

1 **Base-resolution mapping reveals distinct m¹A methylome in**
2 **nuclear- and mitochondrial-encoded transcripts**

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1 SUMMARY

2 Gene expression can be post-transcriptionally regulated via dynamic and reversible
3 RNA modifications. *N*¹-methyladenosine (m¹A) is a recently identified mRNA
4 modification; however, little is known about its precise location, regulation and
5 function. Here, we develop a base-resolution m¹A profiling method, based on m¹A-
6 induced misincorporation during reverse transcription, and report distinct classes of
7 m¹A methylome in the human transcriptome. m¹A in 5'-UTR, particularly those at the
8 first nucleotide of mRNA, associate with increased translation efficiency. A different
9 subset of m¹A exhibit a GUUCRA tRNA-like motif, are evenly distributed in the
10 transcriptome and are dependent on the methyltransferase TRMT6/61A. Additionally,
11 we show for the first time that m¹A is prevalent in the mitochondrial-encoded
12 transcripts. Manipulation of m¹A level via TRMT61B, a mitochondria-localizing m¹A
13 methyltransferase, demonstrates that m¹A in mitochondrial mRNA interferes with
14 translation. Collectively, our approaches reveal distinct classes of m¹A methylome
15 and provide a resource for functional studies of m¹A-mediated epitranscriptomic
16 regulation.
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1 INTRODUCTION

2 More than 100 different types of post-transcriptional modifications have been
3 identified so far (Machnicka et al., 2013). Recent breakthroughs in sequencing
4 technologies have greatly advanced our understanding to the location, regulation,
5 and function of RNA modifications in the transcriptome (Frye et al., 2016; Fu et al.,
6 2014; Helm and Motorin, 2017; Li et al., 2016b), leading to the emerging field of
7 epitranscriptomics (He, 2010; Saletore et al., 2012). One such example is *N*¹-
8 methyladenosine (m¹A), a prevalent modification in non-coding RNA (ncRNA) and
9 messenger RNA (mRNA) (Anderson and Droogmans, 2005; Roundtree et al., 2017).
10 m¹A was first documented more than 50 years ago (Dunn, 1961); later it was found
11 to be a primordial RNA modification across the three major phylogenetic domains
12 (Machnicka et al., 2013). In human cells, m¹A is found at position 9 and 58 of human
13 mitochondrial and cytoplasmic tRNAs, catalyzed by TRMT10C, TRMT61B and
14 TRMT6/61A, respectively (Chujo and Suzuki, 2012; Ozanick et al., 2005; Vilaro et
15 al., 2012); it is also present at position 1322 of 28S rRNA, catalyzed by NML (Waku
16 et al., 2016). Its unique physicochemical property has also endowed m¹A with pivotal
17 roles in maintaining the proper structure and function of these ncRNAs (Roundtree et
18 al., 2017). m¹A in tRNA has also been systematically evaluated by microarray and
19 sequencing (Cozen et al., 2015; Saikia et al., 2010; Zheng et al., 2015); more
20 recently, m¹A58 is shown to be reversible by ALKBH1, demonstrating an example of
21 reversible tRNA modification in translation regulation (Liu et al., 2016). In addition to
22 ncRNAs, m¹A is also found to be a dynamic modification in mammalian mRNA, with

1 strong enrichment in the 5'-UTR (Dominissini et al., 2016; Li et al., 2016a).

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

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

1 **RESULTS**

2 **m¹A-induced misincorporation during reverse transcription**

3 Because m¹A can cause both truncation and misincorporation during cDNA
4 synthesis (Hauenschild et al., 2015; Zubradt et al., 2017), we first established the
5 truncation and mutation profiles of different reverse transcriptases (RTases). We
6 systematically compared the performance of several commercially available RTases
7 (including AMV, SuperScript II, SuperScript III and TGIRT) under different conditions
8 (Figure S1). We found that m¹A can precisely induce misincorporation at the site of
9 modification, while m¹A-induced truncation is less accurate and can occur to the
10 neighboring nucleotides. In addition, the truncation profile could be complicated by
11 RNA secondary structures and the fragmentation process needed for library
12 preparation. We concluded that the mutation profile contains a higher signal/noise
13 ratio and is more precise in detecting the exact position of m¹A. Among the RTases
14 we tested, TGIRT demonstrated excellent read-through efficiency and relatively high
15 mutation frequency at the site of m¹A (Figure S1B), consistent with the recent DMS-
16 MaPseq and DM-tRNA-seq results (Zheng et al., 2015; Zubradt et al., 2017).
17 Moreover, we employed a ligation-based strand-specific library preparation protocol
18 (Van Nostrand et al., 2016), which ensures that the m¹A-induced mutation is within
19 the sequenced fragment (see Method Details).

20 Because we only observed ~40-50% mutation rate at m¹A1322 in 28S rRNA
21 (Figure S2A), which is known to be of high modification level, we further examined

1 the quantitative capability of TGIRT. We chemically synthesized two model RNA
2 sequences with site-specific m¹A modification. For m¹A sites with ~97-98%
3 modification level (measured by quantitative mass spectrometry) (Figure S2B), we
4 consistently observed ~66-75% misincorporation (Figure S2C); the mismatch rate
5 dropped non-linearly when we gradually lowered the modification level. Even with
6 ~50% m¹A modification, a mismatch rate of only ~9-10% was observed (Figure
7 S2C). These findings suggest that the observed mutation rate is an underestimation
8 of the actual modification level. While the TGIRT-based procedure can still detect
9 m¹A sites of high modification level, sequencing RNA directly with TGIRT may not be
10 able to capture the m¹A sites with averaging modification level in the transcriptome
11 (~20% as previously measured by microarray) (Dominissini et al., 2016). To improve
12 the sensitivity for transcriptome-wide m¹A detection, we decided to couple the
13 TGIRT-based procedure with a pre-enrichment step and an additional *in vitro*
14 demethylation step (Figure 1A). We first show that *in vitro* demethylation reaction
15 mediated by the demethylase AlkB is more efficient than the Dimroth reaction,
16 demonstrating ~98% and ~80% efficiency (Figure S2D), respectively. In addition, the
17 extended treatment of RNA in alkaline condition during the Dimroth reaction leads to
18 excessive RNA degradation (Figure S2E), potentially causing loss of RNA molecules.
19 By integrating the enrichment and demethylation steps, we successfully maximized
20 the dynamic range of m¹A-induced mutational signature for m¹A1322 in 28S rRNA
21 (~47%, ~95% and ~0.9% in the input, (-) and (+) demethylase samples,
22 respectively), allowing sensitive and confident m¹A detection (Figure 1B). We termed

1 our approach misincorporation-assisted profiling of m¹A, or m¹A-MAP.

2

3 **m¹A-MAP detects known and novel m¹A in tRNA**

4 We next applied m¹A-MAP to tRNA. In mammals, m¹A can occur at position 9, 14
5 and 58 of tRNA (Anderson and Droogmans, 2005). m¹A₁₄ has been reported only in
6 tRNA^{Phe} and is considered to be very rare (Machnicka et al., 2013); we did not
7 observe any m¹A modification at position 14 for cytosolic tRNAs in HEK293T cells
8 (Table S1). m¹A₅₈ is conserved across the three domains of life; previous tRNA
9 microarray and sequencing data has reported hypomodified tRNAs at this position
10 (Cozen et al., 2015; Saikia et al., 2010; Zheng et al., 2015). Our results confirmed
11 that m¹A₅₈ is globally present in the cytosolic tRNAs (Figure 1C and S2F). The m¹A₉
12 modification exists only in archaea tRNA or mammalian mitochondrial tRNA;
13 interestingly, we observed a novel m¹A₉ site for cytosolic tRNA^{Asp(GUC)}, representing
14 the first cytosolic tRNA with m¹A modification at position 9 (Figure 1D). Collectively,
15 these observations suggest that m¹A-MAP is highly sensitive in detecting m¹A at
16 single-base resolution.

