A-modified tRNA sample was digested with 1 U of nuclease P1 and 0.5 U of benzonase nuclease in a 20 μl solution including the nuclease P1 buffer supplied with the kit and 20 mM NH<sub>4</sub>OAc (pH 5.2) at 37 °C for 6 h. Then, 0.5 U of calf alkaline phosphatase (CAP) and 0.5 U of bacterial alkaline phosphatase (BAP) with CAP buffer were added to the reaction solution. After complete hydrolysis at 37 °C overnight and filtration, 1 μl of product was subjected to UPLC-MS/MS. m<sup>3</sup>C and t<sup>6</sup>A were detected with Poroshell 120 PFP column (Agilent) and i<sup>6</sup>A was detected with Hypersil GOLD aQ column (Thermo Scientific).

### Western blotting

After co-IP or protein purification, protein samples were separated on a 10% separating gel via SDS-PAGE. Western blotting was performed as described in a previous report (33).

### Gel filtration analyses of mature METTL8-Iso1 and METTL8-Iso4

Purified mature METTL8-Iso1 and METTL8-Iso4 were analyzed by gel filtration chromatography on a Superdex 200 column using running solution (50 mM Tris-HCl (pH 8.0), 150 mM NaCl) at a rate of 0.5 ml/min. Protein standards were also loaded and eluted under the same conditions. A linear calibration curve was obtained by plotting the logarithms of the known molecular masses of the standards versus their elution times.

### Insect cell culture, transfection, expression and determination of the N-terminal sequence of mature METTL8-Iso1

Sf9 and High Five cells were cultured in Sf-900 II SFM medium at 27 °C. The pFastBac2-METTL8-Iso1 precursor was constructed with a C-terminal His<sub>6</sub> tag for gene expression in insect cells by inserting the gene encoding the METTL8-Iso1 precursor between the BamHI and XhoI sites of pFastbac2. Recombinant baculoviruses were generated by transfecting the bacmid into Sf9 cells using Cellfectin II reagent according to the manufacturer's protocol. P1 virus stock was harvested after 6–7 days at 27 °C and used to infect Sf9 cells, which were then incubated for 5–6 days at 27 °C to produce a higher-titer P2 virus stock. The same procedure was used to produce P3 virus stock, which was then stored at 4 °C with protection from light. Gene expression was performed by infecting High Five cells in Sf-900 II SFM medium with P3 virus stock at a 1:200 ratio. Cells were harvested by centrifugation (500 g, 5 min) 66 h after infection, washed in PBS and stored at -80 °C. The High Five cells were disrupted by sonication on ice, and the whole cell lysate was applied to Ni-NTA Superflow resin for mature METTL8-Iso1 purification according to the manufacturer's protocol. The eluted proteins were separated on a 10% separating gel via SDS-PAGE and transferred to PVDF membranes in CAPS transfer buffer (10 mM CAPS, 10% methanol), stained with Ponceau red for 25 min and washed with ultrapure water. The washing steps were repeated several times until the band of interest was clearly visible. The band was excised and sent to BiotechPack Scientific (Beijing, China) for identification of the N-terminal sequence through the Edman degradation method.

## RESULTS

### METTL8 encodes several isoforms via mRNA alternative splicing

Seven protein isoforms of the human METTL8 gene have been deposited in the NCBI database (Supplementary Figure 1). Primary sequence alignment of these isoforms, together with other m<sup>3</sup>C32 methyltransferases, including yeast Trm140 and Trm141 and mammalian METTL2A,

METTL2B and METTL6, showed that isoforms 5–7 are truncated (Figure 1A). Comparison with our recently resolved crystal structure of METTL6 (PDB No. 7EZG) (17) reveals that isoforms 5–7 of METTL8 lack a complete SAM- and tRNA-binding domain. An absolutely conserved residue in METTL6, Thr<sup>217</sup>, has been shown to be critical for m<sup>3</sup>C32 methylation, likely due to impaired tRNA binding (17). Notably, its counterpart was absent in isoforms 5–7 but present in isoforms 1–4 of METTL8 (Thr<sup>337</sup> in isoform 1) (Supplementary Figure 1). Therefore, isoforms 5–7 are unlikely to be m<sup>3</sup>C32-modifying enzymes. Based on these observations, we focus mainly on the presence or absence of isoforms 1–4 in this work. Notably, isoforms 3 and 4 (designated METTL8-Iso3 and -Iso4 hereafter) differ only in the presence of five additional amino acids (<sup>1</sup>MPRDH<sup>5</sup>) in the N-terminus of METTL8-Iso3. Compared with METTL8-Iso3 or -Iso4, isoforms 1 and 2 (METTL8-Iso1 and -Iso2) contain longer extensions of the N-terminus (Figure 1A, Supplementary Figure 1).

*METTL8* contains 12 exons (sequence of each exon is shown in Supplementary Table 2). Retrieved mRNA sequences encoding these protein isoforms in the NCBI database showed that METTL8-Iso1 was potentially encoded by four mRNA sequences (mRNAs 1v1-4) produced by alternative splicing (Figure 1B). mRNA sequences (mRNAs 2–7), sequentially encoding METTL8-Iso2 to METTL8-Iso7, were also generated by mRNA alternative splicing (Figure 1B). To distinguish and confirm the sequences of these mRNAs, we designed three forward primers (F1 complementary to exon 1; F2 to exon 2 and F3 to exon 4) (Figure 1B). Using cDNA from HEK293T cells, in combination with a universal reverse primer (R1 complementary to exon 6), we amplified several fragments. In detail, using F1 and R1, a weak fragment was obtained, which was then sequenced and found to have 341 bp, derived from mRNA 3 and/or mRNA 6, encoding METTL8-Iso3 and/or METTL8-Iso6, respectively (Figure 1C). Two obvious fragments amplified with F2 and R1 were sequenced and found to be 505 and 350 bp, clearly showing the existence of mRNA 1v2 (encoding METTL8-Iso1) and mRNA 4 (encoding METTL8-Iso4). In addition, two weak but detectable fragments of 474 and 629 bp were also obtained and sequenced. The former was definitely derived from mRNA 7, while the latter fragment indicated the existence of mRNA 5 (encoding METTL8-Iso5) and/or mRNA 1v3 (still encoding METTL8-Iso1) (Figure 1C). A 411 bp fragment was obtained with F3 and R1 and sequenced, indicating that the cDNA template was qualified, but it could not be assigned to an mRNA isoform (Figure 1C). The presence of mRNAs 5–7 was further confirmed by amplification with forward primer F4 (complementary to exon 9) and reverse primer R2 (complementary to exon 11), which produced a 449 bp fragment showing that exon 10 was indeed skipped, in addition to a 556 bp fragment that included exon 10 (Figure 1D). Notably, amplification of DNA products of 505, 350 and 474 bp with F2 and R1 primers definitely showed the presence of mRNA 1v2, mRNA 4 and mRNA 7 in human cells.

In summary, the above results showed a complex mRNA alternative splicing landscape of the *METTL8* gene in human HEK293T cells. In detail, at least two distinct mRNAs

(including mRNAs 1v2 and 4) encode two full-length protein isoforms (METTL8-Iso1 and METTL8-Iso4). In addition, at least mRNA 7 encoded a truncated protein lacking a complete SAM- and tRNA-binding domain and was not further investigated in this study.

### Cellular localization of METTL8 protein isoforms

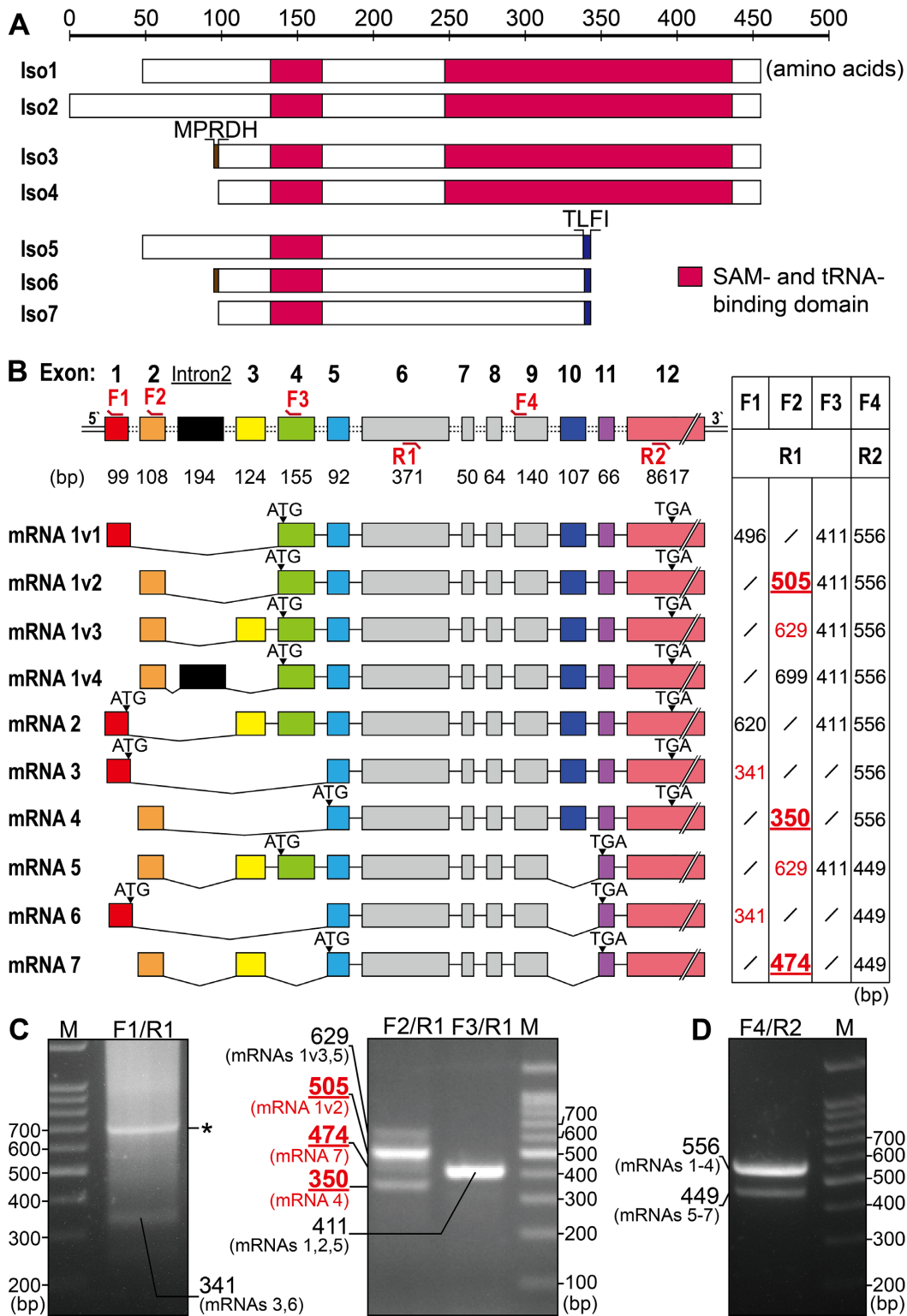
As described above, METTL8-Iso3 and METTL8-Iso4 are nearly the same, with a difference of only five additional amino acids (<sup>1</sup>MPRDH<sup>5</sup>) in the N-terminus of METTL8-Iso3. Compared with METTL8-Iso4, METTL8-Iso1 has a longer N-terminal extension (Met<sup>1</sup>-His<sup>50</sup>, NTD50). MitoProt software (34) predicted no mitochondrial distribution of either METTL8-Iso3 or METTL8-Iso4; however, METTL8-Iso1 exhibited a higher probability (with a score of 0.8052) of import into mitochondria, suggesting that it is a mitochondrial protein, in accordance with a recent proteome analysis reporting mitochondrial localization of METTL8 in human mitochondria (22).

