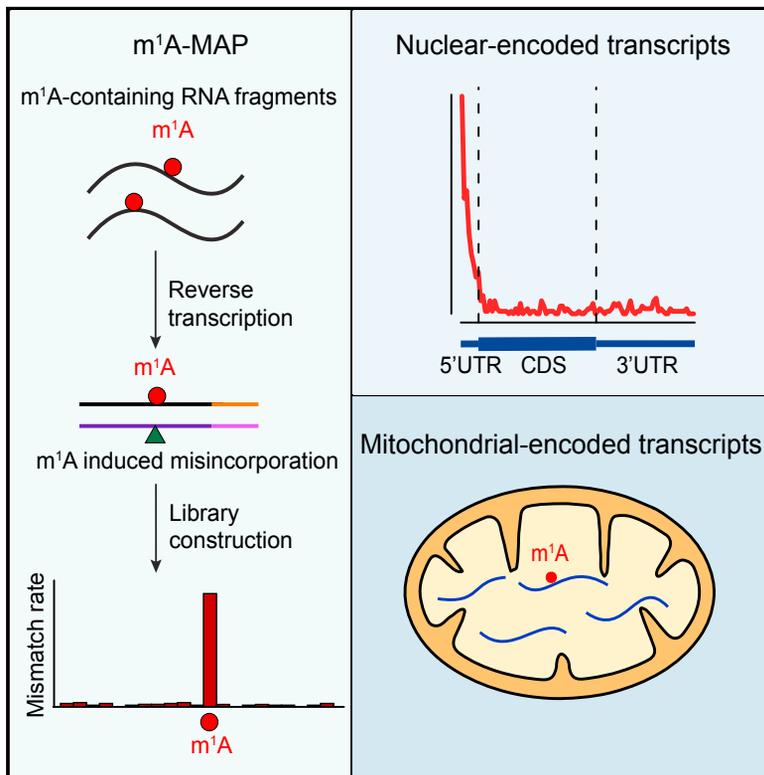


Molecular Cell

Base-Resolution Mapping Reveals Distinct m¹A Methylome in Nuclear- and Mitochondrial-Encoded Transcripts

Graphical Abstract



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In Brief

Li et al. developed a single-nucleotide-resolution technology, named “m¹A-MAP,” to profile m¹A in the human transcriptome, and revealed distinct classes of m¹A methylome in nuclear- and mitochondrial-encoded transcripts.

Highlights

- We report “m¹A-MAP,” a misincorporation-based method, to map m¹A at base resolution
- m¹A-MAP detects m¹A methylation at mRNA cap and 5' UTR
- m¹A-MAP reveals prevalent m¹A modification in mitochondrial-encoded transcripts
- m¹A in different mRNA regions differentially impacts translation



Base-Resolution Mapping Reveals Distinct m¹A Methylome in Nuclear- and Mitochondrial-Encoded Transcripts

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SUMMARY

Gene expression can be post-transcriptionally regulated via dynamic and reversible RNA modifications. N¹-methyladenosine (m¹A) is a recently identified mRNA modification; however, little is known about its precise location and biogenesis. Here, we develop a base-resolution m¹A profiling method, based on m¹A-induced misincorporation during reverse transcription, and report distinct classes of m¹A methylome in the human transcriptome. m¹A in 5' UTR, particularly those at the mRNA cap, associate with increased translation efficiency. A different, small subset of m¹A exhibit a GUUCRA tRNA-like motif, are evenly distributed in the transcriptome, and are dependent on the methyltransferase TRMT6/61A. Additionally, we show that m¹A is prevalent in the mitochondrial-encoded transcripts. Manipulation of m¹A level via TRMT61B, a mitochondria-localizing m¹A methyltransferase, demonstrates that m¹A in mitochondrial mRNA interferes with translation. Collectively, our approaches reveal distinct classes of m¹A methylome and provide a resource for functional studies of m¹A-mediated epitranscriptomic regulation.

INTRODUCTION

More than 100 different types of post-transcriptional modifications have been identified so far (Machnicka et al., 2013). Recent breakthroughs in sequencing technologies have greatly advanced our understanding of the location, regulation, and function of RNA modifications in the transcriptome (Frye et al.,

2016; Fu et al., 2014; Helm and Motorin, 2017; Li et al., 2016b), leading to the emerging field of epitranscriptomics (He, 2010; Saletore et al., 2012). One such example is N¹-methyladenosine (m¹A), a prevalent modification in non-coding RNA (ncRNA) and messenger RNA (mRNA) (Anderson and Droogmans, 2005; Dominissini et al., 2016; Li et al., 2016a; Roundtree et al., 2017). m¹A was first documented more than 50 years ago (Dunn, 1961); later it was found to be a primordial RNA modification across the three major phylogenetic domains (Machnicka et al., 2013). In human cells, m¹A is found at positions 9 and 58 of human mitochondrial and cytoplasmic tRNAs, catalyzed by TRMT10C, TRMT61B, and TRMT6/61A, respectively (Chujo and Suzuki, 2012; Ozanick et al., 2005; Vilardo et al., 2012); it is also present at position 1322 of 28S rRNA, catalyzed by NML (Waku et al., 2016). Its unique physicochemical property has endowed m¹A with pivotal roles in maintaining the proper structure and function of these ncRNAs (Roundtree et al., 2017). m¹A in tRNA has been systematically evaluated by microarray and sequencing (Cozen et al., 2015; Saikia et al., 2010; Zheng et al., 2015); more recently, m¹A58 is shown to be reversible by ALKBH1, demonstrating an example of reversible tRNA modification in translation regulation (Liu et al., 2016). In addition to ncRNAs, m¹A is also found to be a dynamic modification in mammalian mRNA, with strong enrichment in the 5' UTR (Dominissini et al., 2016; Li et al., 2016a).

Despite such rapid progress, a high-resolution profile of the mammalian m¹A methylome is still lacking, significantly limiting our understanding and functional characterization of this newly discovered mRNA modification. Previous m¹A profiling technologies have a resolution of about tens of nucleotides to several hundred nucleotides, primarily determined by the size of RNA fragments in these experiments (Dominissini et al., 2016; Li et al., 2016a). In addition, the methyltransferase(s) and functional consequence of mRNA m¹A modification are poorly understood. Hence, except for a handful positions in rRNA and tRNA, little is known about the precise location, regulation, and function of m¹A in the human transcriptome.

Here, we report a base-resolution method to profile m¹A in the human transcriptome. Our method is based on m¹A-induced misincorporation during reverse transcription and reveals distinct classes of m¹A methylome: a major group of m¹A sites that are enriched in 5' UTR, a small subset of GUUCRA ("R" denotes a purine)-transfer RNA (tRNA)-like m¹A sites with relatively even distribution in the transcriptome, and prevalent m¹A modification in the coding sequence (CDS) of 10 of 13 mitochondrial-encoded transcripts. m¹A sites in the 5' UTR, particularly those located at the first and second nucleotide of mRNA transcripts (or "cap+1" and "cap+2" positions), are associated with increased translation efficiency. In contrast, m¹A in the CDS of mt-mRNA inhibits translation. Collectively, our approaches reveal distinct classes of base-resolution m¹A methylome in the nuclear- and mitochondrial-encoded transcripts and provide an in-depth resource toward elucidating the functions of m¹A methylation in mRNA.

DESIGN

Because m¹A can cause both truncation and misincorporation during cDNA synthesis (Hauenschild et al., 2015; Zubratt et al., 2017), we first established the truncation and mutation profiles of different reverse transcriptases (RTases). We systematically compared the performance of several commercially available RTases (including AMV, SuperScript II, SuperScript III, and TGIRT) under different conditions (Figure S1). We found that m¹A can precisely induce misincorporation at the site of modification, while m¹A-induced truncation is less accurate and can occur to the neighboring nucleotides. In addition, the truncation profile could be complicated by RNA secondary structures and the fragmentation process needed for library preparation. We concluded that the mutation profile contains a higher signal-to-noise ratio and is more precise in detecting the exact position of m¹A. Among the RTases we tested, TGIRT demonstrated excellent readthrough efficiency and relatively high mutation frequency at the site of m¹A (Figure S1B), consistent with the recent dimethyl sulfate mutational profiling with sequencing (DMS-MaPseq) and demethylase-thermostable group II intron RT tRNA sequencing (DM-tRNA-seq) results (Zheng et al., 2015; Zubratt et al., 2017). Moreover, we employed a ligation-based strand-specific library preparation protocol (Van Nostrand et al., 2016), which ensures that the m¹A-induced mutation is within the sequenced fragment (see STAR Methods).

Because we only observed ~40%–50% mutation rate at m¹A1322 in 28S rRNA (Figure S2A), which is known to be of high modification level, we further examined the quantitative capability of TGIRT. We chemically synthesized two model RNA sequences with site-specific m¹A modification. For m¹A sites with ~97%–98% modification level (measured by quantitative mass spectrometry) (Figure S2B), we consistently observed ~66%–75% misincorporation (Figure S2C); the mismatch rate dropped non-linearly when we gradually lowered the modification level. Even with ~50% m¹A modification, a mismatch rate of only ~9%–10% was observed (Figure S2C). These findings suggest that the observed mutation rate is an underestimation of the actual modification level. While the TGIRT-based

procedure can still detect m¹A sites of high modification level, sequencing RNA directly with TGIRT may not be able to capture the m¹A sites with averaging modification level in the transcriptome (~20% as previously measured by microarray) (Dominissini et al., 2016). To improve the sensitivity for transcriptome-wide m¹A detection, we decided to couple the TGIRT-based procedure with an antibody-mediated pre-enrichment step and an additional *in vitro* demethylation step (Figure 1A). We first showed that *in vitro* demethylation reaction mediated by the demethylase AlkB is more efficient than the Dimroth reaction, demonstrating ~98% and ~80% efficiency (Figure S2D), respectively. In addition, the extended treatment of RNA in alkaline condition during the Dimroth reaction leads to excessive RNA degradation (Figure S2E), potentially causing loss of RNA molecules. By integrating the enrichment and demethylation steps, we successfully maximized the dynamic range of m¹A-induced mutational signature for m¹A1322 in 28S rRNA (~47%, ~95%, and ~0.9% in the Input, [–] demethylase, and [+] demethylase samples, respectively), allowing sensitive and confident m¹A detection (Figure 1B). We termed our approach misincorporation-assisted profiling of m¹A, or m¹A-MAP.

RESULTS

m¹A-MAP Detects m¹A in tRNA

We first applied m¹A-MAP to tRNA. In mammals, m¹A can occur at positions 9, 14, and 58 of tRNA (Anderson and Droogmans, 2005). m¹A14 has been reported only in tRNA^{Phe} and is considered to be very rare (Machnicka et al., 2013); we did not observe any m¹A modification at position 14 for cytosolic tRNAs in HEK293T cells (Table S1). m¹A58 is conserved across the three domains of life; previous tRNA microarray and sequencing data have reported hypomodified tRNAs at this position (Cozen et al., 2015; Saikia et al., 2010; Zheng et al., 2015). Our results confirmed that m¹A58 is globally present in the cytosolic tRNAs (Figures 1C and S2F). A recent study reported m¹A at position 9 in cytosolic tRNA^{Asp(GUC)} (Clark et al., 2016); this site is also detected by m¹A-MAP (Figure 1D). Collectively, these observations suggest that m¹A-MAP is highly sensitive in detecting m¹A at single-base resolution.