17

18 **Single-nucleotide resolution m¹A methylome in the transcriptome**

19 We then sought to detect transcriptome-wide m¹A methylome at single-base
20 resolution. We defined two parameters to evaluate the m¹A-MAP data: difference of
21 mismatch rate and fold change of mismatch rate (see Method Details). To minimize

1 the effect of mismatch rate variation during m¹A identification, we rigorously tested
2 our threshold and identified 740 m¹A sites in the 293T transcriptome (Table S1-3). To
3 evaluate potential false positives caused by m¹A-independent mismatch, we
4 performed the opposite calculation and retrieved only 17 such sites (see Method
5 Details). Moreover, we also systematically evaluated the mutation pattern of the
6 identified m¹A sites in the transcriptome. Using m¹A sites in tRNA as positive
7 controls, we found that m¹A-induced mutation is more strongly influenced by its 5'-
8 nucleotide than the 3'-nucleotide; importantly, a similar sequence-dependent feature
9 is also observed for m¹A sites in mRNA (Figure 2A and S3A). Therefore, we
10 conclude that our strict threshold allowed us to confidently detect transcriptome-wide
11 m¹A sites at single-nucleotide resolution.

12 Out of the 740 m¹A modifications in the transcriptome (Figure S3B), 473 sites are
13 located in mRNA and lncRNA molecules (Figure S3C and Table S2). Majority of
14 these sites are within the 5'-UTR (Figure 2B and 2C), consistent with the previous
15 finding (Dominissini et al., 2016; Li et al., 2016a). Our single-base profile also reveals
16 new features of the m¹A methylome: for instance, we found 24 m¹A methylation sites
17 that are present exactly at the first nucleotide of the 5' end of the transcripts (Figure
18 2D and Table S2). Because the first two nucleotides of the 5' end of mRNA are
19 known to contain ribose methylation, it is likely that these transcripts have an m¹A_m
20 modification at the cap+1 site. We also found 3 additional transcripts with m¹A
21 methylation at the second nucleotide, potentially giving rise to m¹A_m at cap+2 as
22 well. For m¹A located in CDS, while we did not observe a tendency for codon

1 position, we did notice a mild preference for codon types, with Arg(CGA) being the
2 most frequently modified by m¹A (Figure S3D). No m¹A is detected for AUG start
3 codons. Representative examples of m¹A sites identified from different mRNA
4 regions are shown (Figure 2D-F and S3E). Two additional sites of high mutation,
5 which are insensitive to the demethylase treatment, also appeared in the *WDR18*
6 and *BRD2* examples (Figure 2D and 2E). By referring to the SNP database, we
7 found that these two positions belong to annotated SNP sites, demonstrating the
8 robustness of our approach in distinguishing true m¹A sites from false signals (SNP,
9 other modifications and etc.).

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

17

18 **A subset of m¹A sites demonstrate a GUUCRA consensus motif**

19 An unbiased motif detection using DREME revealed that a subset of m¹A (53 sites)
20 are found within a strong GUUCRA sequence (Figure 3A). Interestingly, these sites
21 demonstrate a very different distribution pattern: instead of being enriched in the 5'-
22 UTR, these sites are evenly distributed in the transcriptome (Figure 3B). Because

1 this motif is reminiscent of the m¹A-containing TΨC loop in tRNA, we hypothesized
2 that the tRNA methyltransferase complex TRMT6/61A could be responsible for these
3 mRNA m¹A sites. We first performed direct m¹A sequencing (without antibody
4 enrichment) to RNA population below 200nt. We found that the m¹A58 sites within
5 the GUUCNA motif experienced a global decrease of mutation rate in the
6 TRMT6/61A knock-down sample, which was not observed for m¹A58 sites that do
7 not confine to the motif (Figure 3C, S4A and S4B). This result suggests that
8 TRMT6/61A-mediated m¹A methylation is highly sequence-specific, consistent with
9 evidence from crystal structures (Finer-Moore et al., 2015). We then analyzed the
10 secondary structure for the 53 mRNA m¹A sites, and found highly conserved
11 structural features compared to the T-loop of tRNA (Figure S4C and S4D). We also
12 picked 3 m¹A sites (in CDS, 3'-UTR and lncRNA, respectively) and examined their
13 response after TRMT6/61A knock-down (Figure 3D). Our locus-specific approach
14 (see Method Details), which enabled us to interrogate these sites with high
15 sequencing depth, unambiguously demonstrated a decrease in mismatch rate after
16 TRMT6/61A knock-down (Figure 3D). As a comparison, a non-motif m¹A site located
17 in a different structural context demonstrated an unaltered modification status
18 (Figure 3E). Taken together, these observations suggest that in addition to tRNA,
19 TRMT6/61A is also responsible for a subset of m¹A sites in mRNA.

21 **Distinct m¹A methylome in the mitochondrial transcriptome**

22 In addition to the nuclear-encoded transcripts, we also detected prevalent m¹A

1 modification in the mitochondrial (mt) transcriptome. mt-tRNAs are known to contain
2 m¹A at position 9 and 58 (Suzuki et al., 2011), catalyzed by TRMT10C and
3 TRMT61B (Chujo and Suzuki, 2012; Vilardo et al., 2012) , respectively. m¹A-MAP
4 showed that all the 14 mt-tRNAs bearing an adenosine residue at position 9 are m¹A
5 modified; for position 58, m¹A was detected for the 3 known and 2 novel mt-tRNA
6 molecules (Figure 4 and Table S3). For mt-rRNA, the only known m¹A site is at
7 position 947 of 16S rRNA (Bar-Yaacov et al., 2016). Interestingly, we additionally
8 detected 7 and 10 novel m¹A sites on 16S and 12S mt-rRNA, respectively (Figure 4
9 and Table S3). This is very different from cytosolic rRNA, where there is only one
10 m¹A site in 28S rRNA (m¹A1322). Considering the length of these rRNA species, mt-
11 rRNAs are much more heavily modified by m¹A.

12 In human mitochondria, mRNAs are transcribed from the heavy and light strands
13 as polycistronic units (Falkenberg et al., 2007; Mercer et al., 2011). The processed
14 mt-mRNAs lack a cap at the 5' end and contain no or short untranslated regions
15 (Richter-Dennerlein et al., 2015; Rorbach and Minczuk, 2012; Temperley et al.,
16 2010). We identified 22 m¹A sites from 10/13 mitochondrial genes, in which 21 are
17 residing in CDS and 1 is located in the 3'-UTR (Figure 4). This is distinct to the m¹A
18 methylome in the nuclear-encoded transcripts, where m¹A is enriched in the 5'-UTR.
19 In addition, no preference for codon types was observed, yet m¹A appears to be
20 more likely present at the third position of a codon in the CDS of mt-mRNA (Figure
21 S5A). Moreover, we also identified 25 m¹A sites within the intergenic spacers. 24/25
22 m¹A sites are in the light strand (Figure 4B); some of these m¹A sites could be within

1 the 3'-UTR of *MT-ND6*, for which there is no current consensus of its 3' end
2 (Slomovic et al., 2005).