The open reading frames (ORFs) encoding METTL8-Iso1, -Iso3 and -Iso4 could all be readily amplified from HEK293T cDNA. Three ORFs were individually cloned into pEGFP-N2 to express C-terminal EGFP-fusion proteins. Fluorescence analysis showed that the main fractions of both METTL8-Iso3 and METTL8-Iso4 were located in the nucleolus, which was confirmed by their colocalization with fibrillarlin (FBL), a key small nucleolar protein involved in pre-rRNA processing (Supplementary Figure 2A). However, METTL8-Iso1 was obviously distributed in the mitochondrial fraction, as stained by MitoTracker (Figure 2). The mitochondrial localization of METTL8-Iso1 was also confirmed by immunostaining of a C-terminal FLAG-tagged METTL8-Iso1 (METTL8-Iso1-FLAG) using anti-FLAG antibodies (Supplementary Figure 2B). Furthermore, the N-terminal extension of METTL8-Iso1, NTD50, was cloned upstream of EGFP. Consistently, the overexpressed NTD50-EGFP was exclusively targeted to mitochondria, suggesting a high efficiency of NTD50 in promoting the import of a foreign protein into the mitochondria (Figure 2).

In summary, the above data clearly showed that two METTL8 isoforms (METTL8-Iso3 and METTL8-Iso4) are mainly located in the nucleolar fraction, while METTL8-Iso1 is present in the mitochondria, indicating that METTL8-Iso1 is an m<sup>3</sup>C methyltransferase in this organelle.

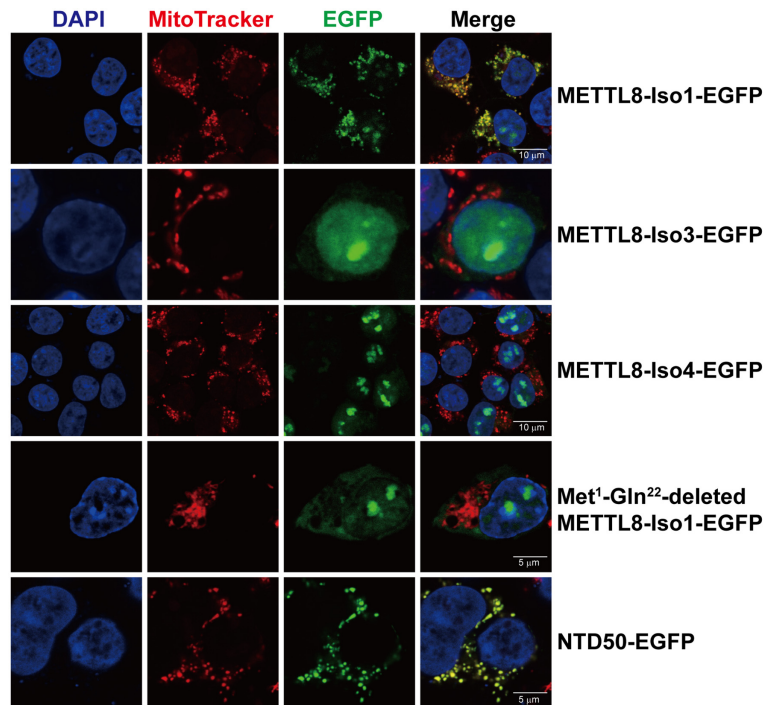
### Purification of the mitochondrial form of METTL8-Iso1

To reconstitute potential mitochondrial tRNA m<sup>3</sup>C32 modification, it was necessary to purify METTL8-Iso1. The MitoProt database predicted that the cleavage site of METTL8-Iso1 after import into the mitochondrial matrix was between Phe<sup>43</sup> and Glu<sup>44</sup>. However, we observed frequent inaccuracy in locating precise cleavage sites during studies of human mitochondrial aminoacyl-tRNA synthetases (such as LARS2, TARS2 and AARS2) (29,35–37) and tRNA modifying enzymes (such as GTPBP3) (38). Instead, we found that N-terminal protein sequencing by automatic Edman degradation after gene expression and pu-



**Figure 1.** Protein and mRNA analyses of METTL8. (A) Schematic showing NCBI-annotated METTL8 protein isoforms. Isoforms 5–7 lack a complete SAM and tRNA binding domain. Isoforms 3 and 4 differ only in the presence of five residues (MPRDH) in the former. The same difference exists between isoforms 5 and 6. A TLF1 motif in isoforms 5–7, which was absent in isoforms 1–4 is also indicated. (B) Schematic showing NCBI-annotated METTL8 mRNA variants with each exon indicated. The black box represents intron 2. mRNAs 1v1-4 encoded the identical METTL8-Iso1 protein isoform, while mRNAs 2–7 encoded isoforms METTL8 Iso2-7. The pairing positions of primers used for mRNA identification are labeled at the top of the relevant exons. Bands that were successfully amplified using F1 and F2 forward primers and DNA sequenced are highlighted in red, while predicted bands not obtained by PCR are marked in black. (C, D) Agarose gel images of PCR products with different primer combinations as indicated. Each lane was confirmed by DNA sequencing. A nonspecific band not derived from the METTL8 gene is marked by an asterisk in (C). Three bold and underlined sizes represent DNA products that could be definitely assigned to specific mRNA isoforms in (B) and (C).





**Figure 2.** Cellular localization of various METTL8 protein isoforms. Different METTL8 protein isoforms and NTD50 were fused with a C-terminal EGFP and expressed, and the cellular distribution of individual proteins was observed. The nuclei and mitochondria were stained with DAPI and MitoTracker, respectively.

rification of the mitochondrial proteins in a Bac-to-Bac baculovirus expression system is an excellent method for establishing the exact MTS (35,38). To this end, the gene encoding the METTL8-Iso1 precursor was expressed with a C-terminal His<sub>6</sub> tag in the baculovirus expression system and subsequently purified by Ni-NTA affinity chromatography (Supplementary Figure 3). The N-terminus of purified METTL8-Iso1 was determined to be SGYHPV, clearly establishing that Met<sup>1</sup>-Gln<sup>22</sup> is the MTS of METTL8-Iso1, in contrast to the predicted peptide (Met<sup>1</sup>-Phe<sup>43</sup>) (Supplementary Figure 1). To validate the mitochondrial targeting capacity of Met<sup>1</sup>-Gln<sup>22</sup>, a gene encoding mature METTL8-Iso1 (with Met<sup>1</sup>-Gln<sup>22</sup> deleted) and a C-terminal EGFP fusion was overexpressed in HEK293T cells. Consistently, the EGFP-fused Met<sup>1</sup>-Gln<sup>22</sup>-truncated METTL8-Iso1 was distributed in both the nucleus and cytoplasm but no longer in the mitochondria (Figure 2), showing loss of mitochondrial localization of Met<sup>1</sup>-Gln<sup>22</sup>-truncated METTL8-Iso1 in the absence of the MTS.

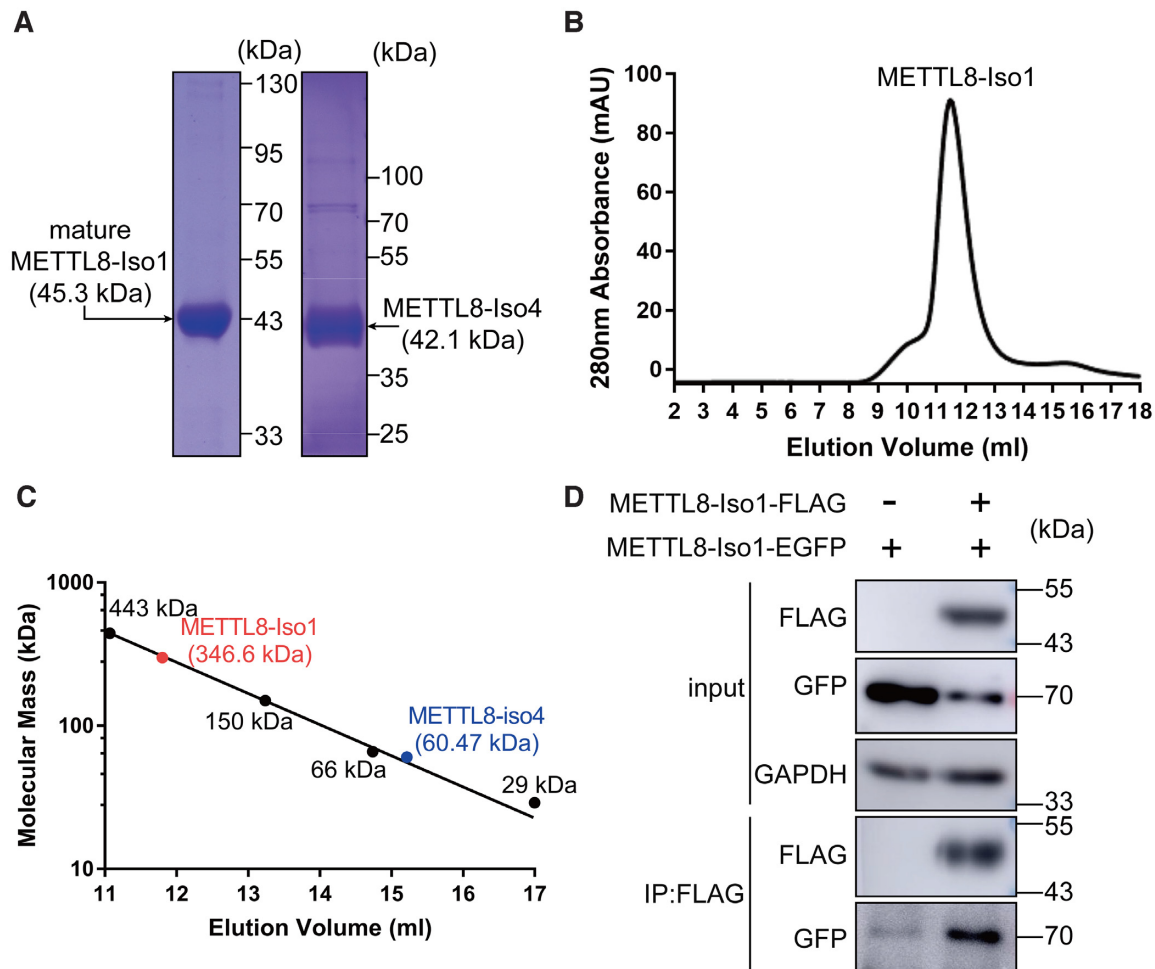
Then, the gene encoding mature METTL8-Iso1 (with N-terminal Met<sup>1</sup>-Gln<sup>22</sup> deleted) was expressed in *E. coli* and purified (Figure 3A). Both METTL2A and METTL6 are shown to be monomers in solution. To understand the quaternary structure of mature METTL8-Iso1, the protein was subjected to gel filtration analysis with Superdex S200 (Figure 3B). The results unexpectedly showed that METTL8-Iso1 eluted in a single peak with an elution volume between those of apoferritin (443 kDa) and yeast alcohol dehydrogenase (150 kDa). Based on the elution volume of the four protein standards, the calculated molecular mass was 346.6 kDa (Figure 3C). The theoretical molecular mass of puri-