Single-Nucleotide Resolution m¹A Methylome in the Transcriptome

We then sought to detect transcriptome-wide m¹A methylome at single-base resolution. We defined two parameters to evaluate the m¹A-MAP data: difference of mismatch rate and fold change of mismatch rate (see STAR Methods). To minimize the effect of mismatch rate variation during m¹A identification, we rigorously tested our threshold and identified 740 m¹A sites in the 293T transcriptome (Tables S1, S2, and S3). To evaluate potential false positives caused by m¹A-independent mismatch, we performed a "reverse calculation" in which we artificially took the mismatch rate in (–) demethylase as the background and (+) demethylase as the signal and retrieved only 17 such sites (see STAR Methods). Moreover, we also systematically evaluated the mutation pattern of the identified m¹A sites in the transcriptome. Using m¹A sites in tRNA as positive controls, we found that m¹A-induced mutation is more strongly influenced by its

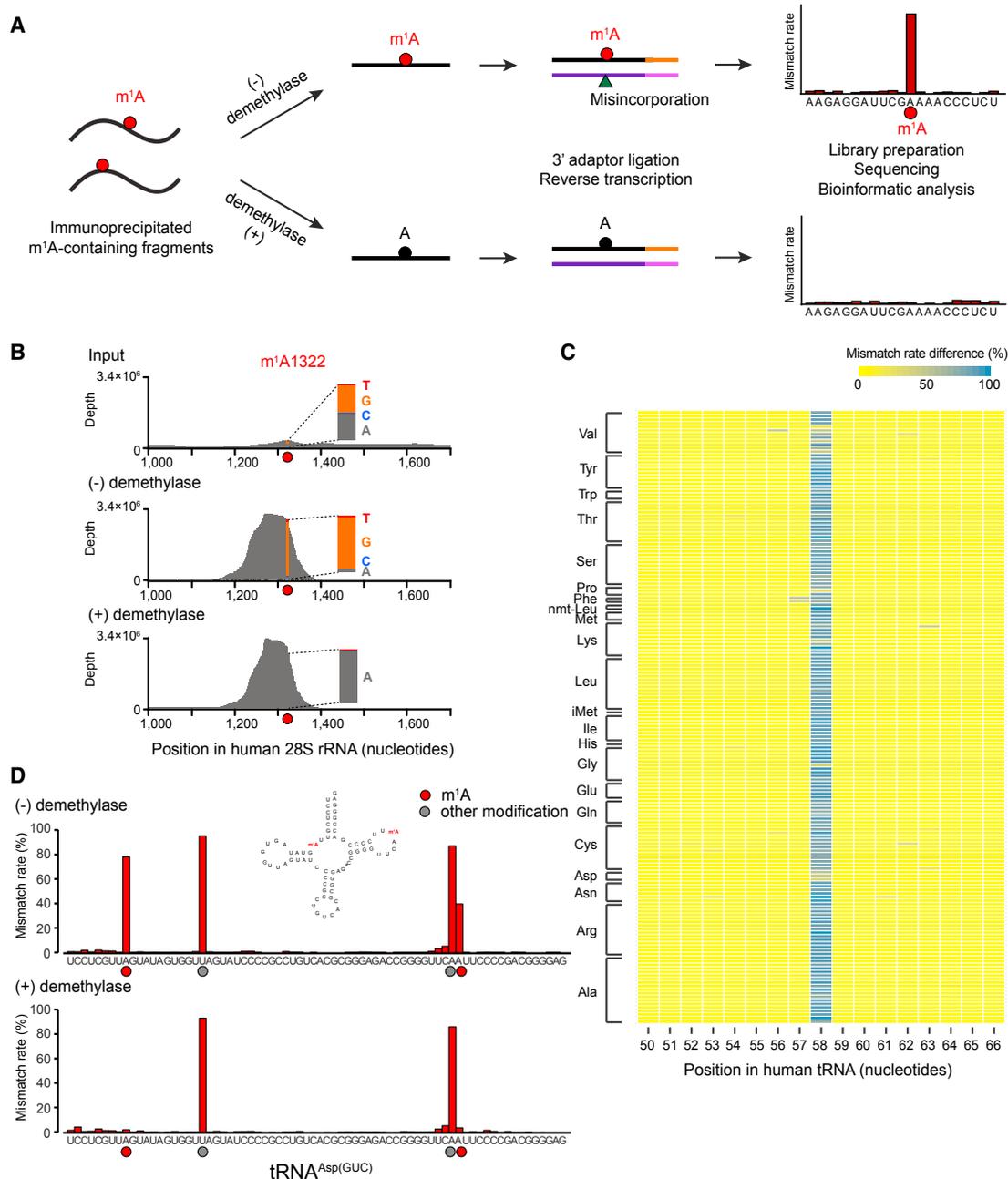


Figure 1. m¹A-MAP Utilizes m¹A-Induced Misincorporation to Detect m¹A Sites at Single-Nucleotide Resolution

(A) Scheme of m¹A-MAP. We optimized the conditions of RT so as to allow efficient misincorporation in cDNA synthesis. The use of an m¹A antibody pre-enriches the m¹A-containing RNA fragments, thereby maximizing the misincorporation signal, and the use of demethylase treatment improves the confidence of detection. An m¹A modification is identified depending on the difference and fold change of mismatch rate between the (-) demethylase and (+) demethylase samples (see STAR Methods).

(B) m¹A-MAP maximizes the misincorporation signal for m¹A1322 on 28S rRNA.

(C) m¹A-MAP detects m¹A58 for the cytosolic tRNAs. Shown here is the difference of mismatch rate between the (-) and (+) demethylase samples.

(D) m¹A-MAP detects an m¹A site at position 9 in the cytosolic human tRNA^{Asp(GUC)}. The mismatch rate for m¹A58 is also reduced after demethylase treatment, while two other modifications (at positions 20 and 57) are not sensitive to demethylation, representing other types of RNA modifications.

See also Figures S1 and S2.

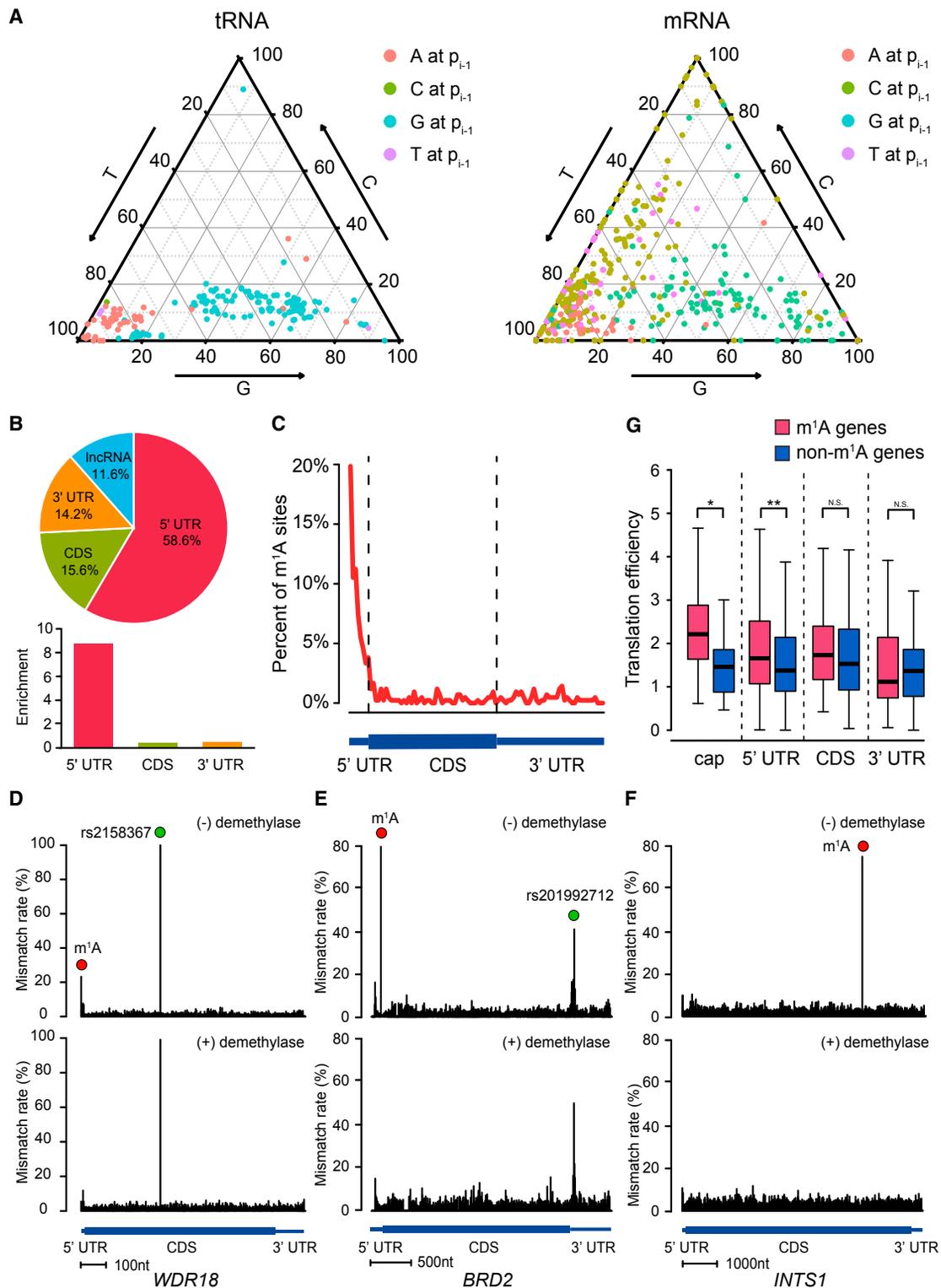


Figure 2. Single-Nucleotide-Resolution m¹A Methylome in the Human Transcriptome

(A) Mutation pattern of m¹A sites in mRNA resembles that in tRNA. Shown here is the sequence-dependent mutation profile of m¹A sites with regard to the immediate 5' nucleotide.

(B) The pie chart shows the percentage of m¹A sites in each non-overlapping segment.

(C) Distribution of m¹A sites across mRNA segments. Each segment was normalized according to its average length in Ref-seq annotation.

(legend continued on next page)

5' nucleotide than the 3' nucleotide; importantly, a similar sequence-dependent feature is also observed for m¹A sites in mRNA (Figure 2A and S3A). Therefore, we conclude that our strict threshold allowed us to confidently detect transcriptome-wide m¹A sites at single-nucleotide resolution.

Out of the 740 m¹A modifications in the transcriptome (Figure S3B), 473 sites are located in mRNA and lncRNA molecules (Figure S3C and Table S2). The majority of these sites are within the 5' UTR (Figures 2B and 2C), consistent with the previous finding (Dominissini et al., 2016; Li et al., 2016a). Our single-base profile also reveals multiple features of the m¹A methylome: for instance, we found 24 m¹A methylation sites that are present exactly at the first nucleotide of the 5' end of the transcripts (Figure 2D and Table S2). Out of the 24 cap+1 m¹A methylation sites, 22 are found in mRNA while the other two are located in lncRNA (Table S2). We further validated these cap+1 m¹A sites using an antibody-independent, locus-specific approach (Figure S3D). We also found three additional transcripts with m¹A methylation at the second nucleotide. For m¹A located in CDS, while we did not observe a tendency for codon position, we did notice a mild preference for codon types, with Arg(CGA) being the most frequently modified by m¹A (Figure S3E). No m¹A is detected for AUG start codons. Representative examples of m¹A sites identified from different mRNA regions are shown (Figures 2D–2F and S3F). Two additional sites of high mutation, which are insensitive to the demethylase treatment, also appeared in the *WDR18* and *BRD2* examples (Figures 2D and 2E). By referring to the SNP database, we found that these two positions belong to annotated SNP sites, demonstrating the robustness of our approach in distinguishing true m¹A sites from false signals (SNP, other modifications, etc.).

Because m¹A is enriched in the 5' UTR, we examined whether m¹A could be involved in translation regulation. We performed ribosome profiling and compared the translation efficiency for transcripts with or without m¹A. We found that m¹A within the 5' UTR positively correlates with the translation efficiency for mRNA (Figure 2G), in agreement with a previous report (Dominissini et al., 2016). This positive correlation is even stronger for m¹A at the cap+1 position, but is not observed for m¹A located in CDS nor 3' UTR. This observation hints that m¹A within different regions of mRNA may have different biological functions.