3

4 **m¹A in mt-mRNA interferes with mitochondrial translation**

5 We next sought to examine the biological consequence of m¹A in the mt-mRNAs.
6 Translation requires accurate base pairing between mRNA codons and the cognate
7 tRNAs; however, m¹A is known to block the canonical A:U base pairing. These facts
8 prompted us to hypothesize that m¹A in mt-mRNA, which are primarily located in
9 CDS, could interfere with translation in mitochondria. We first integrated published
10 mitochondria ribosome profiling data with m¹A-MAP identified m¹A methylome in
11 mitochondria (Rooijers et al., 2013). We found a strong signal of mitochondrial
12 ribosome stalling at the m¹A site on *MT-ND5* (Figure 5A and S5B), whose
13 modification level is the highest among all m¹A sites in mt-mRNA. Due to the
14 difficulty of an *in vitro* mitochondrial translation system (Smits et al., 2010), we
15 sought to enzymatically manipulate the modification level of the mt-mRNAs. Two
16 enzymes are known to introduce m¹A in human mitochondria: TRMT10C generates
17 m¹A as well as m¹G at position 9 in mitochondrial tRNAs, while TRMT61B is
18 responsible for m¹A at position 58 in some mitochondrial tRNAs and position 947 in
19 16S mt-rRNA (Bar-Yaacov et al., 2016; Chujo and Suzuki, 2012; Vilaro et al., 2012).
20 Because TRMT10C is a subunit of the mitochondrial RNase P complex and is not
21 specific for adenosine, we focused on TRMT61B. We utilized a qPCR-based assay
22 to quantitatively evaluate the modification status of the m¹A sites in mt-mRNAs (see

1 Method Details); we found that while TRMT61B knock-down mildly reduced the m¹A
2 level (Figure S5C and S5D), TRMT61B overexpression greatly increased the m¹A
3 modification level of several mt-mRNAs (Figure 5B and S5D). This observation
4 suggests that in addition to mt-tRNA and mt-rRNA, TRMT61B could also target mt-
5 mRNA. Because of the high efficiency of TRMT61B overexpression in increasing the
6 m¹A level, we used mass spectrometry to quantitatively measure the mitochondrial
7 protein level upon TRMT61B overexpression (Figure S5E). Indeed, TRMT61B
8 overexpression led to a reduced protein level for *MT-CO2* and *MT-CO3* (Figure 5C
9 and S5F), which are targets of TRMT61B. We further confirmed this observation for
10 the MT-CO2 protein using Western blot (Figure 5D). Collectively, these results
11 suggest that m¹A in mt-mRNA interferes with translation in mitochondria.

12

13 **DISCUSSION**

14 In this study, we developed a single-nucleotide resolution method for transcriptome-
15 wide identification of m¹A in human cells. m¹A-MAP utilizes the m¹A-induced
16 misincorporation in cDNA synthesis to achieve base-resolution m¹A detection. This
17 enabled us to identify m¹A modification not only at the mRNA cap, but also within a
18 GUUCRA tRNA-like sequence motif. In principle, such misincorporation-dependent
19 strategy could be applied to estimate the modification status of RNA sites of interest.
20 However, our results in both rRNA and model RNA sequences strongly suggest that
21 such estimation should be done with caution: TGIRT underestimates m¹A
22 modification level and m¹A-induced mismatch decreases in a non-linear fashion as

1 the modification level decreases. Additionally, the sequence context of RNA has also
2 been reported to affect the mutation rate (Hauenschild et al., 2015; Zubradt et al.,
3 2017). Hence, while direct sequencing (without enrichment) could still detect m¹A
4 sites with high modification level, m¹A sites with averaging modification level or those
5 located within a non-optimal context for mismatch induction could be missed.
6 Therefore, coupling the pre-enrichment step to the mutational signature is necessary
7 to improve the detection sensitivity. In addition, to achieve high confidence, we
8 employed an *in vitro* demethylation step, which enabled us to distinguish true m¹A
9 sites from false signals (SNP, other modifications, and etc.) in the transcriptome. The
10 combined use of mutational signature, the pre-enrichment step and the *in vitro*
11 demethylation step enabled m¹A-MAP to achieve high sensitivity and confidence.

12 Our study revealed that two known m¹A modification machinery, TRMT6/61A and
13 TRMT61B, could work on mRNA as well. The hetero complex TRMT6/61A
14 recognizes the sequence and structure of the tRNA T-loop and installs an m¹A at the
15 58 position (Finer-Moore et al., 2015). Consistent with this knowledge, we found that
16 the TRMT6/61A-dependent mRNA m¹A sites are also confined to a hairpin structure
17 mostly frequently with a 7nt loop, reminiscent of the tRNA T-loop. In contrast, we did
18 not find an obvious sequence context for the m¹A in mt-mRNA. In fact, TRMT61B
19 appears to be a promiscuous enzyme that also modifies mt-tRNA and mt-rRNA; the
20 substrate specificity and the underlying mechanism of TRMT61B in the human
21 mitochondria remains to be determined. In addition, the fact that both TRMT6/61A
22 and TRMT61B are known tRNA modification enzymes also reminds us of the

1 modification machinery for other mRNA modifications. For instance, eukaryotic Ψ
2 synthases PUS1, PUS7 and TRUB1 can work on both tRNA and mRNA (Carlile et
3 al., 2014; Li et al., 2015; Lovejoy et al., 2014; Safra et al., 2017; Schwartz et al.,
4 2014a); yet the modification complex for m^6A , consisting at least METTL3,
5 METTL14, WTAP and KIAA1429, appears to be dedicated to mRNA (Bokar et al.,
6 1997; Liu et al., 2014; Ping et al., 2014; Schwartz et al., 2014b). In the case of m^1A ,
7 the enzyme(s) responsible for the majority of the modification sites in mRNA remains
8 to be identified. It would be interesting to see if such machinery is specific for mRNA
9 or promiscuous for multiple RNA substrates.

10 Our base-resolution m^1A profiles reveal distinct m^1A methylome in the human
11 transcriptome. m^1A is enriched in the 5'-UTR; and only 5'-UTR m^1A sites, but not
12 those in CDS or 3'-UTR, are correlated with higher translation efficiency. Different
13 from the m^1A sites in the nuclear-encoded transcripts, m^1A in mt-mRNA are primarily
14 located in CDS and inhibit translation. In addition, we also identified a notable group
15 of m^1A methylation adjacent to the mRNA cap, raising the possibility of m^1A_m
16 modification at the first position of RNA transcripts. A related but different
17 modification, m^6A_m , is also known to be present at this position and is recently
18 reported to improve mRNA stability (Mauer et al., 2017). While m^1A -MAP could not
19 discriminate m^1A from m^1A_m , neither m^6A nor m^6A_m should induce misincorporation
20 during reverse transcription, nor become sensitive to demethylation by AlkB. Hence,
21 methylation to the N1 and N6 position of adenosine within the cap should represent
22 distinct types of modifications.

1 Our results also revealed m¹A in mt-mRNA for the first time and showed that
2 such methylation interferes with translation. By manipulating the m¹A level via
3 TRMT61B, we monitored the corresponding changes of the mitochondrial protein
4 level by both quantitative mass spectrometry and Western blot. TRMT61B modifies
5 both mt-tRNA and mt-rRNA (Bar-Yaacov et al., 2016; Chujo and Suzuki, 2012); and
6 these m¹A sites are thought to be beneficial for their functions in translation. Because
7 m¹A sites in mt-mRNA have an opposing effect in translation, changes of protein
8 synthesis level after TRMT61B knock-down could be a mixed result of contrary m¹A
9 sites in these different components (rRNA, tRNA and mRNA) of translation.
10 Conversely, because 16S rRNA and mt-tRNA^{Leu(UUR)} are already of high m¹A level, we
11 envisioned that TRMT61B overexpression should lead to a greater increase of m¹A
12 level in mRNA than in these ncRNAs. In this simplified scenario, we indeed observed
13 greatly increased modification level for the mt-mRNA and detected reduced protein
14 level for two mitochondrial proteins, whose mRNA transcripts are m¹A modified.
15 While our MS experiments detected the overall protein level, future experiments
16 measuring the nascent protein level upon TRMT61B overexpression or knock-down
17 could provide more detailed information regarding protein synthesis. In terms of the
18 mechanism of m¹A-mediated translational suppression, multiple possibilities need to
19 be considered. For instance, due to the presence of a base-pairing interfering
20 modification in the CDS, m¹A could serve as a road block for the mitochondrial
21 ribosome. In fact, recent *in vitro* translation experiments on synthetic RNA
22 sequences have shown that m¹A in CDS represses translation; the effect is stronger

1 when m¹A is at codon position 1 and 2, while m¹A at the third position also mitigates
2 translation (You et al., 2017). Thus, not only the density but also the exact position of
3 m¹A in mt-mRNA could influence protein synthesis. In addition, microRNA has been
4 shown to enhance translation in mitochondria (Zhang et al., 2014). We analyzed the
5 published CLASH results in which microRNA and their direct mRNA targets are
6 captured (Helwak et al., 2013); interestingly, we found two m¹A sites that are located
7 within the experimentally verified targets of microRNA (Figure S5G). In fact, these
8 m¹A sites reside exactly within mRNA sequences that form base-pairing with the
9 seed regions of microRNAs. More m¹A sites in mt-mRNA were found within the
10 predicted mt-mRNA targets of the microRNA seed regions (Figure S5H). While both
11 the speculated mechanisms point to a suppressive role of m¹A in mitochondria
12 translation, alternative hypothesis and mechanism should also be tested in future
13 experiments. Nevertheless, our discovery that m¹A in mt-mRNA interferes with
14 translation improves our understanding of translation regulation in human
15 mitochondria.