fied METTL8-Iso1 with a C-terminal His<sub>6</sub> tag is 45.3 kDa; these results suggested that mature METTL8-Iso1 exists as a polymer (likely octamer) in solution. We further purified METTL8-Iso4 from *E. coli* (Figure 3A), and the same analysis revealed that the molecular mass of purified METTL8-Iso4 was 60.47 kDa (Figure 3C), suggesting that METTL8-Iso4 (theoretical molecular mass 42.1 kDa) is possibly a monomer in solution. The clear difference between the quaternary structures of mature METTL8-Iso1 and METTL8-Iso4 is unanticipated considering that the former is only 28 aa longer than the latter at the N-terminus (Supplementary Figure 1). The reason for this difference is unclear and needs to be studied further.

To further confirm that METTL8-Iso1 forms a polymer *in vivo*, genes encoding METTL8-Iso1-FLAG and METTL8-Iso1-EGFP were coexpressed in HEK293T cells. Coimmunoprecipitation (co-IP) analysis using anti-FLAG antibodies showed that METTL8-Iso1-EGFP indeed coprecipitated with METTL8-Iso1-FLAG (Figure 3D), suggesting that METTL8-Iso1 forms polymers *in vivo*.

### m<sup>3</sup>C32 biogenesis by METTL8-Iso1 is not reliant on t<sup>6</sup>A37 modification

After obtaining purified METTL8-Iso1, we subsequently explored whether METTL8-Iso1 is able to modify the hmtRNA<sup>Thr</sup> transcript or requires a preformed t<sup>6</sup>A moiety as a determinant, as revealed by the modification of cytoplasmic tRNA<sup>Thr</sup> by METTL2A (16). Indeed, METTL8-Iso1 obviously generated m<sup>3</sup>C modification in the hmtRNA<sup>Thr</sup> transcript (Figure 4A). To understand



**Figure 3.** METTL8-Iso1 forms polymers. (A) SDS-PAGE analyses of purified recombinant mature METTL8-Iso1 and METTL8-Iso4 from the *E. coli* expression system. (B) Gel filtration analysis of purified mature METTL8-Iso1. (C) Determination of the molecular mass of mature METTL8-Iso1 and METTL8-Iso4 based on the elution volumes of four protein standards, namely, apoferritin (443 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa). (D) The METTL8-Iso1-FLAG and METTL8-Iso1-EGFP genes were coexpressed in HEK293T cells. METTL8-Iso1-EGFP was precipitated by METTL8-Iso1-FLAG in a co-IP assay.

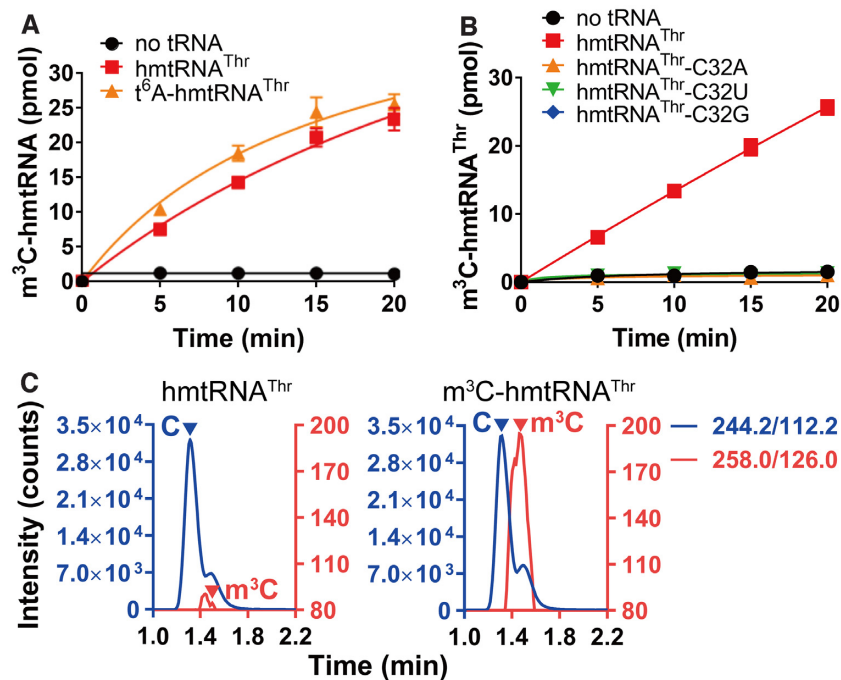
whether position 32 was modified, we mutated C32 to three other nucleotides, generating the hmtRNA<sup>Thr</sup>-C32A, -C32U and -C32G mutants (Figure 4B). The three mutants were unable to be modified by METTL8-Iso1, suggesting that position 32 was modified. The modified hmtRNA<sup>Thr</sup> transcript was further hydrolyzed to mononucleosides and analyzed by UPLC-MS/MS, confirming the formation of m<sup>3</sup>C modification (Figure 4C). These data showed that METTL8-Iso1 indeed modified the hmtRNA<sup>Thr</sup> transcript with m<sup>3</sup>C at position 32.

We further prepared t<sup>6</sup>A37-modified hmtRNA<sup>Thr</sup> (t<sup>6</sup>A-hmtRNA<sup>Thr</sup>) using yeast Sua5 and KEOPS complex (39), which was confirmed by UPLC-MS/MS analysis after digestion of t<sup>6</sup>A-hmtRNA<sup>Thr</sup> into mononucleosides (Supplementary Figure 4). We found that METTL8-Iso1 introduced comparable amounts of the m<sup>3</sup>C moiety into t<sup>6</sup>A-hmtRNA<sup>Thr</sup> and the hmtRNA<sup>Thr</sup> transcript (Figure 4A). These data clearly showed that t<sup>6</sup>A formation at position 37 has little stimulatory effect on m<sup>3</sup>C32 biogenesis by METTL8-Iso1 *in vitro*, which is in sharp contrast to its role in modification by METTL2A (16).

### Mechanism of hmtRNA<sup>Thr</sup> recognition by METTL8-Iso1

After establishing that the hmtRNA<sup>Thr</sup> transcript is a qualified METTL8-Iso1 substrate, we further studied how it is selected among the mitochondrial tRNAs. Our previous studies on the METTL8 homologs METTL2A and METTL6 revealed that the anticodon loop is a key element for m<sup>3</sup>C32 formation by both enzymes (16). Based on these results, we targeted the anticodon loop of hmtRNA<sup>Thr</sup>. We mutated each nucleotide in the anticodon loop (U33, U34, G35, U36, A37 and A38) into three other nucleotides (Figure 5A). hmtRNA<sup>Thr</sup>-U33G was not modified by METTL8-Iso1, while hmtRNA<sup>Thr</sup>-U33A and hmtRNA<sup>Thr</sup>-U33C were modified with comparable efficiency to wild-type hmtRNA<sup>Thr</sup> (Figure 5B). For position 34, hmtRNA<sup>Thr</sup>-U34A and hmtRNA<sup>Thr</sup>-U34G were not modified, while the modification efficiency of hmtRNA<sup>Thr</sup>-U34C was even higher than that of wild-type hmtRNA<sup>Thr</sup> (Figure 5C). Strikingly, none of the G35 mutants were modified. Position 35 has no interaction with other nucleotides in tRNAs. Mutation at this residue has little pos-





**Figure 4.** Reconstitution of  $m^3C$ 32 modification activity of METTL8-Iso1 for hmtRNA<sup>Thr</sup>. Time-course curves of the  $m^3C$ 32 modification of hmtRNA<sup>Thr</sup> and t<sup>6</sup>A-hmtRNA<sup>Thr</sup> (A) or hmtRNA<sup>Thr</sup>, hmtRNA<sup>Thr</sup>-C32A, -C32U and -C32G (B) by mature METTL8-Iso1. (C) UPLC-MS/MS analysis of the digested product of METTL8-Iso1-modified hmtRNA<sup>Thr</sup>.  $m/z$  value 244.2 to 112.2 (C) and  $m/z$  value 258.0 to 126.0 ( $m^3C$ ) are shown on the right. Controls in (A) and (B) (no tRNA) represent assays without tRNA added. The data represent the averages of three independent experiments and the corresponding standard error of the mean (SEM) in (A) and (B).

sibility to influence tRNA folding and structure. Therefore, no modification of all G35 mutants clearly revealed that G35 functions as a positive determinant of  $m^3C$ 32 biogenesis by METTL8-Iso1 (Figure 5D). For position 36, only hmtRNA<sup>Thr</sup>-U36A was obviously modified, but with a reduced (approximately half) efficiency (Figure 5E). The results from mutants of A37 showed that hmtRNA<sup>Thr</sup>-A37G was only slightly modified, while hmtRNA<sup>Thr</sup>-A37U and hmtRNA<sup>Thr</sup>-A37C displayed higher or comparable modification efficiency than wild-type hmtRNA<sup>Thr</sup> (Figure 5F). Finally, hmtRNA<sup>Thr</sup>-A38G was not modified, while hmtRNA<sup>Thr</sup>-A38U and hmtRNA<sup>Thr</sup>-A38C exhibited reduced modification efficiency (Figure 5G).