A Subset of m¹A Sites Demonstrate a GUUCRA Consensus Motif

An unbiased motif detection using DREME revealed that a subset of m¹A (53 sites) are found within a strong GUUCRA sequence (Figure 3A). Interestingly, these sites demonstrate a very different distribution pattern: instead of being enriched in the 5' UTR, these sites are evenly distributed in the transcriptome (Figure 3B). Because this motif is reminiscent of the m¹A-containing TΨC loop in tRNA, we hypothesized that the tRNA meth-

yltransferase complex TRMT6/61A could be responsible for these mRNA m¹A sites. We first performed direct m¹A sequencing (without antibody enrichment) on a RNA population below 200 nt to examine the substrate specificity of TRMT6/61A. We found that the m¹A58 sites within the GUUCRA motif experienced a global decrease of mutation rate in the TRMT6/61A knockdown sample, which was not observed for m¹A58 sites that do not conform to the motif (Figures 3C, S4A, and S4B). This result suggests that TRMT6/61A-mediated m¹A methylation is highly sequence specific, consistent with evidence from crystal structures (Finer-Moore et al., 2015). We then analyzed the secondary structure for the 53 mRNA m¹A sites and found highly conserved structural features compared to the T-loop of tRNA (Figures S4C and S4D). We also picked three m¹A sites (in CDS, 3' UTR, and lncRNA, respectively) and examined their response after TRMT6/61A knockdown using a locus-specific approach (Figure 3D). This approach is based on targeted amplification using specific primers (see STAR Methods), enabling us to interrogate these sites with high sequencing depth. The results unambiguously demonstrated a decrease in the mismatch rate for these three sites after TRMT6/61A knockdown (Figure 3D). As a comparison, a non-motif m¹A site located in a different structural context demonstrated an unaltered modification status (Figure 3E). Taken together, these observations suggest that in addition to tRNA, TRMT6/61A is also responsible for a subset of m¹A sites in mRNA.

Distinct m¹A Methylome in the Mitochondrial Transcriptome

In addition to the nuclear-encoded transcripts, we also detected prevalent m¹A modification in the mitochondrial transcriptome. mt-tRNAs are known to contain m¹A at positions 9 and 58 (Suzuki et al., 2011), catalyzed by TRMT10C and TRMT61B (Chujo and Suzuki, 2012; Vilardo et al., 2012), respectively. m¹A-MAP showed that all the 14 mt-tRNAs bearing an adenosine residue at position 9 are m¹A modified; for position 58, m¹A was detected for the three known and two additional mt-tRNA molecules (Figures 4A and 4B and Table S3). For mt-rRNA, the only known m¹A site is at position 947 of 16S rRNA (Bar-Yaacov et al., 2016). Interestingly, we additionally detected seven and ten m¹A sites in 16S and 12S mt-rRNA, respectively (Figure 4 and Table S3). This is very different from cytosolic rRNA, where only one m¹A site in 28S rRNA (m¹A1322) was found. Unlike m¹A947 in 16S mt-rRNA and m¹A1322 in 28S rRNA (both are highly modified), the newly detected m¹A sites are relatively low in modification level (Table S3). We further validated the presence of m¹A in 12S mt-rRNA by both quantitative MS and a primer extension assay (Figures 4C, S5A, and S5B).

In human mitochondria, mRNAs are transcribed from the heavy and light strands as polycistronic units (Falkenberg et al., 2007; Mercer et al., 2011). The processed mt-mRNAs

(D–F) Representative views of a typical m¹A site at the cap+1 position (the first transcribed nucleotide of mRNA) of *WDR18* (D), the 5' UTR of *BRD2* (E), and the CDS of *INTS1* mRNA (F). The demethylation-insensitive signals in (D) and (E), which are indicated as green dots, are known SNPs. The scale bars are indicated at the bottom of each panel.

(G) m¹A in the cap (24 sites at the cap+1 and three sites at the cap+2 position) and 5' UTR positively correlates with increased translation efficiency. Transcripts with comparable expression level but without m¹A sites were chosen as the negative control for each category.

See also Figure S3.

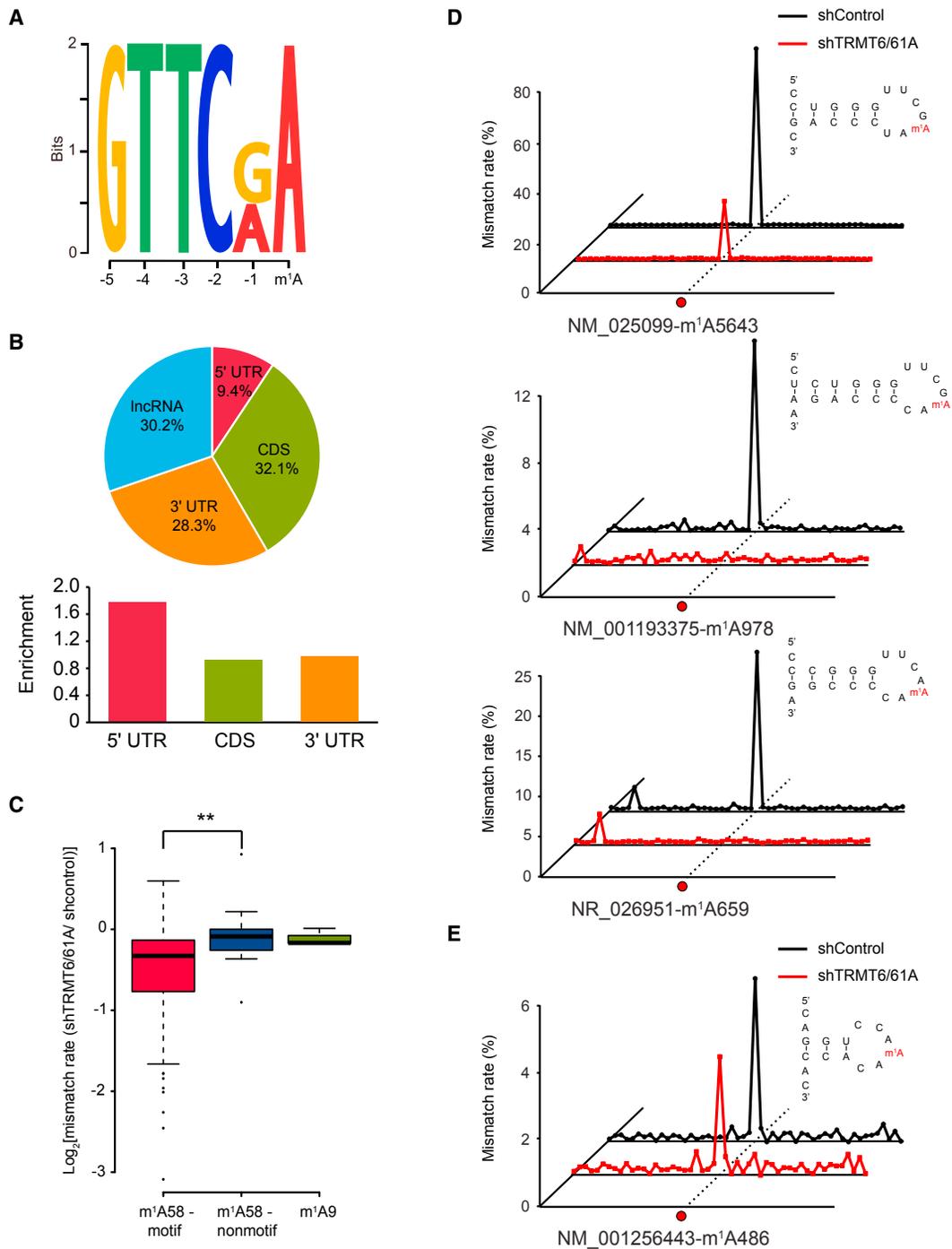


Figure 3. The tRNA Methyltransferase Complex TRMT6/61A Catalyzes a Subset of m¹A Methylation in mRNA

(A) Motif analysis revealed a GUUCRA tRNA-like consensus for a group of m¹A in mRNA. E-value = 4.8e-009.

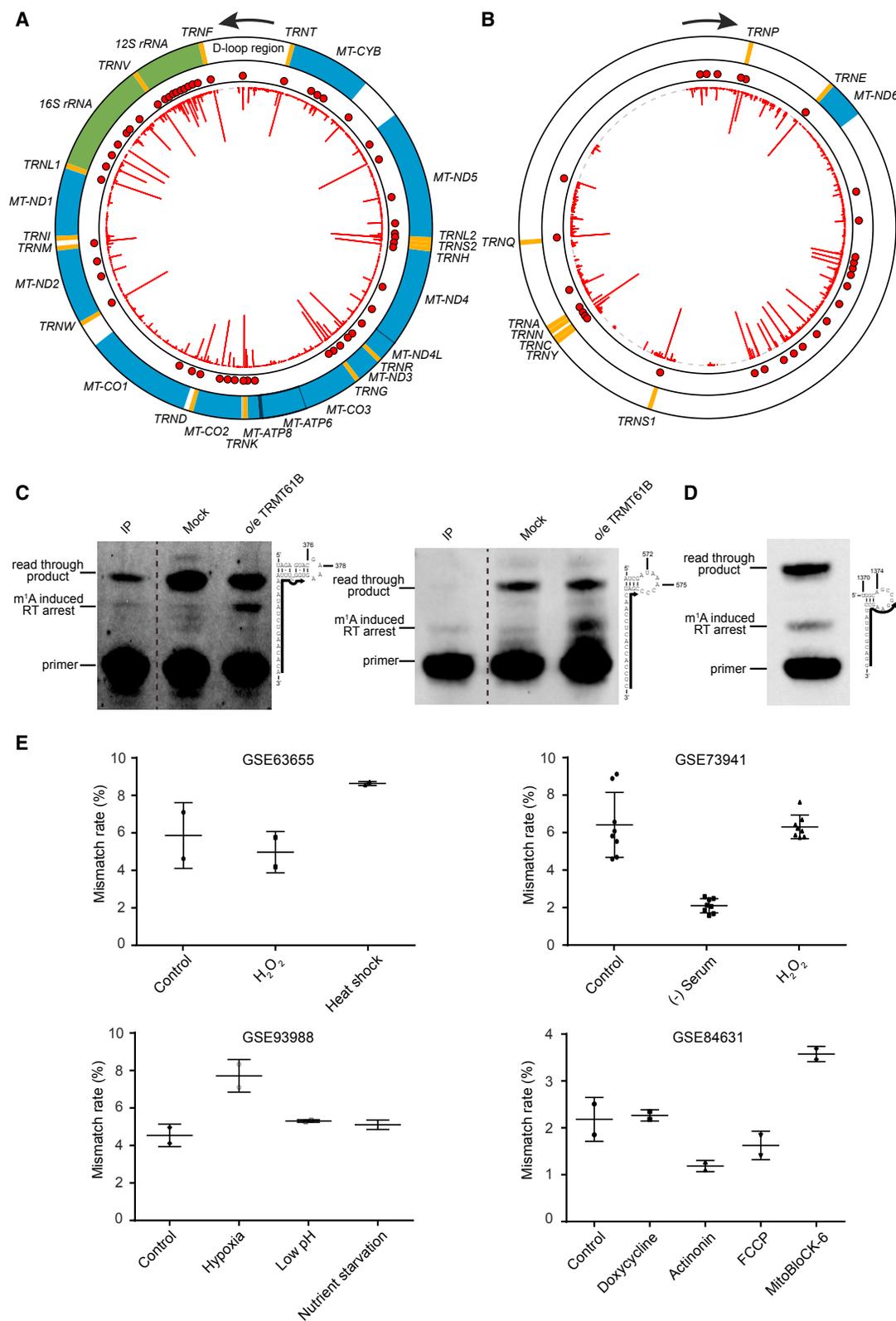
(B) Pie chart showing the percentage of m¹A sites in each non-overlapping segment. Compared to the non-motif m¹A sites in mRNA, these sites are evenly distributed in the transcriptome.

(C) TRMT6/61A specifically targets m¹A within the consensus sequence, while it doesn't work on m¹A in non-motif sequence of the T-loop nor m¹A at the 9th position of tRNA. p value < 0.005.

(D) Representative views of three mRNA targets of TRMT6/61A. The predicted RNA secondary structures are also shown, revealing a conserved stem-loop structure that harbors the GUUCRA motif.

(E) An example of non-motif mRNA m¹A site, showing similar mismatch rates in the control and TRMT6/61A knockdown samples.

See also Figure S4.



lack a cap at the 5' end and contain no or short untranslated regions (Richter-Dennerlein et al., 2015; Rorbach and Minczuk, 2012; Temperley et al., 2010). We identified 22 m¹A sites from 10 of 13 mitochondrial genes, in which 21 are residing in CDS and one is located in the 3' UTR (Figures 4A and 4B). This is distinct to the m¹A methylome in the nuclear-encoded transcripts, where m¹A is enriched in the 5' UTR. In addition, no preference for codon types was observed, yet m¹A appears to be more likely present at the third position of a codon in the CDS of mt-mRNA (Figure S5C). By analyzing the published CLASH (cross-linking, ligation and sequencing of hybrids) results in which microRNA and their direct mRNA targets are captured (Helwak et al., 2013), we found two m¹A sites that are located within the experimentally verified targets of microRNA (Figure S5D). In fact, these m¹A sites reside exactly within mRNA sequences that form base-pairing with the seed regions of microRNAs. More m¹A sites in mt-mRNA were found within the predicted mt-mRNA targets of the microRNA seed regions (Figure S5D). Lastly, we also identified 25 m¹A sites within the intergenic spacers. Twenty-four of 25 m¹A sites are in the light strand (Figure 4B); some of these m¹A sites could be within the 3' UTR of *MT-ND6*, for which there is no current consensus of its 3' end (Slomovic et al., 2005).