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

21

22 **AUTHOR CONTRIBUTIONS**

23

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

6

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- 6

1 **METHOD DETAILS**

2 **Cell Culture and Antibodies**

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

11

12 **RNA isolation**

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

19

20 **shRNA knock down of TRMT6/61A**

21 The oligoes targeting TRMT6 and TRMT61A were annealed and cloned into the pLKO vector

1 according to the TRC shRNA library protocol (<http://www.broadinstitute.org/rnai/public/>),
2 respectively. The oligo sequences were listed below: TRMT6-FWD:
3 CCGGGGGAAAGTTCTGAGTATTTATCTCGAGATAAATACTCAGAACTTTCCCTTTTTG;
4 TRMT6-RVS:
5 AATTCAAAAAGGGAAAGTTCTGAGTATTTATCTCGAGATAAATACTCAGAACTTTCCC;
6 TRMT61A-FWD:
7 CCGGGAGGCCAGAGGCACCTTATATCTCGAGATATAAGGTGCCTCTGGCCTCTTTTTG;
8 TRMT61A -RVS:
9 AATTCAAAAAGAGGCCAGAGGCACCTTATATCTCGAGATATAAGGTGCCTCTGGCCTC. A
10 scrambled shRNA was used as the mock control. Lentiviruses were packaged by co-transfecting
11 HEK293T cells with pLKO-TRMT6, pLKO-TRMT61A, pCMV-dR8.91 and VSV-G plasmids,
12 following the instructions from Broad Institute. The supernatants from transfected cells were
13 harvested after 2 days and used to infect HEK293T cells followed by puromycin selection for 5
14 days. Knock-down efficiency was verified by Western blot and qPCR. qPCR primers were listed
15 as follows: TRMT6-qFWD: CTGTCTTTGCTGGACTTTGTGGC; TRMT6-qRVS:
16 AGACAGCCTGAGGTTGATGACC; TRMT61A-qFWD: TCCTCTACTCCACAGACATCGC;
17 TRMT61A-qRVS: CAATGGTGCGGATGATGGCGTG.

18

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

20 200 ng isolated RNA or 100 ng model RNA oligo was digested into nucleosides by 0.5 U
21 nuclease P1 (Sigma, N8630) in 20 µL buffer containing 10 mM ammonium acetate, pH 5.3 at 42

1 °C for 6 h, followed by the addition of 2.5 μ L 0.5 M MES buffer, pH 6.5 and 0.5 U alkaline
2 phosphatase (Sigma, P4252). The mixture was incubated at 37 °C for another 6 h and diluted to
3 50 μ L. 5 μ L of the solution was injected into LC-MS/MS. The nucleosides were separated by
4 ultra-performance liquid chromatography on a C18 column, and then detected by triple-
5 quadrupole mass spectrometer (AB SCIEX QTRAP 5500) in the positive ion multiple reaction-
6 monitoring (MRM) mode. The mass transitions of m/z 282.0 to 150.1 (m¹A), m/z 282.0 to 150.1
7 (m⁶A), m/z 268.0 to 136.0 (A) were monitored and recorded. Concentrations of nucleosides in
8 RNA samples were deduced by fitting the signal intensities into the stand curves.

9

10 **Synthetic m¹A RNA model sequences**

11 Two pairs of synthetic m¹A and A RNA oligoes were used in this study. The oligo sequences were
12 listed as follows: m¹A-1: CGCGGCUCGm¹AGCCCGCGUGCGGGCCUCUUUCAGGCCGCU; A-
13 1: CGCGGCUCGAGCCCGCGUGCGGGCCUCUUUCAGGCCGCU; m¹A-2:
14 CGGCGGCCCGGGACCGm¹AGACCCGGCCCCGGCUCCCC; A-2:
15 CGGCGGCCCGGGACCGAGACCCGGCCCCGGCUCCCC. The m⁶A contamination in the m¹A
16 oligoes caused during the oligo purification process was measured using quantitative LC-MS/MS.
17 The m¹A RNA oligoes and A RNA oligoes were mixed at the ratio: 100%, 75%, 50%, 25%,
18 12.5%, 6.25% and 0%, respectively. The mixed m¹A /A oligoes were subjected to library
19 construction using specific RT primers as listed:

20 RT-m¹A /A-1: ACACGACGCTCTTCCGATCTagcggcctgaaagaggc;

21 RT-m¹A /A-2: ACACGACGCTCTTCCGATCTggggagccggggcc.

1

2 **Cloning, Expression and Purification of AlkB**

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

10

11 ***In vitro* Demethylation treatment**

12 *In vitro* demethylation treatment mediated by the demethylase AlkB: 10 µg full length polyA⁺ RNA
13 was fragmented at 95 °C for 5 min using magnesium RNA fragmentation buffer (NEB) and
14 fragmented polyA⁺ RNA was desalted and concentrated by ethanol precipitation. 10 µg (~0.2
15 nmol) fragmented polyA⁺ RNA was denatured at 65 °C for 5 min, and then demethylated in a 500
16 µL demethylation mixture containing 0.4 nmol purified AlkB, 50 mM MES, pH 6.5, 283 µM of
17 (NH₄)₂Fe(SO₄)₂·6H₂O, 300 µM 2-ketoglutarate, 2 mM L-ascorbic acid, 1 U/µL SUPERaseIn
18 RNase Inhibitor (Invitrogen). The demethylation reaction was incubated for 2 h at 37 °C and
19 quenched by the addition of 5 mM EDTA. The demethylated RNA was then purified by phenol
20 chloroform extraction.

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

5 **m¹A-MAP**

6 40 µg polyA⁺ RNA was fragmented into ~150 nt using magnesium RNA fragmentation buffer
7 (NEB). m¹A-containing RNA fragments were enriched by m¹A immunoprecipitation as previous
8 described (Li et al., 2016a). 10 ng (~0.2 pmol) of the immunoprecipitated m¹A-containing RNA
9 fragments were subjected to the AlkB demethylation treatment. RNA fragments were
10 demethylated in a 20 µL demethylation mixture containing 0.4 pmol purified AlkB, 50 mM MES,
11 pH 6.5, 283 µM of (NH₄)₂Fe(SO₄)₂·6H₂O, 300 µM 2-ketoglutarate, 2 mM L-ascorbic acid, 0.4
12 U/µL RNase inhibitor and then incubated 2 h at 37 °C. The demethylation reaction was quenched
13 by the addition of 5 mM EDTA, and demethylated RNA was purified by phenol chloroform
14 extraction and ethanol precipitation.