Altogether, the observation of a lack of reliance on t<sup>6</sup>A37 enables us to establish a full understanding of the roles of individual nucleotides in the anticodon loop in  $m^3C$ 32 biogenesis of mitochondrial tRNA. Our results showed that G35 is a critical determinant, and the sequence requirement of the anticodon loop is determined as follows: C32-(U/A/C)33-(C > U)34-G35-(U > A)36-(U > A = C > G)37-(A > U > C)38.

#### The newly evolved A29-C41 mismatch fine-tunes the $m^3C$ 32 level of hmtRNA<sup>Thr</sup>

An evolutionary analysis in our previous study identified that an A29-C41 mismatch was newly evolved in the mitochondrial tRNA<sup>Thr</sup> of human and gorilla but not those of other mammals, for unknown reasons (24). Based on the key role of the anticodon stem in  $m^3C$ 32 biogenesis by METTL2A, we wondered whether an A29-C41 mis-

match in hmtRNA<sup>Thr</sup> could influence  $m^3C$ 32 modification by METTL8-Iso1. We mutated either A29 to G29 or C41 to U41 (Supplementary Figure 5A). The resultant hmtRNA<sup>Thr</sup>-A29G and hmtRNA<sup>Thr</sup>-C41U have an anticodon stem with full Watson-Crick base pairing (G29-C41 or A29-U41). Tests of modification by METTL8-Iso1 indeed showed that both mutants were modified with a significantly higher efficiency (Supplementary Figure 5B).

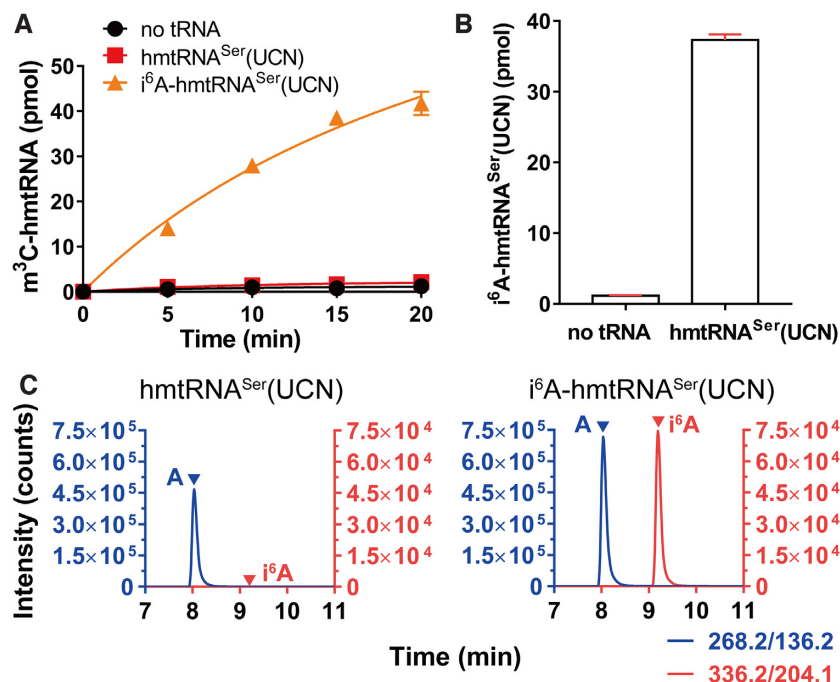
In agreement with these observations, the disease-causing G30A mutation, leading to disruption of the anticodon stem (24), abolished  $m^3C$ 32 modification (Supplementary Figure 5C). Our previous result also showed that in the context of the G30A mutation, an additional C40U mutation is able to restore the tRNA structure, thermal stability and aminoacylation capacity (24); however, hmtRNA<sup>Thr</sup>-G30A/C40U was still a poor substrate of METTL8-Iso1 (Supplementary Figure 5C). In addition, when we mutated A39 to G39, changing the U31-A39 Watson-Crick base pair to the U31-G39 wobble base pair, hmtRNA<sup>Thr</sup>-A39G was modified, but with reduced efficiency (Supplementary Figure 5C).

Taken together, these results suggest that the anticodon stem plays an important role in determining  $m^3C$ 32 biogenesis and that the newly evolved A29-C41 mismatch is able to fine-tune the  $m^3C$ 32 levels in hmtRNA<sup>Thr</sup>.

#### i<sup>6</sup>A37 is a positive determinant of $m^3C$ 32 biogenesis in hmtRNA<sup>Ser</sup>(UCN)

After establishing that METTL8-Iso1 is active in catalyzing hmtRNA<sup>Thr</sup>  $m^3C$ 32 formation, we investigated its





**Figure 6.** i<sup>6</sup>A modification is a prerequisite for m<sup>3</sup>C32 biogenesis in hmtRNA<sup>Ser</sup>(UCN) by METTL8-Iso1. (A) Time-course curves of the m<sup>3</sup>C32 modification of hmtRNA<sup>Ser</sup>(UCN) and i<sup>6</sup>A-hmtRNA<sup>Ser</sup>(UCN) by METTL8-Iso1. Negative controls (no tRNA) represent assays without tRNA added. (B) *EcMiaA* was able to efficiently modify hmtRNA<sup>Ser</sup>(UCN). (C) UPLC-MS/MS analysis of the digested product of *EcMiaA*-modified hmtRNA<sup>Ser</sup>(UCN). *m/z* value 268.2 to 136.2 (A) and *m/z* value 336.2 to 204.1 (i<sup>6</sup>A) are shown on the right. Data represent averages of three (A) or two (B) independent experiments and the corresponding SEM.

cipitated with METTL8-Iso1-FLAG. Furthermore, the addition of RNase to the whole cell lysate prior to co-IP had no effect on the copurification of both enzymes (Figure 7A). These results revealed that METTL8-Iso1 interacted with SARS2 in an RNA-independent mode, in contrast to the METTL2A-SARS1 interaction, which requires tRNA as a scaffold (13,16).

To understand whether the presence of SARS2 was able to stimulate the activity of METTL8-Iso1, mature SARS2 was purified (Supplementary Figure 6). We incubated increasing concentrations of SARS2 relative to METTL8-Iso1, and m<sup>3</sup>C32 modification activity assays showed that SARS2 only slightly elevated the modification of i<sup>6</sup>A-hmtRNA<sup>Ser</sup>(UCN) (Figure 7B). The highest activity of METTL8-Iso1 was observed at a relative molar ratio of 1:2 (METTL8-Iso1 to SARS2), and further elevation of the SARS2 concentration (molar ratio of METTL8-Iso1 to SARS2 of 1:5) provided little stimulatory activity. Again, the hmtRNA<sup>Ser</sup>(UCN) transcript was not a substrate of SARS2 and METTL8-Iso1 (Figure 7C), suggesting that the interaction of METTL8-Iso1 with SARS2 did not abrogate the prerequisite for i<sup>6</sup>A37 modification.

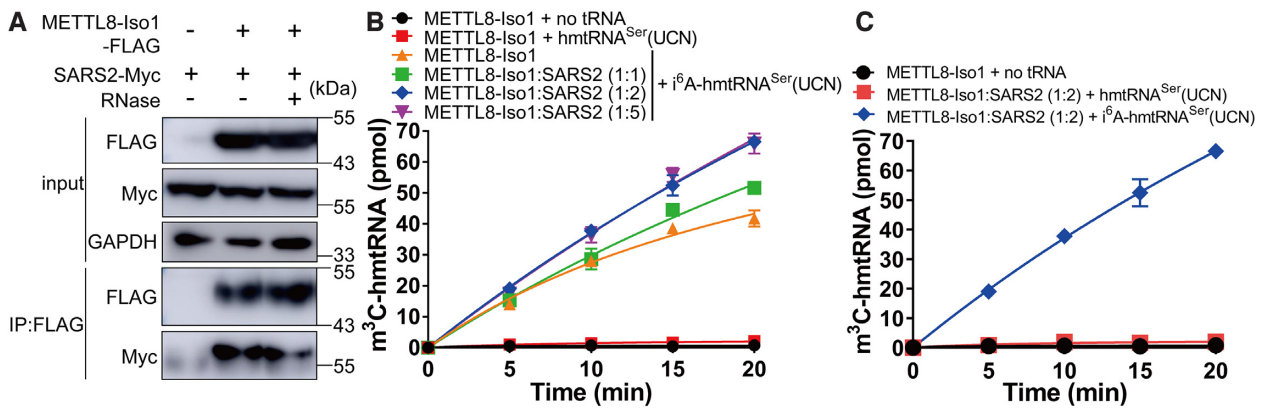
Altogether, these results showed that SARS2 interacted with METTL8-Iso1 directly but only slightly stimulated its modification activity.

### G35 is important but no longer a determinant in hmtRNA<sup>Ser</sup>(UCN)

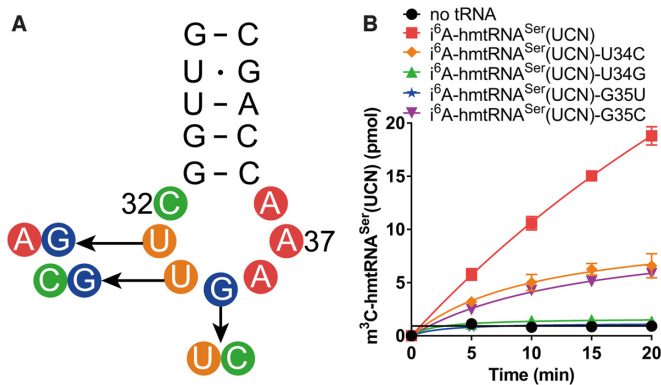
Considering the divergence in the requirement of t<sup>6</sup>A37 or i<sup>6</sup>A37 between hmtRNA<sup>Thr</sup> and hmtRNA<sup>Ser</sup>(UCN)

by METTL8-Iso1, we wanted to know whether there is difference in the role of nucleotides in the anticodon loop of hmtRNA<sup>Ser</sup>(UCN) in m<sup>3</sup>C32 biogenesis. Due to the preparation of the i<sup>6</sup>A37 modification, A36-A37-A38 could not be mutated (32). Therefore, we constructed six mutants of hmtRNA<sup>Ser</sup>(UCN), including hmtRNA<sup>Ser</sup>(UCN)-U33A, -U33G, -U34C, -U34G, -G35U and -G35C (Figure 8A). *EcMiaA* was unable to efficiently introduce i<sup>6</sup>A to hmtRNA<sup>Ser</sup>(UCN)-U33A and -U33G for unknown reasons; therefore, these two mutants were further excluded from the following assays. hmtRNA<sup>Ser</sup>(UCN)-U34C, -U34G, -G35U and -G35C exhibited slightly decreased i<sup>6</sup>A37 modification levels (within 2-fold) (Supplementary Figure 7A). After obtaining i<sup>6</sup>A-modified tRNA mutants, assays of m<sup>3</sup>C32 modification by mature METTL8-Iso1 showed that hmtRNA<sup>Ser</sup>(UCN)-U34C and -G35C were obviously modified, albeit with reduced efficiencies, which might be attributable to a small decrease in i<sup>6</sup>A modification levels (Supplementary Figure 7A). In sharp contrast, both hmtRNA<sup>Ser</sup>(UCN)-U34G and -G35U were not modified (Figure 8B), in spite of their comparable i<sup>6</sup>A modification efficiencies with those of hmtRNA<sup>Ser</sup>(UCN)-U34C and -G35C (Supplementary Figure 7A). Notably, hmtRNA<sup>Thr</sup>-U34C displayed a higher modification efficiency than wild-type hmtRNA<sup>Thr</sup>, and none of the hmtRNA<sup>Thr</sup> G35 mutants were modified (Figure 5C and D). These data collectively revealed divergent recognition of hmtRNA<sup>Thr</sup> and hmtRNA<sup>Ser</sup>(UCN) by METTL8-Iso1 and showed that G35 in hmtRNA<sup>Ser</sup>(UCN), while important, is not a determinant of m<sup>3</sup>C32 modification.