We further validated m¹A1374 in the *MT-ND5* mRNA and observed a clear band corresponding to m¹A1374-induced reverse transcription (RT) arrest in the Input sample (without immunoprecipitation) using the primer extension assay (Figure 4D). Because the modification level of this site appears to be the highest among the m¹A sites in mt-mRNAs (Table S3), we also surveyed publicly available RNA-seq data for the presence of m¹A at this position (Kondo et al., 2017; Li et al., 2015, 2016a; Quirós et al., 2017). Although the RT conditions differ greatly among the datasets, we were able to consistently find a mutation rate of ~2%–9% for this position (Figure 4E). Interestingly, the modification level of m¹A1374 appears to be dynamically regulated by different stress conditions (Figure 4E). For instance, its modification level is upregulated under heat shock and hypoxia conditions, while it is downregulated upon serum starvation and treatment with actinonin, a drug that alters mitochondrial proteostasis. Hence, similar to the nuclear genome-encoded mRNA m¹A methylome (Dominissini et al., 2016; Li et al., 2016a), m¹A in mt-mRNA is also dynamically regulated.

m¹A in mt-mRNA Interferes with Mitochondrial Translation

We next sought to examine the biological consequence of m¹A in the mt-mRNAs. Translation requires accurate base pairing be-

tween mRNA codons and the cognate tRNAs; however, m¹A is known to block the canonical A:U base pairing. These facts prompted us to hypothesize that m¹A in mt-mRNA, which are primarily located in CDS, could interfere with translation in mitochondria. We first integrated published mitochondria ribosome profiling data with a m¹A-MAP-identified m¹A methylome in mitochondria (Rooijers et al., 2013). We found a strong signal of mitochondrial ribosome stalling at the m¹A site on *MT-ND5* (Figures 5A and S5E), whose modification level is the highest among all m¹A sites in mt-mRNA. Due to the difficulty of an *in vitro* mitochondrial translation system (Smits et al., 2010), we sought to enzymatically manipulate the modification level of the mt-mRNAs. Two enzymes are known to introduce m¹A in human mitochondria: TRMT10C generates m¹A as well as m¹G at position 9 in mitochondrial tRNAs, while TRMT61B is responsible for m¹A at position 58 in some mitochondrial tRNAs and position 947 in 16S mt-rRNA (Bar-Yaacov et al., 2016; Chujo and Suzuki, 2012; Vilardo et al., 2012). Because TRMT10C is a subunit of the mitochondrial RNase P complex and is not specific for adenosine, we focused on TRMT61B. We utilized sequencing and a qPCR-based assay to quantitatively evaluate the modification status of the m¹A sites in mt-mRNAs (see STAR Methods). While TRMT61B knockdown mildly reduced the m¹A level (Figures S5F and S5G), TRMT61B overexpression could greatly increase the m¹A modification level (Figures S5F and S5H). More specifically, we found that five (*MT-CO1*, *MT-CO2*, *MT-CO3*, *MT-CYB*, and *MT-ND4L*) out of the ten m¹A-modified mt-mRNAs were targeted by TRMT61B (Figure 5B and Table S4). This observation suggests that in addition to mt-tRNA and mt-rRNA, TRMT61B could also modify mt-mRNA. Because of the high efficiency of TRMT61B overexpression in increasing the m¹A level, we used mass spectrometry to quantitatively measure the mitochondrial protein level upon TRMT61B overexpression (Figure S5I). Indeed, TRMT61B overexpression led to a reduced protein level for *MT-CO2* and *MT-CO3* (Figures 5C and S5J), which are components of Complex IV; importantly, for *MT-ATP6* and *MT-ATP8* that are not targeted by TRMT61B, their protein levels remain unaffected. We also used metabolic labeling to further demonstrate that TRMT61B overexpression-induced translation repression is specific to mt-mRNA targets of TRMT61B, but not to non-targets of TRMT61B (Figure 5D). We further confirmed this observation using western blot for *MT-CO2* and *MT-ATP6*, a target and non-target of TRMT61B, respectively (Figure 5E). Of note, upon TRMT61B knockdown, the protein productions for several non-targets of TRMT61B were reduced (Figure S5K), presumably due to the decreased modification level in tRNA and rRNA m¹A sites (which are originally highly modified and beneficial

(B) m¹A methylome of the light strand.

(C) Detection of two novel m¹A sites at position 378 and 575 in 12S mt-rRNA using a primer extension assay. In the immunoprecipitated samples (denoted as "IP"), the m¹A modification at the two sites can be unambiguously detected. In the mock control samples (without immunoprecipitation), the bands corresponding to m¹A378- and m¹A575-induced reverse transcription (RT) arrest were weak, indicating a relatively low stoichiometry of these sites. Upon TRMT61B overexpression, strong bands were observed, suggesting that these two sites can be targeted by TRMT61B. The primers are shown as solid lines next to the RNA sequence.

(D) Detection of m¹A1374 in *MT-ND5* mt-mRNA using the primer extension assay. A strong band that corresponds to m¹A1374-induced RT arrest can be found in the wild-type sample without immunoprecipitation, indicating a high modification level of this site. The primer is shown as solid lines next to the RNA sequence.

(E) m¹A1374 in *MT-ND5* is dynamically regulated by different stress conditions. Mismatch signal of m¹A1374 could be consistently detected in multiple publicly available RNA-seq datasets and is regulated upon environmental stress.

See also Figure S5.

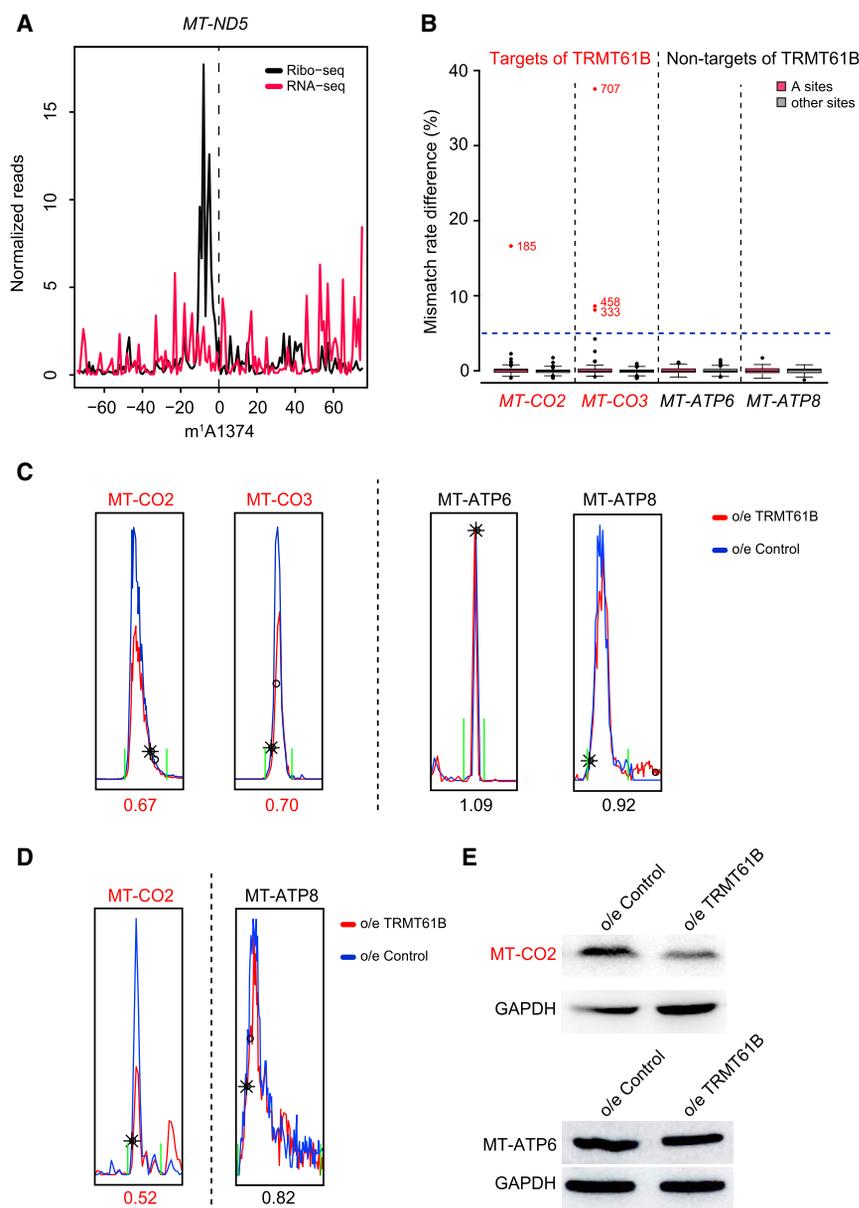


Figure 5. m¹A in the Mitochondrial mRNA Interferes with Translation

(A) Mitochondrial ribosome stalling at the m¹A site of the *MT-ND5* mRNA. The density of the 5' end of footprints was calculated for each position surrounding the m¹A site. Ribosome profiling and RNA-seq data were taken from a published study (see STAR Methods).

(B) TRMT61B overexpression greatly increased the m¹A modification level for several sites in *MT-CO2* and *MT-CO3*, while the modification status of *MT-ATP6* and *MT-ATP8* remained unchanged. The red dots represent the targets of TRMT61B in *MT-CO2* and *MT-CO3*.

(C) Extracted ion chromatograms of *MT-CO2* and *MT-CO3* (two targets of TRMT61B) showed decreased protein level upon TRMT61B overexpression, while those of *MT-ATP8* and *MT-ATP6* (non-targets of TRMT61B) remained unchanged.

(D) Extracted ion chromatograms of nascent *MT-CO2* and *MT-ATP8* upon TRMT61B overexpression. The nascent protein level of *MT-CO2* decreased while that of *MT-ATP8* remained unaffected.

(E) Western blot of *MT-CO2* and *MT-ATP6* upon TRMT61B overexpression.

See also Figure S5.

sequences strongly suggest that such an estimation should be done with caution: TGIRT underestimates m¹A modification level, and m¹A-induced mismatch decreases in a non-linear fashion as the modification level decreases. Additionally, the sequence context of RNA has also been reported to affect the mutation rate (Hauenschild et al., 2015; Zubradt et al., 2017). Hence, while direct sequencing (without enrichment) could still detect m¹A sites with high modification levels, m¹A sites with average modification levels or those located within a non-optimal context for mismatch induction could be missed. Therefore, coupling

the pre-enrichment step to the mutational signature is necessary to improve detection sensitivity. In addition, to achieve high confidence, we employed an *in vitro* demethylation step, which enabled us to distinguish true m¹A sites from false signals (SNP, other modifications, etc.) in the transcriptome. The combined use of mutational signature, the pre-enrichment step, and the *in vitro* demethylation step enabled m¹A-MAP to achieve high sensitivity and confidence. Of note, AlkB also demethylates several other methylations in RNA (e.g., m³C and m¹G) (Cozen et al., 2015; Zheng et al., 2015); hence, with the help of specific antibodies for these methylations, our strategy could in principle be applied to these modifications as well.

DISCUSSION

for translation) (Bar-Yaacov et al., 2016; Chujo and Suzuki, 2012). Collectively, these results suggest that m¹A in mt-mRNA interferes with translation in mitochondria.