15 Fragmented polyA⁺ RNA (as “input”), immunoprecipitated RNA [as (-) demethylase sample]
16 and demethylated immunoprecipitated RNA [as (+) demethylase samples] were subjected to
17 library construction. The library construction was performed according to the eCLIP library
18 construction protocol with several modifications (Van Nostrand et al., 2016). For
19 dephosphorylation of 3' ends, RNA samples were treated with PNK (NEB) and incubated at
20 37 °C for 1 h and then heat-inactivation of PNK at 65 °C for 20 min. RNA samples were purified
21 by ethanol precipitation and then subjected to 3' RNA linker ligation using T4 RNA ligase2,

1 truncated KQ (NEB) at 25 °C 2 h. The 3' RNA linker sequence was listed: 5'rAPP-
2 AGATCGGAAGAGCGTCGTG-3SpC3. The excess RNA adaptor was digested by adding 1 µL 5'
3 Deadenylase (NEB) into the ligation mix, incubating at 30 °C for 1 h and then adding 1 µL RecJf
4 (NEB), incubating at 37 °C for another 1 h. These enzymes were subjected to heat-inactivation at
5 70 °C for 20 min and RNA was purified by ethanol precipitation. RNA pellets were dissolved in 10
6 µL H₂O and then 1 µL 2 µM RT primer (ACACGACGCTCTTCCGATCT) was added. RNA-primer
7 mix was denatured at 80 °C for 2 min and then chilling on ice. RT reaction buffer (50 mM Tri-HCl
8 pH 8.3, 75 mM KCl, and 3 mM MgCl₂, final), dNTPs (1 mM, final), DTT(5 mM, final), RNase
9 Inhibitor (1 U/µL, final) and 1 µL TGIRT (InGex) were added into the denatured RNA-primer mix
10 and reverse transcription was carried at 57 °C for 2 h. Excess RT primer was digested by the
11 addition of 1 µL Exonuclease I (NEB) and incubation at 37 °C for 30 min. cDNA was purified
12 using silane beads (Invitrogen) and then ligated to 5' adaptor (5Phos-
13 NNNNNNNNNNAGATCGGAAGAGCACACGTCTG-3SpC3). Ligation was performed with T4
14 RNA ligase 1, high concentration (NEB) at 25 °C overnight. The cDNA was purified using silane
15 beads and then amplified by PCR with primers (5'-
16 AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT-3';
17 5'-
18 CAAGCAGAAGACGGCATACGAGATXXXXXXGTGACTGGAGTTCAGACGTGTGCTCTTCCGA
19 TC-3', XXXXXX represents index sequence). PCR products were purified by 8% TBE gel and
20 sequenced on Illumina Hiseq X10 with paired-end 2×150 bp read length.

21

1 **Locus-specific m¹A detection**

2 200 ng polyA⁺ RNA was isolated from TRMT6/61A stable knock-down cells and mock control
3 cells respectively, and then were fragmented into 150 nt using magnesium RNA fragmentation
4 buffer (NEB). Fragmented RNA was ligated to 3' RNA linker and then reverse transcription was
5 carried out with TGIRT using the same RT condition as that of m¹A-MAP. The regions containing
6 m¹A were amplified by PCR using specific primers. And these amplicons from the same sample
7 were mixed together and then subjected to DNA library construction using NEBNext® Ultra™ II
8 DNA Library Prep Kit for Illumina® (E7645) according to the manufacturer's instructions. These
9 libraries were deep sequenced on Illumina HiSeq X10 with paired-end 2×150 bp read length.

10 Thus, these regions of particular interest were covered with very high sequencing depth. Specific
11 PCR primers were listed:

12 NM_025099-m¹A5643-FWD: AAAAAGCTCGGTCCGGGTTTC;

13 NM_025099-m¹A5643-RVS: TTAGCCGCAAATCACGCTG;

14 NM_001193375-m¹A978-FWD: ACACCTGTCCAAGCCCTAAT;

15 NM_001193375-m¹A978-RVS: CTGAGGGGCCCTTATTCCCA;

16 NR_026951-m¹A659-FWD: ACGTCGGCTCGTTGGTCTAG;

17 NR_026951-m¹A659-RVS: ACAGTCAAGCCTCCTGCAGC;

18 NM_001256443-m¹A486-FWD: CAAGGTTCCAGGCGAAGGG;

19 NM_001256443-m¹A486-RVS: AGCCGGGGTCTCTGTGG.

1

2 **siRNA Knock-down and overexpression of TRMT61B**

3 Two synthesized duplex RNAi oligoes targeting TRMT61B mRNA sequences were used: 5'-

4 GGAUAUCAACCCAGGUGAUTT-3' and 5'-GCGUGAUUCAUGGAAAUUATT-3' ; a scrambled

5 duplex RNAi oligo (5'-UCCUCCGAACGUGUCACGUTT) was used as a mock control. The

6 siRNA oligo was transfected into HEK293T by Lipofectamine RNAiMAX (Invitrogen) according to

7 the manufacturer's instructions and cells were harvested 48 h after transfection. Knockdown

8 efficiency was verified by qPCR. qPCR primers were listed as follows: TRMT61B-qFWD:

9 CAGGAGCAACCGAAGACAT; TRMT61B-qRVS:ATATACAGCACATACACCACCAT;

10 TRMT61B was cloned into pcDNA3.1 using the following primers: FWD:

11 TCGCGAAACACTATGCTAATGGC; RVS: GTTAAGTTGTGGTTTGACCTTCCTC. The empty

12 pcDNA3.1 vector was used as the mock control. The plasmid was transfected into HEK293T by

13 Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions and cells were

14 harvested 48 h after transfection.

15

16 **Ribosome profiling**

17 2 Plates of 15-cm HEK293T cells were grown to 90% confluency; CHX was then added to the

18 medium at a final concentration of 100 µg/mL for 7 min. The cells were then harvested and lysed

19 with 1 mL lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 100 mM KCl, 1% Triton X-100, 2

20 mM DTT, 100 µg/mL CHX, 0.5 U/µL RNase inhibitor, 1×complete protease inhibitor). The cell

1 lysates were centrifuged at 15,000g for 15 min and the supernatant was collected followed by
2 measuring the OD260 of cell lysates. 100 μ L lysates were kept as input sample and 1 mL TRIzol
3 was added to purify the RNA. 1 μ L Micrococcal Nuclease (NEB) per 25OD was added to the
4 remaining cell lysates and allowed to incubate at 25 °C for 20 min. The digested cell lysates were
5 used for performing ribosome foot-printing. Lysates were fractioned on 10/50% w/v sucrose
6 gradients using the SW-40Ti rotor at 27,500rpm for 4h. 80S monosome fractions were collected
7 followed by the addition of equal volume of extraction buffer (1% SDS, 40 mM EDTA). RNA was
8 isolated by phenol-chloroform extraction. RNA fragments between 28–30 nt were selected using
9 15% Urea-PAGE. Recovered RNA fragments were subjected to library construction. In brief,
10 RNA samples were dephosphorylated with PNK (NEB) and ligated to 3' RP linker (5'rAPP-
11 CTGTAGGCACCATCAAT-3SpC3) using T4 RNA ligase2, truncated KQ (NEB). Reverse
12 transcription was carried using Superscript III (Invitrogen) with RP-RT primer (5Phos-
13 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGC-SpC18-CACTCA-
14 SpC18-TTCAGACGTGTGCTCTTCCGATCTATTGATGGTGCCTACAG). cDNA was circ-ligated
15 with CircLigase II (Epicentre) and then amplified by PCR with primers (5'-
16 AATGATACGGCGACCACCGAGATCTACAC-3'; 5'-
17 CAAGCAGAAGACGGCATAACGAGATXXXXXXGTGACTGGAGTTCAGACGTGTGCTCTTCCGA
18 TC-3', XXXXXX represents index sequence). PCR products were purified by 8% TBE gel and
19 sequenced on Illumina Hiseq 2500 with single end reads (50 bp).