**Figure 7.** SARS2 interacts with METTL8-Iso1 directly but does not determine the  $m^3C32$  modification activity of METTL8-Iso1. (A) Genes encoding METTL8-Iso1-FLAG and SARS2-Myc were coexpressed in HEK293T cells. SARS2-Myc was precipitated by METTL8-Iso1-FLAG using anti-FLAG antibodies. The addition of RNase had no effect on the METTL8-Iso1 and SARS2 interaction. (B) Time-course curves of the  $m^3C32$  modification of  $i^6A$ -hmtRNA<sup>Ser</sup>(UCN) by METTL8-Iso1 and different concentrations of SARS2 as indicated (METTL8-Iso1 to SARS2 mole ratio 1:1, 1:2, or 1:5). Two negative controls (modification by METTL8-Iso1 alone without (METTL8-Iso1 + no tRNA) or with (METTL8-Iso1 + hmtRNA<sup>Ser</sup>(UCN)) tRNA transcript added) were included. (C) Time-course curves of the  $m^3C32$  modification of hmtRNA<sup>Ser</sup>(UCN) transcript by METTL8-Iso1 and SARS2 (1:2). Negative controls (METTL8-Iso1 + no tRNA) represent assays without tRNA added. Modification of  $i^6A$ -hmtRNA<sup>Ser</sup>(UCN) by METTL8-Iso1:SARS2 (1:2) was included as a positive control. Data represent averages of two (B) or three (C) independent experiments and the corresponding SEM.



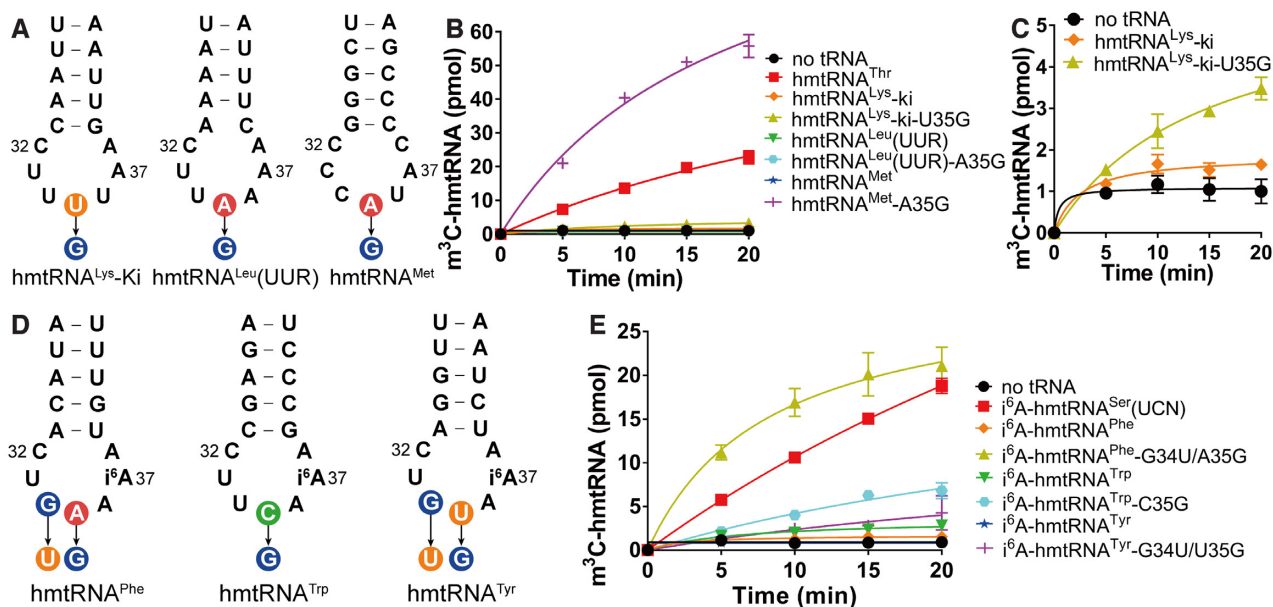
**Figure 8.** G35 is important but is not a determinant in  $i^6A$ -hmtRNA<sup>Ser</sup>(UCN). (A) Point mutations targeting the anticodon loop of hmtRNA<sup>Ser</sup>(UCN). (B) Time-course curves of the  $m^3C32$  modification of  $i^6A$ -hmtRNA<sup>Ser</sup>(UCN)-U34C, -U34G, -G35U and -G35C. Negative controls (no tRNA) represent assays without tRNA added. Positive controls ( $i^6A$ -hmtRNA<sup>Ser</sup>(UCN)) represent modification of  $i^6A$ -hmtRNA<sup>Ser</sup>(UCN). Data represent averages of two independent experiments and the corresponding SEM.

### Converting other mitochondrial tRNAs to be substrates of METTL8-Iso1

Based on the requirement for C32-(U/A/C)33-(C > U)34-G35-(U > A)36-(U > A = C > G)37-(A > U > C)38 in the anticodon of hmtRNA<sup>Thr</sup> for  $m^3C32$  formation by METTL8-Iso1, we checked the mitochondrial tRNA sequences and found that several tRNAs harbor a similar anticodon loop sequence lacking only the G35 nucleotide, such as hmtRNA<sup>Lys</sup>, hmtRNA<sup>Leu</sup>(UUR) and hmtRNA<sup>Met</sup> (Figure 9A). Thus, we mutated base 35 of these tRNAs to G35 to produce hmtRNA<sup>Lys</sup>-Ki-G35, hmtRNA<sup>Leu</sup>(UUR)-G35 and hmtRNA<sup>Met</sup>-G35 (Figure 9A). The wild-type tRNAs and their mutants were subsequently modified by METTL8-Iso1, and the results showed that hmtRNA<sup>Met</sup>-

G35 was modified with an obviously higher efficiency than hmtRNA<sup>Thr</sup> (Figure 9B). In parallel, hmtRNA<sup>Lys</sup>-Ki-G35 was also modified, but the efficiency was quite low (Figure 9B and C). However, METTL8-Iso1 was unable to introduce  $m^3C$  modification into tRNA<sup>Leu</sup>(UUR)-G35 (Figure 9B). These results suggested that METTL8 discriminates among some mitochondrial tRNAs via the presence or absence of G35, at least for hmtRNA<sup>Lys</sup> and hmtRNA<sup>Met</sup>. Differences in modification efficiency among various tRNAs were probably due to differences in the sequences and/or conformations of the anticodon stem, which has been proven to be an important element for  $m^3C$  biogenesis in hmtRNA<sup>Thr</sup>. On other hand, modifications in the anticodon loop of hmtRNA<sup>Lys</sup> (5-taurinomethyl-2-thiouridine at position 34,  $\tau m^5s^2U34$ ) and hmtRNA<sup>Met</sup> (5-formylcytidine at position 34,  $f^5C34$ ) (9) may also facilitate discrimination *in vivo*.

Several human mitochondrial tRNAs harbor the  $i^6A37$  modification, including hmtRNA<sup>Tyr</sup>, hmtRNA<sup>Phe</sup> and hmtRNA<sup>Trp</sup>, in addition to hmtRNA<sup>Ser</sup>(UCN) (9,21) (Figure 9D). This poses the question of how METTL8-Iso1 discriminates hmtRNA<sup>Ser</sup>(UCN) from these  $i^6A37$ -harboring tRNAs. *EcMiaA* was able to modify hmtRNA<sup>Tyr</sup>, hmtRNA<sup>Phe</sup> and hmtRNA<sup>Trp</sup> albeit with variable efficiencies (Supplementary Figure 7B). We obtained  $i^6A$ -hmtRNA<sup>Tyr</sup>,  $i^6A$ -hmtRNA<sup>Phe</sup> and  $i^6A$ -hmtRNA<sup>Trp</sup> using *EcMiaA* and found that METTL8-Iso1 was indeed unable to modify  $i^6A$ -hmtRNA<sup>Tyr</sup> and  $i^6A$ -hmtRNA<sup>Phe</sup> (Figure 9E). However,  $i^6A$ -hmtRNA<sup>Trp</sup> was modified, albeit with a much lower efficiency than  $i^6A$ -hmtRNA<sup>Ser</sup>(UCN). Among these three tRNAs, only hmtRNA<sup>Trp</sup> harbors C35 (A35 in hmtRNA<sup>Phe</sup> and U35 in hmtRNA<sup>Tyr</sup>). These results were consistent with our earlier data showing that  $i^6A$ -modified tRNA<sup>Ser</sup>(UCN)-G35C (with C35) could be modified by METTL8-Iso1 (Figure 8B). Furthermore, we constructed hmtRNA<sup>Phe</sup>-G34U/A35G, hmtRNA<sup>Tyr</sup>-G34U/U35G and hmtRNA<sup>Trp</sup>-C35G mutants to mimic the anticodon loop of hmtRNA<sup>Ser</sup>(UCN)



**Figure 9.** Discrimination of hmtRNA<sup>Thr</sup> and hmtRNA<sup>Ser</sup>(UCN) from nonsubstrate tRNAs by METTL8-Iso1. Point mutations targeting the anticodon loop of hmtRNA<sup>Lys</sup>, hmtRNA<sup>Leu</sup>(UUR) and hmtRNA<sup>Met</sup> (A) or hmtRNA<sup>Phe</sup>, hmtRNA<sup>Trp</sup> and hmtRNA<sup>Tyr</sup> (D). Time-course curves of the m<sup>3</sup>C32 modification of hmtRNA<sup>Thr</sup> (positive control), hmtRNA<sup>Lys</sup>-Ki, hmtRNA<sup>Lys</sup>-Ki-U35G, hmtRNA<sup>Leu</sup>(UUR), hmtRNA<sup>Leu</sup>(UUR)-A35G, hmtRNA<sup>Met</sup>, and hmtRNA<sup>Met</sup>-A35G (B); Modification of hmtRNA<sup>Lys</sup>-Ki and hmtRNA<sup>Lys</sup>-Ki-U35G are shown independently in (C) for clarity. Time-course curves of the m<sup>3</sup>C32 modification of i<sup>6</sup>A-hmtRNA<sup>Ser</sup>(UCN) (positive control), i<sup>6</sup>A-hmtRNA<sup>Phe</sup>, i<sup>6</sup>A-hmtRNA<sup>Phe</sup>-G34U/A35G, i<sup>6</sup>A-hmtRNA<sup>Trp</sup>, i<sup>6</sup>A-hmtRNA<sup>Trp</sup>-C35G, i<sup>6</sup>A-hmtRNA<sup>Tyr</sup>, and i<sup>6</sup>A-hmtRNA<sup>Tyr</sup>-G34U/U35G (E). Negative controls (no tRNA) represent assays without tRNA added in (B, C, E). Data represent averages of three (B and C) or two (E) independent experiments and the corresponding SEM.