In this study, we developed a single-nucleotide-resolution method for transcriptome-wide identification of m¹A in human cells. m¹A-MAP utilizes the m¹A-induced misincorporation in cDNA synthesis to achieve base-resolution m¹A detection. This enabled us to identify m¹A modification not only at the mRNA cap, but also within a GUUCRA tRNA-like sequence motif. In principle, such a misincorporation-dependent strategy could be applied to estimate the modification status of RNA sites of interest. However, our results in both rRNA and model RNA

Our study revealed that two known m¹A modification machineries, TRMT6/61A and TRMT61B, could work on mRNA as well. The hetero complex TRMT6/61A recognizes the sequence and

structure of the tRNA T-loop and installs an m¹A at the 58 position (Finer-Moore et al., 2015). Consistent with this knowledge, we found that the TRMT6/61A-dependent mRNA m¹A sites are also confined to a hairpin structure mostly with a 7-nt loop, reminiscent of the tRNA T-loop. In contrast, we did not find an obvious sequence context for the m¹A in mt-mRNA. In fact, TRMT61B appears to be a promiscuous enzyme that also modifies mt-tRNA and mt-rRNA; the substrate specificity and the underlying mechanism of TRMT61B in the human mitochondria remains to be determined. In addition, the fact that both TRMT6/61A and TRMT61B are known tRNA modification enzymes also reminds us of the modification machinery for other mRNA modifications. For instance, eukaryotic Ψ synthases PUS1, PUS7, and TRUB1 can work on both tRNA and mRNA (Carile et al., 2014; Li et al., 2015; Lovejoy et al., 2014; Safra et al., 2017; Schwartz et al., 2014a), yet the modification complex for m⁶A, consisting of at least METTL3, METTL14, WTAP, and KIAA1429, appears to be dedicated to mRNA (Bokar et al., 1997; Liu et al., 2014; Ping et al., 2014; Schwartz et al., 2014b). In the case of m¹A, the enzyme(s) responsible for the majority of the modification sites in mRNA remain to be identified. It would be interesting to see if such machinery is specific for mRNA or promiscuous for multiple RNA substrates.

Our base-resolution m¹A profiles reveal distinct m¹A methylome in the human transcriptome. m¹A is enriched in the 5' UTR, and only 5' UTR m¹A sites, but not those in CDS nor 3' UTR, are correlated with higher translation efficiency. Different from the m¹A sites in the nuclear-encoded transcripts, m¹A in mt-mRNA are primarily located in CDS and inhibit translation. Interestingly, m¹A in mt-mRNA can be dynamically regulated, as shown in the case of m¹A in *MT-ND5*. Of note, m¹A517 in *MT-ND2* overlaps with a known site of non-synonymous mutation that is associated with oral cancer (<https://www.mitomap.org/MITOMAP>). In addition, we also identified a notable group of m¹A methylation adjacent to the mRNA cap; because the first two nucleotides of the 5' end of mRNA are known to contain ribose methylation, future experiments will be needed to examine whether they are m¹A or m¹A_m modifications. A related but different modification, m⁶A_m, is also known to be present at this position. Yet m⁶A_m does not induce misincorporation during reverse transcription, nor does it become sensitive to demethylation by AlkB, as we showed in Figure S3D. Hence, m¹A-MAP is capable of specific detection of methylation at the N1—but not the N6—position of adenosine. Whether the positive association of cap and 5' UTR m¹A with translation is due to improved mRNA stability (similar to m⁶A_m) (Mauer et al., 2017; Schwartz et al., 2014b) or other mechanisms remains to be further studied.

Our results also revealed prevalent m¹A methylation in mt-mRNA; this is due to the utilization of a stranded library preparation strategy in this study as compared to the previous methods (Dominissini et al., 2016; Li et al., 2016a). Similar stranded library preparation strategy has also enabled the identification of pseudouridylation in mt-mRNA (Antonicka et al., 2017). We also showed that such methylation interferes with translation. By manipulating the m¹A level via TRMT61B, we monitored the corresponding changes of the mitochondrial protein level by both quantitative mass spectrometry and western blot. TRMT61B

modifies both mt-tRNA and mt-rRNA (Bar-Yaacov et al., 2016; Chujo and Suzuki, 2012), and these m¹A sites are thought to be beneficial for their functions in translation. Because m¹A sites in mt-mRNA have an opposing effect in translation, changes of protein synthesis level after TRMT61B knockdown could be a mixed result of contrary m¹A sites in these different components (rRNA, tRNA, and mRNA) of translation. Conversely, because 16S rRNA and mt-tRNA^{Leu(UUR)} are already of high m¹A level, we envisioned that TRMT61B overexpression should lead to a greater increase of m¹A level in mRNA than in these ncRNAs. In this simplified scenario, we indeed observed greatly increased modification levels for several mt-mRNAs upon TRMT61B overexpression and detected reduced protein levels for such mt-mRNA transcripts. Importantly, protein levels for non-targets of TRMT61B, which are either devoid of m¹A or contain TRMT61B-independent m¹A sites, remain unaffected upon TRMT61B overexpression. Hence, TRMT61B overexpression-mediated translation repression is caused by mt-mRNA m¹A and is specific to its mt-mRNA targets. For the mt-mRNAs that contain TRMT61B-independent m¹A sites, additional methyltransferase(s), for instance, TRMT10C (also localized in the mitochondria), may be involved. In terms of the mechanism of m¹A-mediated translational suppression, multiple possibilities need to be considered. For instance, due to the presence of a base-pairing interfering modification in the CDS, m¹A could serve as a road block for the mitochondrial ribosome. In fact, recent *in vitro* translation experiments on synthetic RNA sequences have shown that m¹A in CDS represses translation; the effect is stronger when m¹A is at codon positions 1 and 2, while m¹A at the third position also mitigates translation (You et al., 2017). Thus, not only the stoichiometry but also the exact position of m¹A in mt-mRNA could influence protein synthesis. In addition, we showed that the presence of m¹A in mt-mRNA could interfere with base pairing to microRNA, which is thought to enhance translation in mitochondria (Zhang et al., 2014). While both the speculated mechanisms point to a suppressive role of m¹A in mitochondria translation, alternative hypotheses and mechanisms should also be tested in future experiments. Nevertheless, our discovery that m¹A in mt-mRNA interferes with translation improves our understanding of translation regulation in human mitochondria.

In summary, our study demonstrated distinct classes of m¹A methylome in the nuclear- and mitochondrial-encoded transcripts. Our single-nucleotide-resolution m¹A technology allowed the comprehensive profiling of m¹A in the human transcriptome, providing a reference and resource for future investigations to elucidate the biological functions and mechanisms of this epitranscriptomic mark.

Limitations

Our current procedure of m¹A-MAP favors sensitivity and accuracy over the quantitative ability, due to the involvement of a pre-enrichment step using an m¹A antibody. For the m¹A sites located in abundant RNAs (rRNA, tRNA, and a limited subset of mRNA), their modification levels could be estimated by comparing the mismatch rates in the Input sample with a stoichiometry curve (similar to Figure S2C). However, for the majority of mRNA molecules in the transcriptome, only limited sequencing

reads could be obtained by transcriptome-wide sequencing experiments. Thus, stoichiometry for m¹A sites in less abundant mRNA molecules may not be reliably estimated by their mismatch rates in the Input sample. To address this limitation, the modification level of m¹A-MAP-identified m¹A sites can be estimated via additional locus-specific sequencing to the Input samples, which would allow sufficient sequencing depth while remaining cost effective. The exact stoichiometry of these sites can then be determined by fitting the mismatch rates in the locus-specific sequencing into the stoichiometry curve of synthetic spike-in RNAs that contain the exact sequence of the RNA molecules of interest.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, five tables, and detailed m¹A-MAP protocols and can be found with this article online at <https://doi.org/10.1016/j.molcel.2017.10.019>.

AUTHOR CONTRIBUTIONS

X.L., X.X., and C.Y. conceived the base-resolution method and performed sequencing; Y.C., M.Z., K.W., J.L., and D.Y. performed MS analysis and cell biology experiments under the guidance of C.Y., C.W., and X.-W.C. X.L., K.W., and J.Z. performed ribosome profiling and translation experiments. X.X. designed and performed the bioinformatics analysis with the help of Y.M. C.Y. and S.-B.Q. supervised the project. All authors commented on and approved the paper.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-m ¹ A antibody	Medical and Biological Laboratories	Cat#D345-3
Rabbit polyclonal anti-TRMT6 antibody	Santa Cruz Biotechnology	Cat#sc-271752; RRID: AB_10707666
Mouse monoclonal anti-GAPDH antibody	CWBiotech	Cat#CW0100M
Mouse monoclonal anti-β-Actin antibody	CWBiotech	Cat#CW0096M; RRID: AB_2665433
Rabbit Polyclonal anti-MT-CO2 antibody	Proteintech Group	Cat#55070-1-AP; RRID: AB_10859832
Rabbit Polyclonal anti-MT-ATP6 antibody	Proteintech Group	Cat#55313-1-AP
Anti-mouse-IgG-HRP	CWBiotech	Cat#CW0102
Anti-rabbit-IgG-HRP	CWBiotech	Cat#CW0103
Chemicals, Peptides, and Recombinant Proteins		
DMEM	GIBCO	Cat#11965
FBS	GIBCO	Cat#10438-026
Pen/Strep	GIBCO	Cat#15140
TRIzol Reagent	Invitrogen	Cat#15596018
Nuclease P1	Sigma	Cat#N8630
Alkaline phosphatase	Sigma	Cat#P4252
Magnesium RNA fragmentation buffer	NEB	Cat#E6150S
SUPERaseIn RNase Inhibitor	Invitrogen	Cat#AM2694
RiboLock RNase Inhibitor	Thermo Fisher	Cat#EO0381
T4 Polynucleotide Kinase	NEB	Cat#M0201S
T4 RNA Ligase 2, truncated KQ	NEB	Cat#M0373S
5' Deadenylase	NEB	Cat#M0331S
RecJf	NEB	Cat#M0264S
Exonuclease I	NEB	Cat#M0293S
T4 RNA Ligase 1 (ssRNA Ligase), High Concentration	NEB	Cat#M0437M
Lipofectamine RNAiMAX Transfection Reagent	Invitrogen	Cat#13778030
Lipofectamine 2000 Transfection Reagent	Invitrogen	Cat#11668027
Micrococcal Nuclease	NEB	Cat#M0247S
CircLigase II	Epicenter	Cat#CL9021K
Superscript III	Invitrogen	Cat#18080093
TGIRT-III	InGex	Cat#TGIRT50
Light formaldehyde (CH ₂ O)	Sigma	Cat#252549
Heavy formaldehyde (¹³ CD ₂ O)	Sigma	Cat#596388
Trypsin Gold, Mass Spectrometry Grade	Promega	Cat#V5280
L-Azidohomoalanine	Click Chemistry Tools	Cat#1066-25
Emetine dihydrochloride hydrate	Sigma	Cat#E2375
Pierce Streptavidin Agarose	Thermo Fisher	Cat#20347
Iodoacetamide	Sigma	Cat#V900335
Cy3 Alkyne	Click Chemistry Tools	Cat#TA117
DMEM, High Glucose, no Glutamine, no methionine, no cystine	GIBCO	Cat#21013024
Critical Commercial Assays		
MEGAclear Transcription Clean-Up Kit	Ambion	Cat#AM1908
NEBNext Ultra II DNA Library Prep Kit for Illumina	NEB	Cat#E7645