20

21 **qPCR-based m¹A level evaluation**

1 20 µg polyA⁺ RNA was isolated from HEK293T cells with TRMT61B overexpression, knock-down
2 and the corresponding mock controls, respectively. RNA was fragmented into ~150 nt using
3 magnesium RNA fragmentation buffer (NEB) and concentrated by ethanol precipitation.
4 Fragmented RNA (as input) was denatured and incubated with 2 µg anti-m¹A antibody in IPP
5 buffer (150 mM NaCl, 0.1% NP-40, 10 mM Tris, pH 7.4) at 4°C overnight. 20 µL Protein A/G
6 UltraLink Resin (Pierce) was added to the RNA antibody mixture and incubated for additional 3 h
7 at 4°C. Resins were washed with twice with IPP buffer, once with low salt buffer (75 mM NaCl,
8 0.1% NP-40, 10 mM Tris, pH 7.4), once with high salt buffer (200 mM NaCl, 0.1% NP-40, 10 mM
9 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).
10 m¹A-containing RNA was eluted from resins with 3 mg/mL *N*¹-methyladenosine
11 (Berry&Associates) in IPP buffer and purified by phenol chloroform extraction and ethanol
12 precipitation. Input and immunoprecipitated RNAs were reverse transcribed into cDNA using
13 Superscript III (Invitrogen) and quantified by qPCR using SYBR GREEN mix (Takara) on Roche
14 Lightcycler 96 real-time PCR system. The m¹A-IP/input ratio of target regions in the TRMT61B
15 overexpression, knock-down and the corresponding control samples were calculated,
16 respectively. The primers used for qPCR were listed below:

17 MT-CO1-qFWD: CCTATCATCTGTAGGCTCATTC;

18 MT-CO1-qRVS: GGAGGGTTCTTCTACTATTAGGAC;

19 MT-CO2-qFWD: ACAGATGCAATTCCCGGACG;

20 MT-CO2-qRVS: CCACAGATTTTCAGAGCATTGACC;

- 1 MT-CO3-qFWD: CGCCTGATACTGGCATTTTG;
- 2 MT-CO3-qRVS: GACCCTCATCAATAGATGGAGAC;
- 3 MT-CYB-qFWD: CAACCCCCTAGGAATCACCTC;
- 4 MT-CYB-qRVS: GAGGGCGTCTTTGATTGTGTAG;
- 5 16S rRNA-qFWD: ATGAATGGCTCCACGAGGG;
- 6 16S rRNA-qRVS: CTTGCTGTGTTATGCCCCG.

7

8 **Reductive dimethylation labeling**

9 Mitochondria was isolated from TRMT61B overexpression and mock control HEK293T cell lines
10 according to the manufacturer's (Thermo Fisher) and lysed with RIPA buffer followed by
11 sonication. After extraction, total proteins from different cell lines were quantified with the BCA
12 protein assay kit (Thermo Fisher). Equal amount of proteins from two cell lines were digested by
13 trypsin on-column in 100 mM TEAB buffer and subjected to reductive dimethylation labeling. 4 μ L
14 of 4% (w/w) light or heavy formaldehyde was added to 100 μ L of trypsin digested samples
15 prepared from TRMT61B overexpression and mock control HEK293T cells, respectively. In the
16 meantime, 4 μ L of 0.6 M sodium cyanoborohydride was added and the samples were incubated
17 at room temperature for 1h. The dimethylation labeling reaction was quenched by the addition of
18 1% (w/w) ammonia and 5% (w/w) formic acid. Finally, light and heavy labeled peptide samples
19 were mixed, concentrated by vacuum, and analyzed on a Q Exactive mass spectrometer

1 (Thermo Fisher).

2

3 **LC-MS/MS and data analysis**

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

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

17

18 **Pre-processing of raw sequencing data**

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

11

12 **Reads mapping and PCR duplication removing**

13 Processed reads were mapped to human transcriptome or mitochondrial genome using BWA-
14 MEM with default parameters (version 0.7.15-r1140) (Li and Durbin, 2009). Reference
15 transcriptome was prepared based on the Refseq annotation of human (hg19) downloaded from
16 the table browser of UCSC database. The redundant sequences with the same Refseq id were
17 removed. Transfer RNA (tRNA) sequences were also downloaded from UCSC table browser and
18 integrated into the transcriptome. Mitochondrial genome and corresponding annotation were
19 downloaded from NCBI (NC_012920.1). Reads mapping to an identical position of reference
20 were considered as PCR duplications if their 10 nt random barcodes were the same, and only

1 one of these reads was kept. Performances related to the processing of sam/bam file were done
2 with the help of SAMtools (Li et al., 2009) (<http://samtools.sourceforge.net/>).

3

4 **Identification of m¹A sites**

5 Mismatch rate of each nucleotide in the reference sequences was calculated for both (-) and (+)
6 demethylase samples. Two parameters were defined to evaluate the dynamic change of
7 mismatch rate in the (-) demethylase and (+) demethylase samples: difference of mismatch rate
8 (Diff) and fold change of mismatch rate (FC). Diff was calculated by subtracting the mismatch
9 rate in the (+) demethylase from that in the (-) demethylase samples, while FC was calculated by
10 dividing the mismatch rate in the (-) demethylase by that in the (+) demethylase sample. FC was
11 artificially set to “1000” if the mismatch rate in the (+) demethylase sample was “0”. A position
12 was identified as an m¹A site when the following criteria were met: a) $FC \geq 3$; b) $Diff \geq 10\%$; c)
13 the number of reads with a mismatch at the position was no less than 5; d) criteria (a-c) were all
14 fulfilled in both replicates.

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

18 $Diff_{\text{opposite}} = (+)\text{demethylase} - (-)\text{demethylase}$; $FC_{\text{opposite}} = (+)\text{demethylase}/(-)\text{demethylase}$.

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

1

2 **Motif discovery and GO enrichment analysis**

3 For the analysis of sequence consensus, 15 nt of sequence neighboring each m¹A site was
4 retrieved. These sequences were then subjected to DREME algorithm in MEME suite (Version
5 4.12.0) for the discovery of enriched motifs (Bailey et al., 2009). The shuffled input sequences
6 were used as the background to eliminate potential false positives caused by the nucleotide
7 composition.

8 Gene Ontology (GO) enrichment analyses were performed using DAVID web-based tool
9 (<http://david.abcc.ncifcrf.gov/>) (Huang da et al., 2009).

10

11 **Secondary structure and minimum free energy analysis**

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

19

1 **Ribosome profiling data analysis and TE calculation**

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

6 For the analysis of influence of m¹A on mitochondrial gene translation, mitochondrial
7 ribosome profiling data were downloaded from the GEO Datasets (GSE48933) (Rooijers et al.,
8 2013). The depth of reads covered for each nucleotide along the mitochondrial transcripts was
9 retrieved using Samtools depth tool.