(Figure 9D). All these mutants were efficiently i<sup>6</sup>A-modified by *EcMiaA* compared with wild-type tRNAs (Supplementary Figure 7B). Indeed, METTL8-Iso1 generated even more m<sup>3</sup>C32 in i<sup>6</sup>A-hmtRNA<sup>Phe</sup>-G34U/A35G than in i<sup>6</sup>A-hmtRNA<sup>Ser</sup>(UCN). Likewise, the modification efficiency of i<sup>6</sup>A-hmtRNA<sup>Trp</sup>-C35G was significantly higher than that of i<sup>6</sup>A-hmtRNA<sup>Trp</sup> (Figure 9E). Notably, hmtRNA<sup>Phe</sup>-G34U/A35G exhibited only a small increase in i<sup>6</sup>A modification level in comparison with wild-type hmtRNA<sup>Phe</sup>; similarly, hmtRNA<sup>Trp</sup>-C35G displayed only a small decrease in i<sup>6</sup>A modification level when compared with wild-type hmtRNA<sup>Trp</sup> (Supplementary Figure 7B). Additionally, despite the hypomodification of i<sup>6</sup>A-hmtRNA<sup>Tyr</sup>, the modification of i<sup>6</sup>A-hmtRNA<sup>Tyr</sup>-G34U/U35G was obvious. Possibly, the significant increase in i<sup>6</sup>A modification of hmtRNA<sup>Tyr</sup>-G34U/U35G (Supplementary Figure 7B) also partially contributed to stimulated m<sup>3</sup>C modification (Figure 9E). All these results clearly revealed how METTL8-Iso1 efficiently discriminates hmtRNA<sup>Ser</sup>(UCN) from other tRNAs in the presence of i<sup>6</sup>A37, mainly based on the tRNA anticodon loop. *In vivo*, m<sup>3</sup>C32 is absent in hmtRNA<sup>Trp</sup> (9). Our observation that i<sup>6</sup>A-hmtRNA<sup>Trp</sup> could be m<sup>3</sup>C-modified by METTL8-Iso1 *in vitro* suggested that other modifications in hmtRNA<sup>Trp</sup>, especially those at the anticodon loop, including  $\tau$ m<sup>5</sup>s<sup>2</sup>U at position 34 and/or downstream modification of i<sup>6</sup>A at position 37 (2-methylthio-N<sup>6</sup>-isopentenyladenosine, ms<sup>2</sup>i<sup>6</sup>A) (9), may prevent hmtRNA<sup>Trp</sup> from being modified by METTL8-Iso1 *in vivo*.

## DISCUSSION

Although the types of tRNA modifications in both mammalian cytoplasmic and mitochondrial tRNAs have been

remarkably expanded due to the rapid development of RNA mass spectrometry methodologies in recent years, the identification and confirmation of the corresponding tRNA-modifying enzymes has lagged behind. Indeed, genes have not yet been identified for ~23% of the >100 human tRNA modifying enzymes that are predicted to exist (40). Among these missing genes, the mitochondrial tRNA m<sup>3</sup>C32 methyltransferase has long been uncertain. The identification of Mettl2 and Mettl6 as mouse tRNA m<sup>3</sup>C32-modifying enzymes and the exclusion of Mettl8 from tRNA modification led to the hypothesis that METTL2A, METTL2B and METTL6 in human cells are missing enzymes (13,40). However, the exclusive cytoplasmic localization of METTL2A/2B and METTL6 makes them unlikely to be responsible for mitochondrial tRNA modification (16). A recent study revealed that METTL8 is the missing mitochondrial m<sup>3</sup>C modifying enzyme (23). Here, we provide further evidence, based on mRNA and protein analyses and reconstituted activity, that a METTL8 isoform mediates hmtRNA<sup>Thr</sup> and hmtRNA<sup>Ser</sup>(UCN) m<sup>3</sup>C32 biogenesis.

Observations of mRNA modification (13), the involvement of METTL8 in rRNA-mediated R-loop biogenesis (14), and the presence of METTL8 in the mitochondrial proteome (22) led to confusion about the exact localization of METTL8 and its function in RNA posttranscriptional modification. We demonstrated that the METTL8 gene generates multiple protein isoforms through alternative mRNA splicing and subsequent translation. The longest isoform, METTL8-Iso1, contains a 22-amino acid MTS that targets it to the mitochondria. Other isoforms, including METTL8-Iso4, are localized to the nucleolus, consistent with results obtained by others (14). The distribution

of METTL8 in the nucleolus suggests its possible role in R-loop biogenesis by modifying some yet unidentified nucleic acid constituents (Figure 10). It is puzzling that METTL8-Iso3, if present, differs from METTL8-Iso4 in only the very N-terminal five residues (<sup>1</sup>MPRDH<sup>5</sup>). Both localized in the nucleolus, but whether they exhibit similar or differential methyltransferase activity is unclear at present. Moreover, METTL8-Iso1 contains an in-frame Met residue (Met<sup>51</sup>) when compared with the initiating Met of METTL8-Iso4. We cannot absolutely exclude the possibility that mRNA 1v2 (encoding METTL8-Iso1) is able to generate METTL8-Iso4 via translational re-initiation. However, fluorescence reporter assays show that ectopic expression of mRNA 1v2 in HEK293T cells drives mitochondrial localization, suggesting that this possibility, if it occurs, is low. In addition, it is worth noting that truncated METTL8 isoforms terminating in the middle of the SAM-binding site due to skipping of exon 10 were also potentially translated in human cells. The biological significance of these noncanonical METTL8 isoforms, which likely cannot catalyze m<sup>3</sup>C32 biogenesis due to their incomplete SAM- and tRNA-binding domain, needs to be further explored. Possibly, METTL8-Iso4 and/or the noncanonical METTL8 isoforms are involved in mRNA m<sup>3</sup>C modification.

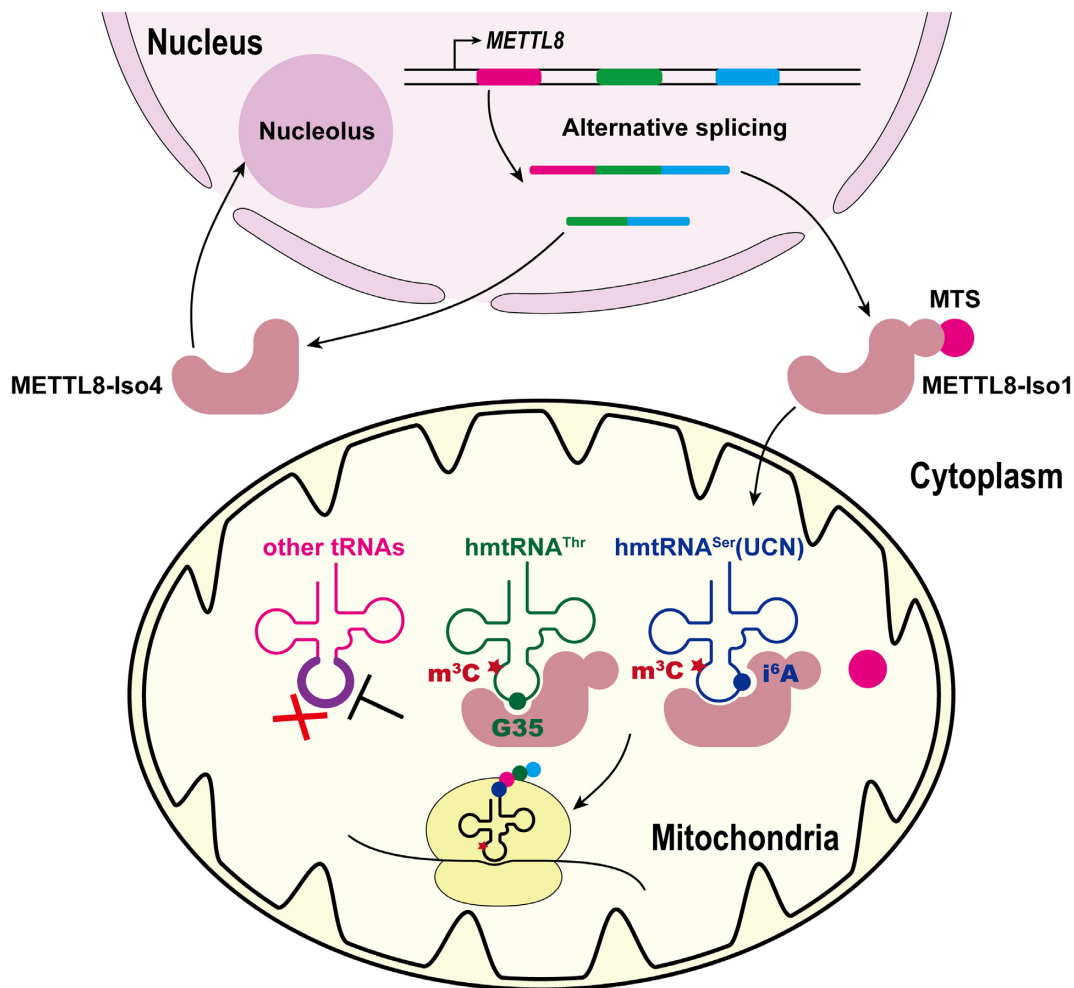
Regarding the modification mechanism, METTL8-Iso1 exhibits both similarity to and divergence from both METTL2A and METTL6. For the modification of cytoplasmic tRNA<sup>Thr</sup>, METTL2A is completely reliant on the preformed t<sup>6</sup>A37 modification (16) catalyzed by YRDC and KEOPS in the cytoplasm (31,39,41); however, METTL8-mediated mitochondrial m<sup>3</sup>C32 biogenesis is independent of the formation of the t<sup>6</sup>A modification. On the other hand, both METTL8 and METTL2A require an anticodon loop, especially the positive determinant G35, for efficient m<sup>3</sup>C32 formation. Anticodon stems also contribute to m<sup>3</sup>C32 modification by both enzymes (16). For the modification of cytoplasmic tRNA<sup>Ser</sup>(GCU), METTL6 requires the presence of SARS1 to generate m<sup>3</sup>C32. SARS1 binds the long variable stem and loop region of cytoplasmic tRNA<sup>Ser</sup>(GCU). Indeed, Ue3 and the U-Ae12 base pair of tRNA<sup>Ser</sup>(GCU) are two elements in the variable stem that are critical determinants for efficient m<sup>3</sup>C32 modification by METTL6-SARS1 (16). However, we showed here that METTL8-Iso1 alone is able to mediate mitochondrial tRNA modification, suggesting that the activity of METTL8-Iso1 is not dependent on other helper proteins. Furthermore, METTL6 requires cytoplasmic tRNA<sup>Ser</sup>(GCU) as a scaffold to interact with SARS1; indeed, SARS2 interacts with METTL8-Iso1 but in a tRNA-independent manner, and the presence of SARS2 did not significantly stimulate METTL8-Iso1 activity. Notably, both mitochondrial tRNA<sup>Ser</sup> isoacceptors are unique due to the absence of the long variable arm, suggesting that SARS2 probably does not play a determinative role by binding to a long variable arm. This might explain why METTL8-Iso1 activity is independent of the presence of SARS2. Finally, we have proposed that the lack of the N-terminal domain of METTL6 is responsible for its reliance on the presence of SARS1 (16). METTL8-Iso1, like METTL2A, contains a long N-terminal domain compared with METTL6. The presence of this domain also