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mitochondria Isolation Kit for Cultured Cells	Thermo Fisher	Cat#89874
BCA protein assay kit	Thermo Fisher	Cat#23225
Dynabeads Oligo(dT)25	Invitrogen	Cat#61005
Dynabeads MyOne Silane	Invitrogen	Cat#37002D
Deposited Data		
Raw and processed data	This paper	GEO: GSE102040
Mitochondrial ribosome profiling data	Rooijers et al., 2013	GEO: GSE48933
RNA-seq data	Li et al., 2015	GEO: GSE63655
m ¹ A-ID-seq data	Li et al., 2016a	GEO: GSE73941
RNA-seq datasets in pancreatic carcinoma cells	Kondo et al., 2017	GEO: GSE93988
RNA-seq data of mitochondrial stress	Quirós et al., 2017	GEO: GSE84631
miRNA CLASH data	Supplementary table of Helwak et al., 2013	http://www.sciencedirect.com/science/article/pii/S009286741300439X
Transcriptome reference under hg19 Refseq annotation	UCSC table browser	http://genome.ucsc.edu/cgi-bin/hgTables
Mendeley Data dataset	This paper	https://doi.org/10.17632/bf8cshtzmx.1
Mitochondrial genome of human	NCBI	NCBI: NC_012920.1
Experimental Models: Cell Lines		
Human: HEK293T cells	ATCC(CRL-11268)	N/A
Oligonucleotides		
See method details for TRMT61B siRNA oligoes	This paper	N/A
See method details for synthetic m ¹ A and A RNA model sequences	This paper	N/A
See method details for PCR primers	This paper	N/A
See method details for qPCR Primers	This paper	N/A
See method details for TRMT6/61A shRNA sequences	This paper	N/A
See Table S5 for cap m ¹ A detection primers	This paper	N/A
See method details for internal m ¹ A detection primers	This paper	N/A
See method details for 12S mt-rRNA pull down probes	This paper	N/A
See method details for primer extension primers	This paper	N/A
See method details for the oligoes used in library construction	This paper	N/A
Recombinant DNA		
pLKO-TRMT6	This paper	N/A
pLKO-TRMT61A	This paper	N/A
pcDNA3.1- TRMT61B	This paper	N/A
Software and Algorithms		
Trim_galore	Babraham Institute	http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/
BWA	Li et al., 2009	http://bio-bwa.sourceforge.net/
Samtools	Li et al., 2009	http://samtools.sourceforge.net/
DREME (Web-based)	Bailey et al., 2009	http://meme-suite.org/tools/dreme
Other		
See the analyses of m ¹ A-MAP data in the methods details	This paper	N/A
See the analyses of ribosome profiling data in the methods details	This paper	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
See the m ¹ A sites identified in tRNA, mRNA and mitochondrial encoded transcripts in Tables S1 , S2 , and S3 , respectively	This paper	N/A
See the m ¹ A sites targeted by TRMT61B in the mitochondrial-encoded transcripts in Table S4	This paper	N/A
Detailed protocol for m ¹ A-MAP	This paper	Methods S1

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests may be directed to and will be fulfilled by the Lead Contact, Chengqi Yi (chengqi.yi@pku.edu.cn).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

HEK293T was maintained at 37°C in DMEM medium (GIBCO) supplemented with 10% FBS (GIBCO) and 1% penicillin/streptomycin (GIBCO).

METHOD DETAILS**Cell Culture and Antibodies**

HEK293T (ATCC, CRL-11268) was used in this study and maintained in DMEM medium (GIBCO) supplemented with 10% FBS (GIBCO) and 1% penicillin/streptomycin (GIBCO). Monoclonal mouse anti-m¹A antibody was purchased from MBL (D345-3). Polyclonal rabbit anti-TRMT6 antibody was purchased from Santa Cruz Biotechnology (sc-271752). MT-CO2 antibody (55070-1-AP) and MT-ATP6 antibody (55313-1-AP) was purchased from Proteintech Group. Monoclonal mouse anti-β-Actin antibody was purchased from CWBiotech (CW0096). Monoclonal mouse anti-GAPDH antibody was purchased from CWBiotech (CW0100M). The secondary antibodies used were anti-mouse-IgG-HRP (CW0102; CWBiotech) and anti-rabbit-IgG-HRP (CW0103; CWBiotech).

RNA isolation

Total RNA was isolated from cells using TRIzol, according to the manufacturer's instructions (Invitrogen). An additional DNase I treatment step was performed to avoid DNA contamination. For polyA⁺ RNA isolation, small RNA was first depleted using MEGAclear Transcription Clean-Up Kit (Ambion), followed by two successive rounds of polyA⁺ selection using oligo(dT)₂₅ Dynabeads (Invitrogen). For small RNA isolation, RNA smaller than 200 nt was recovered from the flow-through fraction in the small RNA depletion step by ethanol precipitation.

shRNA knockdown of TRMT6/61A

The oligoes targeting TRMT6 and TRMT61A were annealed and cloned into the pLKO vector according to the TRC shRNA library protocol (<https://www.broadinstitute.org/mai/public/>), respectively. The oligo sequences were listed below:

```
TRMT6-FWD: CCGGGGGAAAGTTCTGAGTATTTATCTCGAGATAAATACTCAGAACTTTCCCTTTTTG
TRMT6-RVS: AATTCAAAAAGGGAAAGTTCTGAGTATTTATCTCGAGATAAATACTCAGAACTTTCCC
TRMT61A-FWD: CCGGGAGGCCAGAGGCACCTTATATCTCGAGATATAAGGTGCCTCTGGCCTCTTTTTG
TRMT61A-RVS: AATTCAAAAAGAGGCCAGAGGCACCTTATATCTCGAGATATAAGGTGCCTCTGGCCTC
```

A scrambled shRNA was used as the mock control. Lentiviruses were packaged by co-transfecting HEK293T cells with pLKO-TRMT6, pLKO-TRMT61A, pCMV-dR8.91 and VSV-G plasmids, following the instructions from Broad Institute. The supernatant from transfected cells was harvested after 2 days and used to infect HEK293T cells followed by puromycin selection for 5 days. Knockdown efficiency was verified by western blot and qPCR. qPCR primers were listed as follows: TRMT6-qFWD: CTGTCTTTGCTGGACTTTGTGGC; TRMT6-qRVS: AGACAGCCTGAGGTTGATGACC; TRMT61A-qFWD: TCCTCTACTCCACAGACATCGC; TRMT61A-qRVS: CAATG GTGCGGATGATGGCGTG.

Quantification of m¹A and m⁶A level by LC-MS/MS

200 ng isolated RNA or 100 ng model RNA oligo was digested into nucleosides by 0.5 U nuclease P1 (Sigma, N8630) in 20 μL buffer containing 10 mM ammonium acetate, pH 5.3 at 42°C for 6 h, followed by the addition of 2.5 μL 0.5 M MES buffer, pH 6.5 and 0.5 U alkaline phosphatase (Sigma, P4252). The mixture was incubated at 37°C for another 6 h and diluted to 50 μL. 5 μL of the solution was injected into LC-MS/MS. The nucleosides were separated by ultra-performance liquid chromatography with a C18 column, and then

detected by triple-quadrupole mass spectrometer (AB SCIEX QTRAP 5500) in the positive ion multiple reaction-monitoring (MRM) mode. The mass transitions of m/z 282.0 to 150.1 (m^1A), m/z 282.0 to 150.1 (m^6A), m/z 268.0 to 136.0 (A) were monitored and recorded. Concentrations of nucleosides in RNA samples were deduced by fitting the signal intensities into the stand curves.

Stoichiometry curve construction using model oligoes

Two pairs of synthetic RNA oligoes containing either m^1A or A were used to examine the capacity of m^1A in causing mis-incorporation during revers transcription in a quantitative manner. The oligo sequences were listed as follows: m^1A -1: CGCGGCUCGm¹AG CCCGCGUGCGGGCCUCUUUCAGGCCGCU; A-1: CGCGGCUCGAGCCCCGUGCGGGCCUCUUUCAGGCCGCU; m^1A -2: CG GCGGCCCGGGACCGm¹AGACCCGGCCCCGGCUCUCCC; A-2: CGGCGGCCCGGGACCGAGACCCGGCCCCGGCUCUCCC. The m^6A contamination in the m^1A oligoes, which was introduced during the oligo purification process, was measured using quantitative LC-MS/MS. The m^1A -oligo was mixed with the A-oligo to ratios: 100%, 75%, 50%, 25%, 12.5%, 6.25% and 0%, respectively. The mixed m^1A/A oligoes were subjected to library construction using specific RT primers as listed below:

RT- m^1A /A-1: ACACGACGCTCTCCGATCTagcggcctgaaagagggc

RT- m^1A /A-2: ACACGACGCTCTCCGATCTggggagccggggcc

Cloning, Expression and Purification of AlkB

A truncated AlkB with deletion of the N-terminal 11 amino acids was cloned into pET30a (Novagen) and transformed to *E. coli* BL21(DE3) followed by culturing in LB medium at 37°C until the OD₆₀₀ reached 0.6–0.8, and further incubating at 30°C for additional 4 h with the addition of 1 mM IPTG. Proteins were purified using Ni-NTA chromatography (GE Healthcare) and gel-filtration chromatography (Superdex 200, GE Healthcare) followed by Mono-Q anion exchange chromatography (GE Healthcare). Such purification procedure effectively avoided RNA contamination from *E. coli* (the expression host).

In vitro Demethylation treatment

We employed two different strategies for the removal of methyl-group at the N1 position of adenosine.

In vitro demethylation mediated by the purified AlkB protein: 10 μ g full-length polyA⁺ RNA was fragmented at 94°C for 5 min using magnesium RNA fragmentation buffer (NEB) and fragmented polyA⁺ RNA was desalted and concentrated by ethanol precipitation. 10 μ g (~0.2 nmol) fragmented polyA⁺ RNA was denatured at 65°C for 5 min, and then subjected to demethylation reaction in a 500 μ L demethylation mixture containing 0.4 nmol purified AlkB, 50 mM MES, pH 6.5, 283 μ M of (NH₄)₂Fe(SO₄)₂·6H₂O, 300 μ M 2-ketoglutarate, 2 mM L-ascorbic acid, 1 U/ μ L SUPERaseIn RNase Inhibitor (Invitrogen). The demethylation reaction was incubated at 37°C for 2 h and quenched by adding 5 mM EDTA. The demethylated RNA was then purified by phenol chloroform extraction.

In vitro demethylation mediated by the Dimroth rearrangement: 10 μ g full-length polyA⁺ RNA was incubated in alkaline buffer (0.1 M Na₂CO₃ /NaHCO₃, 5mM EDTA, pH 10.2) at 65°C for 3 h, and then the treated RNA was purified by ethanol precipitation.

m^1A -MAP

40 μ g polyA⁺ RNA was fragmented to ~150 nt using magnesium RNA fragmentation buffer (NEB). m^1A -containing RNA fragments were enriched through m^1A immunoprecipitation as previous described (Li et al., 2016a). 10 ng (~0.2 pmol) of the immunoprecipitated m^1A -containing RNA fragments were subjected to the AlkB demethylation treatment. Demethylation reaction was performed in a 20 μ L demethylation mixture containing 0.4 pmol purified AlkB, 50 mM MES, pH 6.5, 283 μ M of (NH₄)₂Fe(SO₄)₂·6H₂O, 300 μ M 2-ketoglutarate, 2 mM L-ascorbic acid and 0.4 U/ μ L RNase inhibitor, following with an incubation at 37°C for 2 h. The reaction was quenched by the addition of 5 mM EDTA, and the demethylated RNA was purified by phenol chloroform extraction followed with ethanol precipitation.

Fragmented polyA⁺ RNA (as “Input”), immunoprecipitated RNA [as “(-) demethylase”] and demethylated immunoprecipitated RNA [as “(+) demethylase”] were subjected to library construction. The library construction was performed according to the eCLIP library construction protocol with several modifications (Van Nostrand et al., 2016). For dephosphorylation of 3' ends, RNA samples were treated by PNK (NEB) with an incubation at 37°C for 1 h, and followed by a heat-inactivation of PNK at 65°C for 20 min. The dephosphorylated RNA was purified by ethanol precipitation and then subjected to 3' RNA linker ligation using T4 RNA ligase2, truncated KQ (NEB) at 25°C for 2 h. The 3' RNA linker sequence is: 5'rAPP-AGATCGGAAGAGCGTCGTG-3SpC3. The excess RNA adaptor was digested by adding 1 μ L 5' Deadenylase (NEB) into the ligation mix followed by an incubation at 30°C for 1 h, and then adding 1 μ L RecJf (NEB), incubating at 37°C for another 1 h. The enzymes were then heat-inactivated at 70°C for 20 min, and the RNA was purified by ethanol precipitation. The RNA pellet was dissolved in 10 μ L H₂O and then 1 μ L 2 μ M RT primer (ACACGACGCTCTCCGATCT) was added. RNA-primer mix was denatured at 80°C for 2 min and then chilled on ice immediately. RT reaction buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, and 3 mM MgCl₂, final), dNTPs (1 mM, final), DTT(5 mM, final), RNase Inhibitor (1 U/ μ L, final) and 1 μ L TGIRT (InGex) were added into the denatured RNA-primer mix and reverse transcription was carried out at 57°C for 2 h. Excess RT primer was digested by the addition of 1 μ L Exonuclease I (NEB) and a following incubation at 37°C for 30 min. cDNA was purified using silane beads (Invitrogen) and then ligated to the 5' adaptor (5Phos- NNNNNNNNNAGATCGGAAGAGCA CACGTCTG-3SpC3). Ligation was performed with T4 RNA ligase 1, high concentration (NEB) at 25°C overnight. The ligated cDNA

was purified using silane beads, and then subjected to PCR amplification with the following primers: 5'-AATGATACGGCGACCACC GAGATCTACTCTTTCCCTACACGAC GCTCTCCGATCT-3', 5'-CAAGCAGAAGACGGCATACGAGATXXXXXXGTGACTGGAGT TCAGACGTGTGCTCTCCGATC-3' (XXXXXX represents index sequence). PCR product was purified by 8% TBE gel. The libraries were sequenced on Illumina Hiseq X10 with paired-end 2 × 150 bp read length.