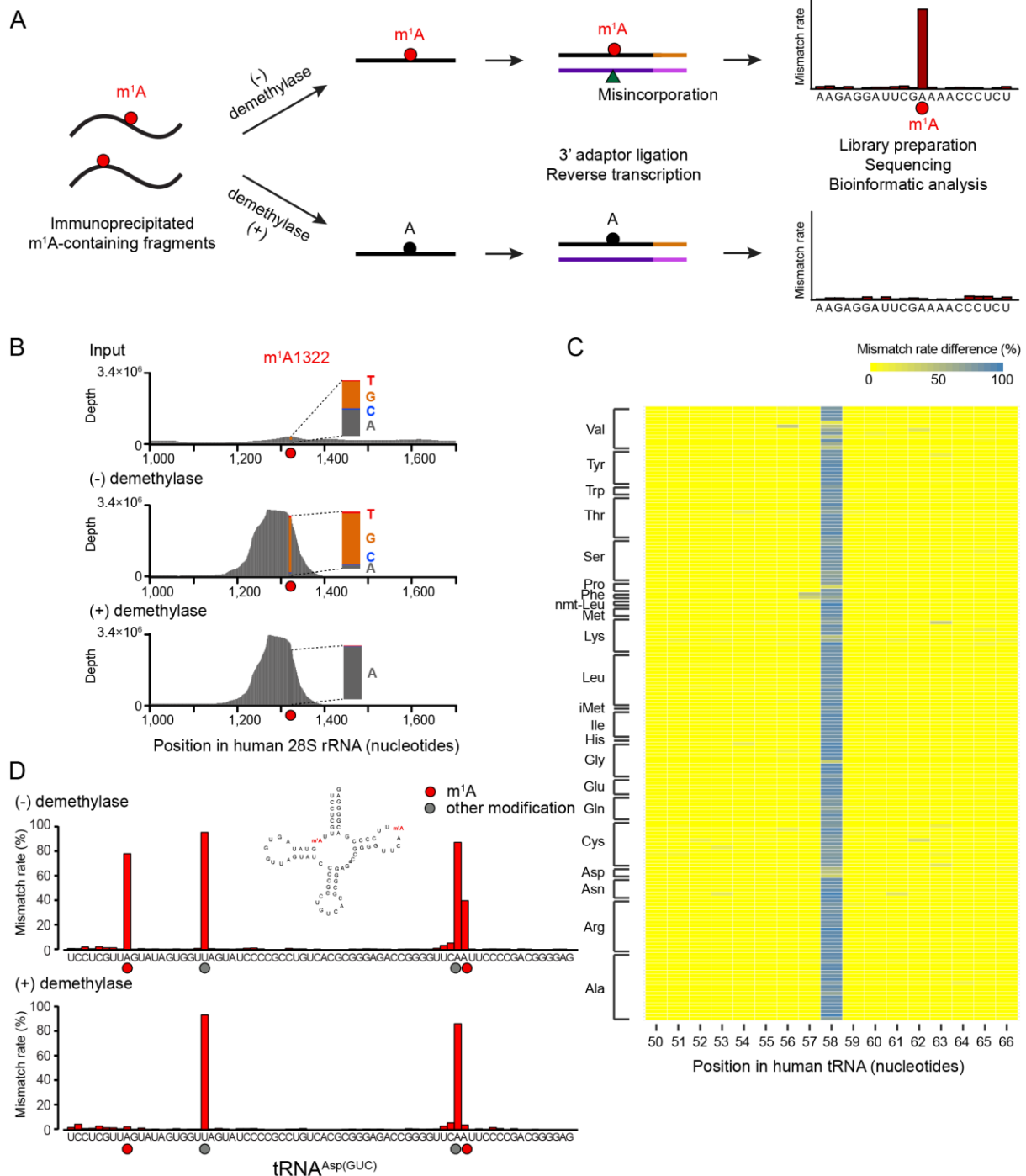
10

11 **miRNA target analysis**

12 The predicted miRNA targeting sites on mitochondrial coding genes were downloaded from the
13 miRWalk database (v2.0) (Dweep and Gretz, 2015), which depends on the match of “seed
14 region” to gene sequence. The minimum length required for the match of seed region was set to
15 7 nt. The experimentally identified miRNA targeting sites were retrieved from the published
16 CLASH results (Helwak et al., 2013).

17

1 Figures



2

3 **Figure 1. m¹A-MAP utilizes m¹A-induced misincorporation to detect m¹A sites at single-**
 4 **nucleotide resolution.**

5 (A) Scheme of m¹A-MAP. We optimized the conditions of RT so as to allow efficient

6 misincorporation in cDNA synthesis. The use of an m¹A antibody pre-enriches the m¹A-

1 containing RNA fragments, thereby maximizing the misincorporation signal; and the use of
2 demethylase treatment improves the confidence of detection. An m¹A modification is called
3 depending on the difference and fold change of mismatch rate between the (-) and (+)
4 demethylase samples (see Method Details).

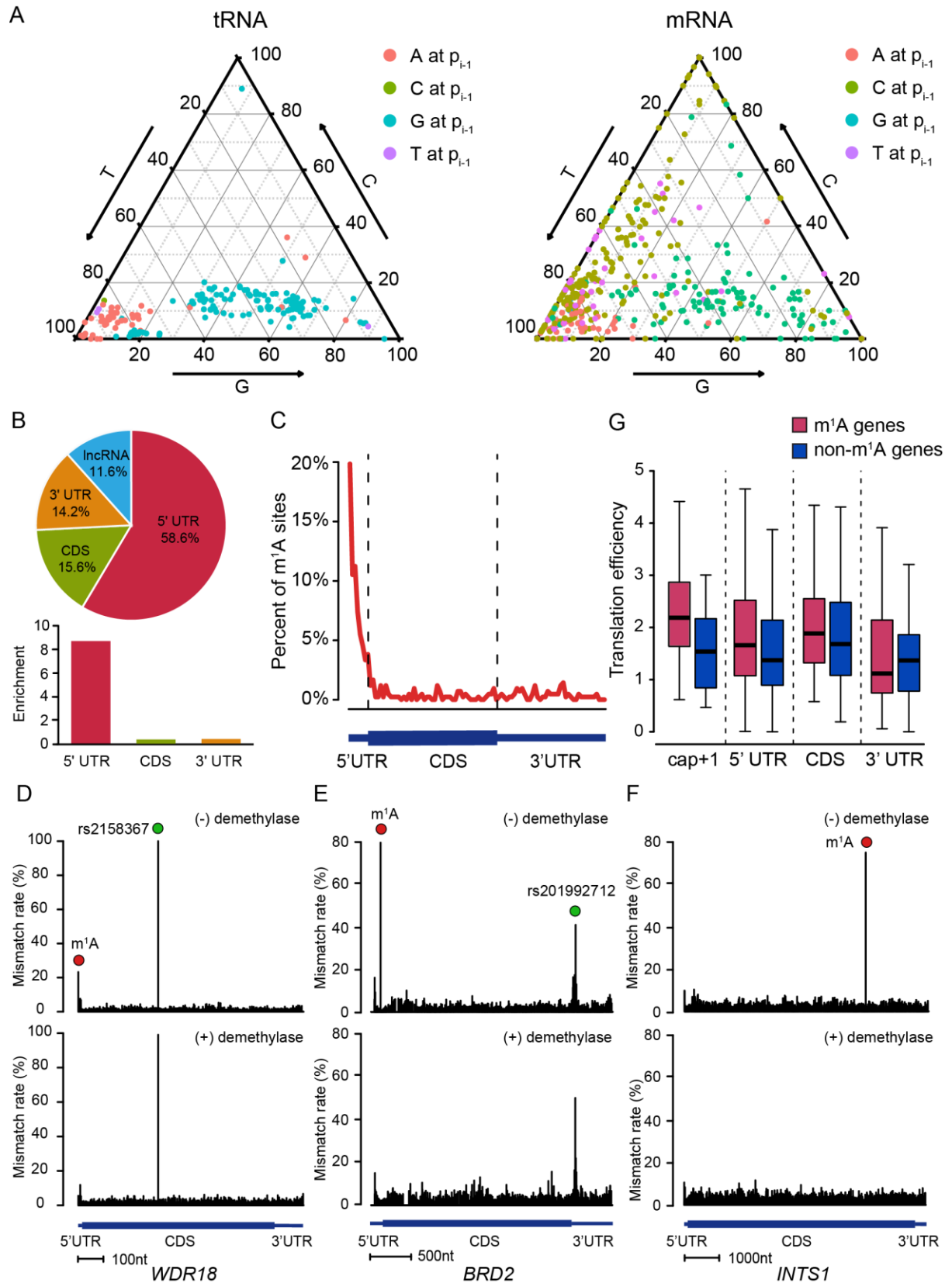
5 **(B)** m¹A-MAP maximizes the misincorporation signal for m¹A1322 on 28S rRNA.

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

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

12 See also Figure S1 and S2.

13



1

2

Figure 2. Single-nucleotide resolution m¹A methylome in the human transcriptome.

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

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

4 (C) Distribution of m¹A sites across mRNA segments. Each segment was normalized according
5 to its average length in Refseq annotation.