likely explains why METTL8-Iso1 alone is an active m<sup>3</sup>C32 methyltransferase.

We also revealed an obvious divergence between the recognition of hmtRNA<sup>Thr</sup> and hmtRNA<sup>Ser</sup>(UCN) by METTL8-Iso1. METTL8-Iso1-mediated m<sup>3</sup>C32 biogenesis depends on i<sup>6</sup>A37 in hmtRNA<sup>Ser</sup>(UCN) but not its equivalent t<sup>6</sup>A37 in hmtRNA<sup>Thr</sup>. On the other hand, G35 plays a determinative role in hmtRNA<sup>Thr</sup> but not in hmtRNA<sup>Ser</sup>(CUN) (Figure 10). Based on these observations, we further determined how METTL8-Iso1 recognized only hmtRNA<sup>Thr</sup> and hmtRNA<sup>Ser</sup>(UCN) in human mitochondria. The most important discriminator seems to be G35. After the introduction of G35, both hmtRNA<sup>Met</sup> and hmtRNA<sup>Lys</sup> were found to be good substrates of METTL8-Iso1, albeit with variable modification efficiencies. On the other hand, with a determinative i<sup>6</sup>A37 modification, METTL8-Iso1 selected hmtRNA<sup>Ser</sup>(UCN), among other i<sup>6</sup>A-modified tRNAs, mainly using the anticodon loop. Indeed, when the anticodon loops of hmtRNA<sup>Phe</sup> and hmtRNA<sup>Tyr</sup> are changed, they become excellent substrates of METTL8-Iso1.

Identification of METTL8 being a mitochondrial m<sup>3</sup>C methyltransferase was first reported by Schöller and colleagues. They further reported that lack of m<sup>3</sup>C impaired mitochondrial translation and respiratory chain complex assembly and activity. In addition, increased METTL8 level was observed in pancreatic cancer (23). After submission of this study, a more recent work by Kleiber *et al.* also reported that METTL8 was responsible for catalyzing m<sup>3</sup>C32 biogenesis of hmtRNA<sup>Thr</sup> and hmtRNA<sup>Ser</sup>(UCN). They revealed the tRNA recognition and selective mechanisms of METTL8 and also found METTL8 knockout led to impaired mitochondrial translation. Both U34 and G35 were found to be recognition elements of METTL8 for hmtRNA<sup>Thr</sup>; however, only the U34A mutant was constructed (42). Our study indeed found hmtRNA<sup>Thr</sup>-U34A was not modified by mature METTL8-Iso1; however, multiple mutations at U34 suggested that it was not a determinant in m<sup>3</sup>C32 modification, because hmtRNA<sup>Thr</sup>-U34C was modified with even a higher efficiency. Notably, both studies used the 407-aa METTL8 (23,42), exactly the METTL8-Iso1 identified in this work. However, our study investigated the splicing pattern of METTL8 mRNA in detail and observed alternative splicing modes generating several protein isoforms. Besides the METTL8-Iso1, METTL8-Iso4 was localized in nucleolus. Our study experimentally identified the MTS (Met<sup>1</sup>-Gln<sup>22</sup>) of METTL8-Iso1, different from the predicted MTS (Met<sup>1</sup>-Arg<sup>20</sup>) in both studies (23,42). In addition, our *in vitro* study showed that t<sup>6</sup>A37 modification of hmtRNA<sup>Thr</sup> only slightly stimulated m<sup>3</sup>C32 modification activity of mature METTL8-Iso1; however, Kleiber *et al.* reported that t<sup>6</sup>A37 increased m<sup>3</sup>C32 modification about 2-fold (42). It is possible that the stimulatory role of t<sup>6</sup>A37 is more pronounced *in vivo*. It is notable that all three studies demonstrated m<sup>3</sup>C32 modification of hmtRNA<sup>Ser</sup>(UCN) was critically reliant on i<sup>6</sup>A37 modification (23,42); however, our data revealed different recognition mechanisms of mature METTL8-Iso1 for hmtRNA<sup>Thr</sup> and hmtRNA<sup>Ser</sup>(UCN). Moreover, we found that SARS2 interacted with METTL8-Iso1 directly but did not determine m<sup>3</sup>C32 modification activity of the latter.





**Figure 10.** Expression of human METTL8 and its selective  $m^3C$  modification of  $hmtRNA^{Thr}$  and  $hmtRNA^{Ser(UCN)}$ . METTL8 generates multiple mRNA isoforms via alternative splicing. Some protein isoforms, including METTL8-Iso4, are distributed in the nucleolus with unclear function. A longer isoform, METTL8-Iso1, containing a 22-amino acid MTS, is imported into mitochondria. Mature METTL8-Iso1 selects  $hmtRNA^{Thr}$  mainly via recognition of the tRNA anticodon loop and stem, particularly G35, and selects  $hmtRNA^{Ser(UCN)}$  via a preformed  $i^6A$  moiety, in addition to the anticodon loop. Non-substrate tRNAs are discriminated by mature METTL8-Iso1 due to unfavorable sequences and/or modifications in the anticodon loop.

In summary, our results clearly revealed that METTL8 generated a mitochondria-localized isoform to mediate human mitochondrial tRNA  $m^3C$ 32 modification. Reconstitution of mitochondrial tRNA  $m^3C$ 32 modification activity provides valuable insights into the substrate recognition and mechanism of mitochondrial  $m^3C$ 32 biogenesis and a full understanding of both the similarities and differences between cytoplasmic and mitochondrial  $m^3C$ 32 modification by multiple  $m^3C$ 32 transferases (16). The roles of the other METTL8 isoforms would benefit from further in-depth investigation.

#### DATA AVAILABILITY

All data presented in this study are available within the Figures and in the Supplementary Data.

#### SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

#### ACKNOWLEDGEMENTS

We thank Dr Howard Gamper (Thomas Jefferson University) for carefully reading the manuscript. We are grateful to Dr Liang Cheng (Institute of Chemistry, CAS) for providing  $i^6A$  standard sample. We thank Dr Mo-Fang Liu in our institute for providing FBL antibody. We also thank the molecular biology core facility of our institute for technical support in UPLC-MS/MS analysis.

#### FUNDING

National Key Research and Development Program of China [2017YFA0504000, 2021YFA1300800, 2021YFC2700903]; Natural Science Foundation of China [91940302, 31870811, 31822015, 81870896, 31670801, 32000889]; Strategic Priority Research Program of the Chinese Academy of Sciences [XDB19010203]; Committee of Science and Technology in Shanghai [22ZR1481300]; Shanghai Key Laboratory of Embryo Original Diseases [Shelab201904]. Funding for open access charge: Natu-

ral Science Foundation of China [31822015, 81870896, 31670801].