Locus-specific m¹A detection

We developed two different strategies for locus-specific detection of m¹A sites at the first nucleotide of mRNA transcripts and those located internally, respectively.

For cap+1 m¹A detection, 2 μg polyA⁺ RNA was fragmented to ~150 nt and then equally divided into two parts. One part of RNA sample was subjected to the AlkB demethylation treatment ["(+)-AlkB"] and the other part was untreated [“(-)-AlkB”]. Demethylation reaction was performed as described above. (+) AlkB and (-) AlkB sample were ligated to 3' RNA linker (5'rAPP-AGATCGGAAGAGCGTCGTG-3SpC3) and then reverse transcription was carried out using TGIRT with the same RT condition as that of m¹A-MAP. The 5' cap-adaptor (5Phos- GTCTCAGCGTCCAGTGCATC-3SpC3) was ligated to the reverse transcribed cDNAs. The regions harboring m¹A were amplified by PCR using a universal forward primer (5'-GATGACACTGGACGCTGAGAC-3') and gene specific reverse primers (listed in Table S5). The amplicons from different m¹A-containing regions were mixed together for (+) AlkB and (-) AlkB sample, respectively, and then subjected to DNA library construction using NEBNext Ultra II DNA Library Prep Kit for Illumina (E7645) according to the manufacturer's instructions. Deep sequencing for the libraries was performed on Illumina Hiseq X10 with paired-end 2 × 150 bp read length. Thus, cap+1 m¹A to be validated can be covered with sufficient reads for the reliable examination of mutation rate.

For internal m¹A detection, 200 ng polyA⁺ RNA was isolated from TRMT6/61A stable knockdown cells and mock control cells respectively, and then was fragmented to 150 nt using magnesium RNA fragmentation buffer (NEB). Fragmented RNA was ligated to 3' RNA linker (5'rAPP-AGATCGGAAGAGCGTCGTG-3SpC3) and then reverse transcription was carried out using TGIRT with the same RT condition as that of m¹A-MAP. The regions harboring m¹A were amplified by PCR using specific primers. The amplicons from different m¹A-containing regions were mixed together for each sample, and then subjected to DNA library construction using NEBNext Ultra II DNA Library Prep Kit for Illumina (E7645) according to the manufacturer's instructions. Deep sequencing for the libraries was performed on Illumina Hiseq X10 with paired-end 2 × 150 bp read length. Thus, the position of m¹A to be validated can be covered with sufficient reads for the examination of mutation rate. Specific PCR primers are listed below:

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NM_025099-m1A5643-FWD: AAAAAGCTCGGTCCGGGTTTC
NM_025099-m1A5643-RVS: TTAGCCGCAAATCACGCTG
NM_001193375-m1A978-FWD: ACACCTGTCCAAGCCCTAAT
NM_001193375-m1A978-RVS: CTGAGGGGCCCTTATTCCCA
NR_026951-m1A659-FWD: ACGTCGGCTCGTTGGTCTAG
NR_026951-m1A659-RVS: ACAGTCAAGCCTCCTGCAGC
NM_001256443-m1A486-FWD: CAAGTTCCAGGCGAAGGG
NM_001256443-m1A486-RVS: AGCCGGGGTCTCTGTGG
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Purification of Mitochondrial 12S mt-rRNA

Total RNA was isolated from cells using TRIzol, according to the manufacturer's instructions (Invitrogen). An additional DNase I treatment step was performed to avoid DNA contamination. Bring 200 μg DNase I treated total RNA to volume of 160 μL in nuclease-free water, and then add 1 μL 12S mt-rRNA probe mix (20 μM each). The probe mix contains 4 3'-biotin labeled oligoes that specifically target 12S mt-rRNA and the sequences were listed as follows: 12S-probe-1: GAGCTAATAGAAAGGCTAGGAC; 12S-probe-2: TCTATTGACTTGGGTTAATC; 12S-probe-3: TTATCGATTACAGAACAGGCT; 12S-probe-4: TCTACTCTTAGTTTACTGCTAA. The RNA mixture was heated at 65°C for 10 min and followed by chilling on the ice. 40 μL 5 × Binding buffer (1.5 M NaCl, 10 mM EDTA, 100 mM Tris, pH 7.4) was added into the RNA mixture and mixed well by pipetting. The RNA mixture was incubated at room temperature for about 30 min. 200 μL Streptavidin C1 Dynabeads (Invitrogen) was resuspended in 200 μL Binding Buffer (300 mM NaCl, 2 mM EDTA, 20 mM Tris, pH 7.4) and then added into the RNA mixture. The RNA beads mixture was incubated at room temperature for 40 min by gently rotating. The beads was washed with Washing Buffer (30 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 7.4, 1% SDS) at 40°C for four times. RNA was eluted from beads by adding 200 μL Elution buffer (5 mM EDTA, 10 mM Tris, pH 7.4, 1% SDS) and incubating at 70°C for 10 min. Isolated RNA was recovered by ethanol precipitation and 12S mt-rRNA was further selected using 4% Urea-PAGE. The purity of isolated 12S mt-rRNA was checked using RNA 6000 Pico Chip on an Agilent 2100 Bioanalyzer (Agilent Technologies). The m¹A methylation level of 12S mt-rRNA was analyzed by quantitative LC-MS/MS.

Primer extension

The primer extension was performed as previously reported (Bar-Yaacov et al., 2016; Chujo and Suzuki, 2012) with several modifications.

To detect m¹A1374 in *MT-ND5*, 2 μg mRNA was mixed with 10 fmol 5' biotin labeled primer (ND5- m¹A1374-primer: CCTGCGAA TAGGCTTCC) in a 5 μL solution containing 10 mM Tris, pH 7.5 and 1 mM EDTA. The RNA mixture was then heated at 80°C for 2 min

and slowly cooled to 25°C over 55 min. The RT mixture (containing 2 μ L 5 \times First-Strand Buffer, 1.5 μ L 25 mM MgCl₂, 0.5 μ L 0.1 M DTT, 0.5 μ L Superscript III, 0.25 μ L RNase inhibitor, 0.25 μ L 1.5 mM ddATP, dTTP, dCTP, and dGTP mix) was then added into the RNA mixture and mixed well. The reverse transcription was carried out at 55°C for 1 h and then the RNA template was digested with RNase H. The reverse transcribed products were separated by 20% Urea-PAGE and then transferred to Nylon membrane, detected by chemiluminescent nucleic acid detection module (Thermo, 89880).

To detect m¹A378 and m¹A575 in 12S mt-rRNA, 50 ng fragmented total RNA or 20 ng immunoprecipitated RNA was mixed with 5 fmol 5' biotin labeled primer (12S-m¹A378-primer: TGTGTTTCAGATATGTTAAAG; 12S-m¹A575-primer: GAGGTGGTGAGGTTGATCG) and then subjected to reverse transcription as described above. The d/ddNTP mix consisted of dATP, dTTP, dCTP and ddGTP for m¹A378 in 12S mt-rRNA; ddATP, dTTP, dCTP and dGTP for m¹A575 in 12S mt-rRNA. The reverse transcription was carried out at 49°C for 12S-m¹A378 and 55°C for 12S-m¹A575. The reverse transcribed products were analyzed as described above.

siRNA Knockdown and overexpression of TRMT61B

Two synthesized duplex RNAi oligoes targeting TRMT61B mRNA sequences were used: 5'-GGAUAUCAACCCAGGUGAUTT-3' and 5'-GCGUGAUUCAUGGAAAUUATT-3'; a scrambled duplex RNAi oligo (5'-UCCUCCGAACGUGUCACGUTT) was used as the mock control. The siRNA oligo was transfected into HEK293T by Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions, and cells were harvested 48 h later after transfection. Knockdown efficiency was examined by qPCR. qPCR primers were listed as follows: TRMT61B-qFWD: CAGGAGCAACCGAAGACAT; TRMT61B-qRVS:ATATACAGCACATACACCACCAT;

For the construction of overexpression plasmid, TRMT61B was cloned into pcDNA3.1 using the following primers: FWD: TCGCGAAACACTATGCTAATGGC; RVS: GTTAAGTTGTGGTTTGACCTTCCTC. The empty pcDNA3.1 vector was used as the mock control. The plasmid was transfected into HEK293T by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions and cells were harvested 48 h later after transfection.

Ribosome profiling

2 plates of 15-cm HEK293T cells were grown to 90% confluency; CHX was then added to the medium at a final concentration of 100 μ g/mL. Cells were treated in the CHX-medium for 7 min before they were harvested and lysed with 1 mL lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 100 mM KCl, 1% Triton X-100, 2 mM DTT, 100 μ g/mL CHX, 0.5 U/ μ L RNase inhibitor, 1 \times complete protease inhibitor). The cell lysates were centrifuged at 15,000 g for 15 min, and the supernatant was collected and sent for the OD260 measuring. 100 μ L lysates were kept as input sample and 1 mL TRIzol was added to purify the RNA. 1 μ L Micrococcal Nuclease (NEB) per 25OD was added to the remaining cell lysates and incubated at 25°C for 20 min. The digested cell lysates were used for performing ribosome foot-printing. The lysates were fractionated on 10/50% w/v sucrose gradients using the SW-40Ti rotor at 27,500rpm for 4h. 80S monosome fraction was collected, followed by the addition of equal volume of extraction buffer (1% SDS, 40 mM EDTA). RNA was isolated by phenol-chloroform extraction. RNA fragments between 28–30 nt were selected using 15% Urea-PAGE. Recovered RNA fragments were subjected to library construction. In brief, RNA samples were dephosphorylated with PNK (NEB) and ligated to 3' RP linker (5'rAPP-CTGTAGGCACCATCAAT-3SpC3) using T4 RNA ligase2, truncated KQ (NEB). Reverse transcription was carried out using Superscript III (Invitrogen) with RP-RT primer (5Phos-AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTGC-3SpC18-CACTCA-SpC18-TTCAGACGTGTGCTCTTCCGATCTATTGATGGTGCC TACAG). cDNA was circ-ligated with CircLigase II (Epicenter) and then amplified by PCR with primers (5'- AATGATACGG CGACCACCGAGATCTACAC-3'; 5'-CAAGCAGAAGACGGCATACGAGATXXXXXXGTGACTGGAGTTCAGACGTGTGCTCTTCCGA TC-3', XXXXXX represents index sequence). PCR products were purified by 8% TBE gel and sequenced on Illumina Hiseq 2500 with single end reads (50 bp).

qPCR-based m¹A level evaluation

20 μ g polyA⁺ RNA was isolated from HEK293T cells with TRMT61B overexpression, knockdown and the corresponding mock controls, respectively. RNA was fragmented into ~150 nt using magnesium RNA fragmentation buffer (NEB) and concentrated by ethanol precipitation. Fragmented RNA (as Input) was denatured and incubated with 2 μ g anti-m¹A antibody in IPP buffer (150 mM NaCl, 0.1% NP-40, 10 mM Tris, pH 7.4) at 4°C overnight. 20 μ L Protein A/G UltraLink Resin (Pierce) was added to the RNA-antibody mixture and incubated at 4°C for additional 3 h. Resins were washed for twice with IPP buffer, once with low salt buffer (75 mM NaCl, 0.1% NP-40, 10 mM Tris, pH 7.4), once with high salt buffer (200 mM NaCl, 0.1% NP-40, 10 mM Tris, pH 7.4) and twice with TEN buffer (10 mM Tris, pH 7.4, 1 mM EDTA, pH 8.0, 0.05% NP-40). m¹A-containing RNA was eluted from resins with 3 mg/mL N¹-methyladenosine (Berry&Associates) in IPP buffer and purified by phenol chloroform extraction and ethanol precipitation. Input and immunoprecipitated samples were reverse transcribed into cDNA using Superscript III (Invitrogen) and quantified by qPCR using SYBR GREEN mix (Takara) on Roche Lightcycler 96 real-time PCR system. The m¹A-IP/Input ratios of the targeted regions were calculated for the TRMT61B overexpression, knockdown and the corresponding control samples, respectively. The primers used for qPCR were listed below:

MT-CO1-qFWD: CCTATCATCTGTAGGCTCATT
 MT-CO1-qRVS: GGAGGGTTCTTCTACTATTAGGAC
 MT-CO2-qFWD: ACAGATGCAATTCCTCCGGACG

MT-CO2-qRVS: CCACAGATTTTCAGAGCATTGACC
 MT-CO3-qFWD: CGCCTGATACTGGCATTGTTG
 MT-CO3-qRVS: GACCCTCATCAATAGATGGAGAC
 MT-CYB-qFWD: CAACCCCTAGGAATCACCTC
 MT-CYB-qRVS: GAGGGCGTCTTTGATTGTGTAG
 16S rRNA-qFWD: ATGAATGGCTCCACGAGGG
 16S rRNA-qRVS: CTTGCTGTGTTATGCCCGC

Reductive dimethylation labeling

Mitochondrion was isolated from TRMT61B overexpression and mock control HEK293T cells according to the manufacturer's (Thermo Fisher) and lysed with RIPA buffer followed by sonication. The extracted proteins from different samples were quantified with the BCA protein assay kit (Thermo Fisher). Equal amount of proteins from two samples were digested by trypsin on-column in 100 mM TEAB buffer and subjected to reductive dimethylation labeling. 4 μ L of 4% (w/w) light or heavy formaldehyde was added to 100 μ L of trypsin digested samples prepared from TRMT61B overexpression and mock control HEK293T cells, respectively. In the meantime, 4 μ L of 0.6 M sodium cyanoborohydride was added and the samples were incubated at room temperature for 1h. The dimethylation labeling reaction was quenched by the addition of 1% (w/w) ammonia and 5% (w/w) formic acid. Finally, light and heavy labeled peptide samples were mixed, concentrated by vacuum, and analyzed on a Q Exactive mass spectrometer (Thermo Fisher).

LC-MS/MS and data analysis

The peptides were analyzed on a Q Exactive mass spectrometer (Thermo Fisher). Under the positive-ion mode, full-scan mass spectra was acquired over the m/z range from 350 to 1800 using the Orbitrap mass analyzer with mass resolution setting of 70000. MS/MS fragmentation was performed in a data-dependent mode, of which the 20 most intense ions were selected from each full-scan mass spectrum for high-energy collision induced dissociation (HCD) and MS2 analysis. MS2 spectra were acquired with a resolution setting of 17500 using the Orbitrap analyzer. Some other parameters in the centroid format: isolation window, 2.0 m/z units; default charge, 2+; normalized collision energy, 28%; maximum IT, 50 ms; dynamic exclusion, 20.0 s.

LC-MS/MS data were analyzed by ProLuCID (Xu et al., 2015) with static modification of cysteine (+57.0215 Da) and variable oxidation of methionine (+15.9949 Da). The searching results were filtered by DTASelect (Tabb et al., 2002) and peptides were also restricted to fully tryptic with a defined peptide false positive rate of 1%. The ratios of reductive dimethylation were quantified by the CIMAGE software as described before (Weerapana et al., 2010).

Pre-processing of raw sequencing data

A random barcode of 10 nt was included in the adaptor that ligates to the 3' end of cDNA and it cannot be precisely located in Read 1. Hence, only Read 2 data of m¹A-MAP was used for subsequent analyses. Raw sequencing reads produced from m¹A-MAP were first subjected to Trim_galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) for quality control and adaptor trimming. The minimum quality threshold was set to 20, and the minimum length required for reads after trimming was 30 nt. The remaining reads were further processed by removing the first 10 nt random barcode in the 5' end. As for the ribosome profiling (Ribo-seq) data and the corresponding RNA-seq data, reads with a quality lower than 20 were discarded, and the adaptor in 3' end was trimmed. Processed reads with a length ranging from 25 nt to 35 nt in the ribosome profiling sample were kept for further analysis.

Reads mapping and PCR duplication removing

Processed reads were mapped to human transcriptome or mitochondrial genome using BWA-MEM with default parameters (version 0.7.15-r1140) (Li and Durbin, 2009). Reference transcriptome was prepared based on the Refseq annotation of human (hg19) downloaded from the table browser of UCSC database. The redundant sequences with the same Refseq id were removed. Transfer RNA (tRNA) sequences were also downloaded from UCSC table browser and integrated into the transcriptome. Mitochondrial genome and corresponding annotation were downloaded from NCBI (NC_012920.1). Reads mapping to an identical position of reference were considered as PCR duplications if their 10 nt random barcodes were the same, and only one of these reads was kept. Performances related to the processing of sam/bam file were done with the help of SAMtools (Li et al., 2009) (<http://samtools.sourceforge.net/>).

Identification of m¹A sites

Mismatch rate of each nucleotide in the reference sequences was calculated for both (-) and (+) demethylase samples. Two parameters were defined to evaluate the dynamic change of mismatch rate in the (-) demethylase and (+) demethylase samples: difference of mismatch rate (Diff) and fold change of mismatch rate (FC). Diff was calculated by subtracting the mismatch rate in the (+) demethylase from that in the (-) demethylase sample, while FC was calculated by dividing the mismatch rate in the (-) demethylase by that in the (+) demethylase sample. FC was artificially set to "1000" if the mismatch rate in the (+) demethylase sample was "0." A position

was identified as an m¹A site when the following criteria were met: a) FC > = 3; b) Diff > = 10%; c) the number of reads with a mismatch at the position was no less than 5; d) criteria (a-c) were all fulfilled in both replicates.

In order to evaluate the frequency of potential false positives caused by m¹A-independent mismatch, we employed a reverse calling procedure. Specifically, the “reverse calculation” of Diff and FC values for each position were performed following the equations below:

$$\text{Diff}_{\text{opposite}} = (+) \text{demethylase} - (-) \text{demethylase}; \text{FC}_{\text{opposite}} = (+) \text{demethylase} / (-) \text{demethylase}.$$

Under such circumstance, only 17 sites passed the above-mentioned threshold, suggesting the m¹A-independent mismatch should minimally interfere with the identification of true m¹A sites.

Identification of TRMT61B targets in mitochondrial-encoded transcripts

For the identification of TRMT61B targeting sites in mitochondrial-encoded RNA, the mismatch rate in the Input sample was first calculated for mock control sample and TRMT61B overexpression sample, respectively. Two parameters were defined to evaluate the increase of mismatch rate from the mock to TRMT61B o/e sample: difference of mismatch rate (Diff) and fold change of mismatch rate (FC). Diff was calculated by subtracting the mismatch rate in the mock sample from that in the TRMT61B o/e sample, while FC was calculated by dividing the mismatch rate in the TRMT61B o/e by that in the mock sample. FC was artificially set to “1000” if the mismatch rate in the mock sample was “0.” A position was identified as TRMT61B target when the following criteria were met: a) FC > = 5; b) Diff > = 5%; c) the site was covered by more than 500 sequencing reads; d) criteria (a-c) were all fulfilled in both replicates.

Motif discovery and GO enrichment analysis

For the analysis of sequence consensus, 15 nt of sequence neighboring each m¹A site was retrieved. These sequences were then subjected to DREME algorithm in MEME suite (Version 4.12.0) for the discovery of enriched motifs (Bailey et al., 2009). The shuffled input sequences were used as the background to eliminate potential false positives caused by the nucleotide composition.

Gene Ontology (GO) enrichment analyses were performed using DAVID web-based tool (<https://david.ncicrf.gov>) (Huang et al., 2009).

Secondary structure and minimum free energy analysis

12 nucleotides of the 5' end and 10 nucleotides of the 3' end of each m¹A site (hence 23 nt sequence in total) were retrieved for local structure analysis. RNAfold (v2.3.4) (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>) was used to predict the secondary structure and calculate the corresponding minimum free energy (MFE). The length of the loop where m¹A resides was determined based on the predicted structure; for an m¹A site that is not located in a loop, this value was set to “0.” The significance test of MFE between m¹A sites within the GUUCRA motif and other m¹A sites was performed using Mann-Whitney U-test.

Ribosome profiling data analysis and TE calculation

Ribo-seq and corresponding RNA-seq reads were aligned to the transcriptome, and RPKM (Reads Per Kilobase per Million mapped reads) of each transcript was calculated. Translation efficiency (TE) was defined for each transcript as the ratio of the RPKM in Ribo-seq to the RPKM in RNA-seq.

For the analysis of influence of m¹A on mitochondrial gene translation, mitochondrial ribosome profiling data were downloaded from the GEO Datasets (GSE48933) (Rooijers et al., 2013). The depth of reads covered for each nucleotide along the mitochondrial transcripts was retrieved using Samtools depth tool.

miRNA target analysis

The predicted miRNA targeting sites on mitochondrial coding genes were downloaded from the miRWalk database (v2.0) (Dweep and Gretz, 2015), which depends on the base-pairing between “seed region” and gene sequence. The minimum length required for the match of seed region was set to 7 nt. The experimentally identified miRNA targeting sites were retrieved from the published CLASH results (Helwak et al., 2013).

Detection of mitochondrial nascent protein synthesis

The TRMT61B knockdown cells and mock control cells were washed with pre-warmed methionine-free medium and incubated in methionine-free medium for 45 min. Then Cells were treated with 50 μg/mL emetine in methionine-free medium for 15 min to inhibit cytoplasmic protein synthesis. Further the medium was replaced with methionine-free medium containing 1 mM methionine analog L-azidohomoalanine (AHA) (Click Chemistry Tools) and 50 μg/mL emetine. After incubation for 1 h, cells were harvested using clod PBS. Total proteins from different cell lines were quantified with the BCA protein assay kit (Thermo Fisher). Equal amount of AHA-containing proteins were labeled with Cy3 (Click Chemistry Tool) via Click Chemistry. The click reaction was performed in RIPA buffer containing 550 μM Cy3-alkyne, 4 mM BTAA-CuSO₄ complex and 151.43 mM sodium ascorbate. The reaction was incubated for 2 h at 25°C with gentle vortex. The labeled proteins were purified by chloroform/methanol precipitation and separated in a 8%–16% gradient SDS-PAGE, and the SDS-PAGE gel was visualized by Typhoon 9500 scan. The gel was also subjected to Coomassie blue staining as loading control.

Enrichment of nascent protein

The TRMT61B overexpression cell lines and mock control cell lines were washed with pre-warmed methionine-free medium and starved of methionine in the methionine-free medium for 1 h. Then the medium was replaced with methionine-free medium containing 1 mM L-azidohomoalanine (AHA). After incubation for 2 h, the cells were harvested using cold PBS. Equal amount of AHA-containing proteins were labeled by biotin-alkyne (Click Chemistry Tool) via click reaction. The purified proteins were incubated with 200 μ L Streptavidin Agarose Resin (Thermo) in RIPA buffer for 3 h at 29°C. Resins were washed 3 times with PBS and 3 additional times with water. Proteins were reduced by incubated at 37°C in the presence of 10mM DTT in 6M Urea in PBS for 30min and later alkylated by adding iodoacetamide (Sigma) to the final concentration of 20 mM for another 30min. Resins were sequentially washed once with PBS and once with 100nM Triethylammonium bicarbonate (TEAB) buffer. Proteins were digested “on bead” with trypsin in 100 mM TEAB and 1 mM CaCl₂ buffer overnight at 37°C. The peptide solution was performed reductive dimethylation labeling and analyzed on a Q Exactive mass spectrometer.

QUANTIFICATION AND STATISTICAL ANALYSIS

p values were calculated using unpaired two-sided Mann-Whitney U-test. *p < 0.05, **p < 0.01. N.S. stands for not significant. Error bars represent mean \pm SD.

DATA AND SOFTWARE AVAILABILITY

Sequencing data have been deposited into the Gene Expression Omnibus (GEO) under the accession number GEO: GSE102040. Original imaging data have been deposited to Mendeley Data and are available at <https://doi.org/10.17632/bf8cshtzmx.1>

ADDITIONAL RESOURCES

A detailed protocol describing m¹A-MAP used in this study is provided in [Methods S1](#).