*Conflict of interest statement.* None declared

## REFERENCES

- McCown,P.J., Ruszkowska,A., Kunkler,C.N., Breger,K., Hulewicz,J.P., Wang,M.C., Springer,N.A. and Brown,J.A. (2020) Naturally occurring modified ribonucleosides. *Wiley Interdiscip. Rev. RNA*, **11**, e1595.
- Suzuki,T. (2021) The expanding world of tRNA modifications and their disease relevance. *Nat. Rev. Mol. Cell. Biol.*, **22**, 375–392.
- 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.
- Zhou,J.B., Wang,E.D. and Zhou,X.L. (2021) Modifications of the human tRNA anticodon loop and their associations with genetic diseases. *Cell Mol. Life Sci.*, **78**, 7087–7105.
- Noma,A., Yi,S., Katoh,T., Takai,Y., Suzuki,T. and Suzuki,T. (2011) Actin-binding protein ABP140 is a methyltransferase for 3-methylcytidine at position 32 of tRNAs in *saccharomyces cerevisiae*. *RNA*, **17**, 1111–1119.
- D’Silva,S., Haider,S.J. and Phizicky,E.M. (2011) A domain of the actin binding protein abp140 is the yeast methyltransferase responsible for 3-methylcytidine modification in the tRNA anti-codon loop. *RNA*, **17**, 1100–1110.
- Lentini,J.M., Alsaif,H.S., Faqeh,E., Alkuraya,F.S. and Fu,D. (2020) *DALRD3* encodes a protein mutated in epileptic encephalopathy that targets arginine tRNAs for 3-methylcytosine modification. *Nat. Commun.*, **11**, 2510.
- Suzuki,T. and Suzuki,T. (2014) A complete landscape of post-transcriptional modifications in mammalian mitochondrial tRNAs. *Nucleic Acids Res.*, **42**, 7346–7357.
- Suzuki,T., Yashiro,Y., Kikuchi,I., Ishigami,Y., Saito,H., Matsuzawa,I., Okada,S., Mito,M., Iwasaki,S., Ma,D. et al. (2020) Complete chemical structures of human mitochondrial tRNAs. *Nat. Commun.*, **11**, 4269.
- Ignatova,V.V., Kaiser,S., Ho,J.S.Y., Bing,X., Stolz,P., Tan,Y.X., Lee,C.L., Gay,F.P.H., Lastres,P.R., Gerlini,R. et al. (2020) METTL6 is a tRNA m<sup>3</sup>C methyltransferase that regulates pluripotency and tumor cell growth. *Sci. Adv.*, **6**, eaaz4551.
- Arimbasseri,A.G., Iben,J., Wei,F.Y., Rijal,K., Tomizawa,K., Hafner,M. and Marañia,R.J. (2016) Evolving specificity of tRNA 3-methyl-cytidine-32 (m<sup>3</sup>C32) modification: a subset of tRNAs<sup>Ser</sup> requires N<sup>6</sup>-isopentenyladenosine A37. *RNA*, **22**, 1400–1410.
- Han,L., Marcus,E., D’Silva,S. and Phizicky,E.M. (2017) *S. cerevisiae* trm140 has two recognition modes for 3-methylcytidine modification of the anticodon loop of tRNA substrates. *RNA*, **23**, 406–419.
- Xu,L., Liu,X., Sheng,N., Oo,K.S., Liang,J., Chionh,Y.H., Xu,J., Ye,F., Gao,Y.G., Dedon,P.C. et al. (2017) Three distinct 3-methylcytidine (m<sup>3</sup>C) methyltransferases modify tRNA and mRNA in mice and humans. *J. Biol. Chem.*, **292**, 14695–14703.
- Zhang,L.H., Zhang,X.Y., Hu,T., Chen,X.Y., Li,J.J., Raida,M., Sun,N., Luo,Y. and Gao,X. (2020) The SUMOylated METTL8 induces R-loop and tumorigenesis via m<sup>3</sup>c. *iScience*, **23**, 100968.
- Cui,J., Liu,Q., Sendinc,E., Shi,Y. and Gregory,R.I. (2021) Nucleotide resolution profiling of m<sup>3</sup>C RNA modification by HAC-seq. *Nucleic Acids Res.*, **49**, e27.
- Mao,X.L., Li,Z.H., Huang,M.H., Wang,J.T., Zhou,J.B., Li,Q.R., Xu,H., Wang,X.J. and Zhou,X.L. (2021) Mutually exclusive substrate selection strategy by human m<sup>3</sup>C RNA transferases METTL2A and METTL6. *Nucleic Acids Res.*, **49**, 8309–8323.
- Chen,R., Zhou,J., Liu,L., Mao,X.L., Zhou,X. and Xie,W. (2021) Crystal structure of human METTL6, the m<sup>3</sup>C methyltransferase. *Commun. Biol.*, **4**, 1361.
- Lin,H., Miyauchi,K., Harada,T., Okita,R., Takeshita,E., Komaki,H., Fujioka,K., Yagasaki,H., Goto,Y.I., Yanaka,K. et al. (2018) CO<sub>2</sub>-sensitive tRNA modification associated with human mitochondrial disease. *Nat. Commun.*, **9**, 1875.
- Zhou,J.B., Wang,Y., Zeng,Q.Y., Meng,S.X., Wang,E.D. and Zhou,X.L. (2020) Molecular basis for t<sup>6</sup>A modification in human mitochondria. *Nucleic Acids Res.*, **48**, 3181–3194.
- Golovko,A., Hjalms,G., Sitbon,F. and Nicander,B. (2000) Cloning of a human tRNA isopentenyl transferase. *Gene*, **258**, 85–93.
- Lamichhane,T.N., Mattijssen,S. and Marañia,R.J. (2013) Human cells have a limited set of tRNA anticodon loop substrates of the tRNA isopentenyltransferase TRIT1 tumor suppressor. *Mol. Cell Biol.*, **33**, 4900–4908.
- Rath,S., Sharma,R., Gupta,R., Ast,T., Chan,C., Durham,T.J., Goodman,R.P., Grabarek,Z., Haas,M.E., Hung,W.H.W. et al. (2021) MitoCarta3.0: an updated mitochondrial proteome now with sub-organellar localization and pathway annotations. *Nucleic Acids Res.*, **49**, D1541–D1547.
- Scholler,E., Marks,J., Marchand,V., Bruckmann,A., Powell,C.A., Reichold,M., Mutti,C.D., Dettmer,K., Feederle,R., Huttelmaier,S. et al. (2021) Balancing of mitochondrial translation through METTL8-mediated m<sup>3</sup>C modification of mitochondrial tRNAs. *Mol. Cell*, **81**, 4810–4825.
- Wang,Y., Zeng,Q.Y., Zheng,W.Q., Ji,Q.Q., Zhou,X.L. and Wang,E.D. (2018) A natural non-Watson-Crick base pair in human mitochondrial tRNA<sup>Thr</sup> causes structural and functional susceptibility to local mutations. *Nucleic Acids Res.*, **46**, 4662–4676.
- Collinet,B., Friberg,A., Brooks,M.A., van den Elzen,T., Henriot,V., Dziembowski,A., Graille,M., Durand,D., Leulliot,N., Saint Andre,C. et al. (2011) Strategies for the structural analysis of multi-protein complexes: lessons from the 3D-Repertoire project. *J. Struct. Biol.*, **175**, 147–158.
- Wang,Y., Zhou,J.B., Zeng,Q.Y., Wu,S.Q., Xue,M.Q., Fang,P.F., Wang,E.D. and Zhou,X.L. (2020) Hearing impairment-associated *KARS* mutations lead to defects in aminoacylation of both cytoplasmic and mitochondrial tRNA<sup>Lys</sup>. *Sci. China Life Sci.*, **63**, 1227–1239.
- Fechter,P., Rudinger,J., Giege,R. and Theobald-Dietrich,A. (1998) Ribozyme processed tRNA transcripts with unfriendly internal promoter for T7 RNA polymerase: production and activity. *FEBS Lett.*, **436**, 99–103.
- Sissler,M., Helm,M., Frugier,M., Giege,R. and Florentz,C. (2004) Aminoacylation properties of pathology-related human mitochondrial tRNA(Lys) variants. *RNA*, **10**, 841–853.
- Zeng,Q.Y., Peng,G.X., Li,G., Zhou,J.B., Zheng,W.Q., Xue,M.Q., Wang,E.D. and Zhou,X.L. (2019) The G3-U70-independent tRNA recognition by human mitochondrial alanyl-tRNA synthetase. *Nucleic Acids Res.*, **47**, 3072–3085.
- El Yacoubi,B., Lyons,B., Cruz,Y., Reddy,R., Nordin,B., Agnelli,F., Williamson,J.R., Schimmel,P., Swairjo,M.A. and de Crécy-Lagard,V. (2009) The universal yrc/Sua5 family is required for the formation of threonylcarbamoyladenine in tRNA. *Nucleic Acids Res.*, **37**, 2894–2909.
- El Yacoubi,B., Hatin,I., Deutsch,C., Kahveci,T., Rousset,J.P., Iwata-Reuyl,D., Murzin,A.G. and de Crécy-Lagard,V. (2011) A role for the universal Kae1/Qri7/YgiD (COG0533) family in tRNA modification. *EMBO J.*, **30**, 882–893.
- Soderberg,T. and Poulter,C.D. (2000) *Escherichia coli* dimethylallyl diphosphate:tRNA dimethylallyltransferase: essential elements for recognition of tRNA substrates within the anticodon stem-loop. *Biochemistry*, **39**, 6546–6553.
- Zhou,X.L., Chen,Y., Zeng,Q.Y., Ruan,Z.R., Fang,P. and Wang,E.D. (2019) Newly acquired N-terminal extension targets threonyl-tRNA synthetase-like protein into the multiple tRNA synthetase complex. *Nucleic Acids Res.*, **47**, 8662–8674.
- Claros,M.G. and Vincens,P. (1996) Computational method to predict mitochondrially imported proteins and their targeting sequences. *Eur. J. Biochem.*, **241**, 779–786.
- Yao,Y.N., Wang,L., Wu,X.F. and Wang,E.D. (2003) The processing of human mitochondrial leucyl-tRNA synthetase in the insect cells. *FEBS Lett.*, **534**, 139–142.
- Wang,Y., Zhou,X.L., Ruan,Z.R., Liu,R.J., Eriani,G. and Wang,E.D. (2016) A human disease-causing point mutation in mitochondrial threonyl-tRNA synthetase induces both structural and functional defects. *J. Biol. Chem.*, **291**, 6507–6520.
- Carapito,C., Kuhn,L., Karim,L., Rompals,M., Rabilloud,T., Schwenger,H. and Sissler,M. (2017) Two proteomic methodologies for defining N-termini of mature human mitochondrial aminoacyl-tRNA synthetases. *Methods*, **113**, 111–119.
- Peng,G.X., Zhang,Y., Wang,Q.Q., Li,Q.R., Xu,H., Wang,E.D. and Zhou,X.L. (2021) The human tRNA taurine modification enzyme

- GTPBP3 is an active GTPase linked to mitochondrial diseases. *Nucleic Acids Res.*, **49**, 2816–2834.
39. Wang, J.T., Zhou, J.B., Mao, X.L., Zhou, L., Chen, M., Zhang, W., Wang, E.D. and Zhou, X.L. (2022) Commonality and diversity in tRNA substrate recognition in t<sup>6</sup>A biogenesis by eukaryotic KEOPs. *Nucleic Acids Res.*, **50**, 2223–2239.
40. de Crecy-Lagard, V., Boccaletto, P., Mangleburg, C.G., Sharma, P., Lowe, T.M., Leidel, S.A. and Bujnicki, J.M. (2019) Matching tRNA modifications in humans to their known and predicted enzymes. *Nucleic Acids Res.*, **47**, 2143–2159.
41. Srinivasan, M., Mehta, P., Yu, Y., Prugar, E., Koonin, E.V., Karzai, A.W. and Sternglanz, R. (2011) The highly conserved KEOPS/EKC complex is essential for a universal tRNA modification, t<sup>6</sup>A. *EMBO J.*, **30**, 873–881.
42. Kleiber, N., Lemus-Diaz, N., Stiller, C., Heinrichs, M., Mai, M.M., Hackert, P., Richter-Dennerlein, R., Hobartner, C., Bohnsack, K.E. and Bohnsack, M.T. (2022) The RNA methyltransferase METTL8 installs m<sup>3</sup>C32 in mitochondrial tRNAs(Thr/Ser(UCN)) to optimise tRNA structure and mitochondrial translation. *Nat. Commun.*, **13**, 209.