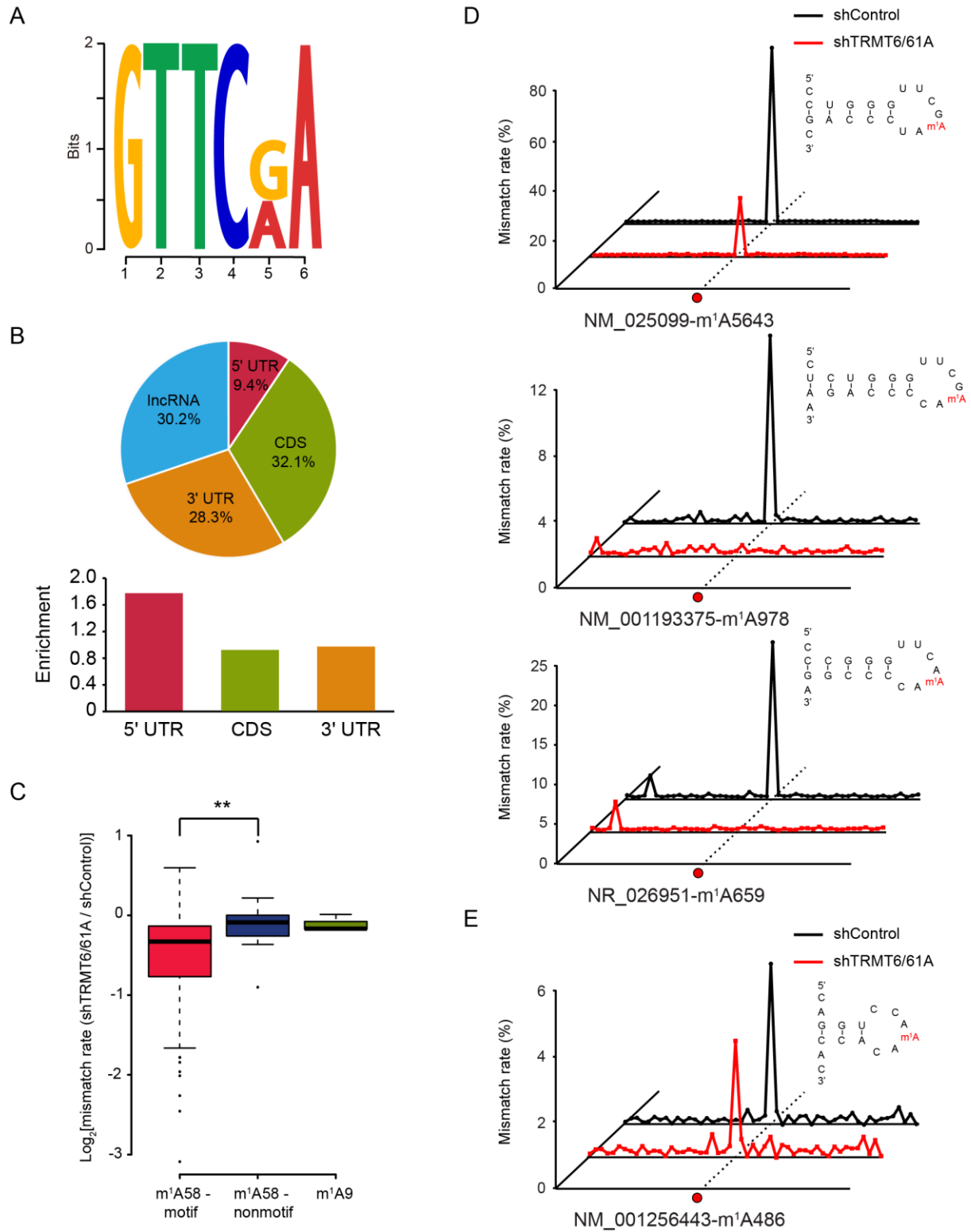
6 (D–F) Representative views of a typical m¹A site at the cap+1 position (the first transcribed
7 nucleotide of mRNA) of *WDR18* (D), the 5'-UTR of *BRD2* (E), and the CDS of *INTS1* mRNA (F).

8 The demethylation-insensitive signals in (D) and (E), which are indicated as green dots, are
9 known SNPs. The scale bars are indicated at the bottom of each panel.

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

13 See also Figure S3.

14

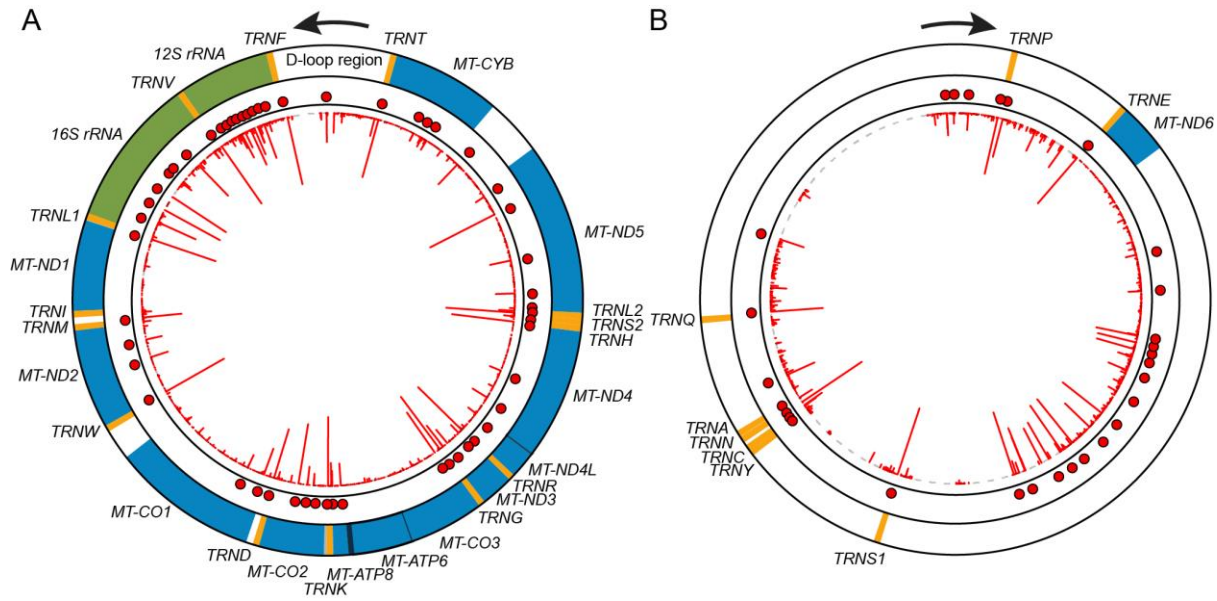


1

2 **Figure 3. The tRNA methyltransferase complex TRMT6/61A catalyzes a subset of m1A**

3 **methylation in mRNA.**

- 1 **(A)** Motif analysis revealed a GUUCRA tRNA-like consensus for a group of m¹A in mRNA, E-
- 2 value=4.8e-009.
- 3 **(B)** Pie chart showing the percentage of m¹A sites in each non-overlapping segment. Comparing
- 4 to the non-motif m¹A sites in mRNA, these sites are evenly distributed in the transcriptome.
- 5 **(C)** TRMT6/61A specifically targets m¹A within the consensus sequence, while doesn't work on
- 6 m¹A in non-motif sequence of the T-loop nor m¹A at the 9th position of tRNA, p-value <0.005.
- 7 **(D)** Representative views of three mRNA targets of TRMT6/61A. The predicted RNA secondary
- 8 structures are also shown, revealing a conserved stem-loop structure that harbors the GUUCRA
- 9 motif.
- 10 **(E)** An example of non-motif mRNA m¹A site, showing similar mismatch rates in the control and
- 11 TRMT6/61A knock-down samples.
- 12 See also Figure S4.



1

2 **Figure 4. Distinct m¹A methylome in the mitochondrial transcriptome.**

3 (A) m¹A methylome of the heavy strand. Orange, green and blue colors represent tRNA, rRNA

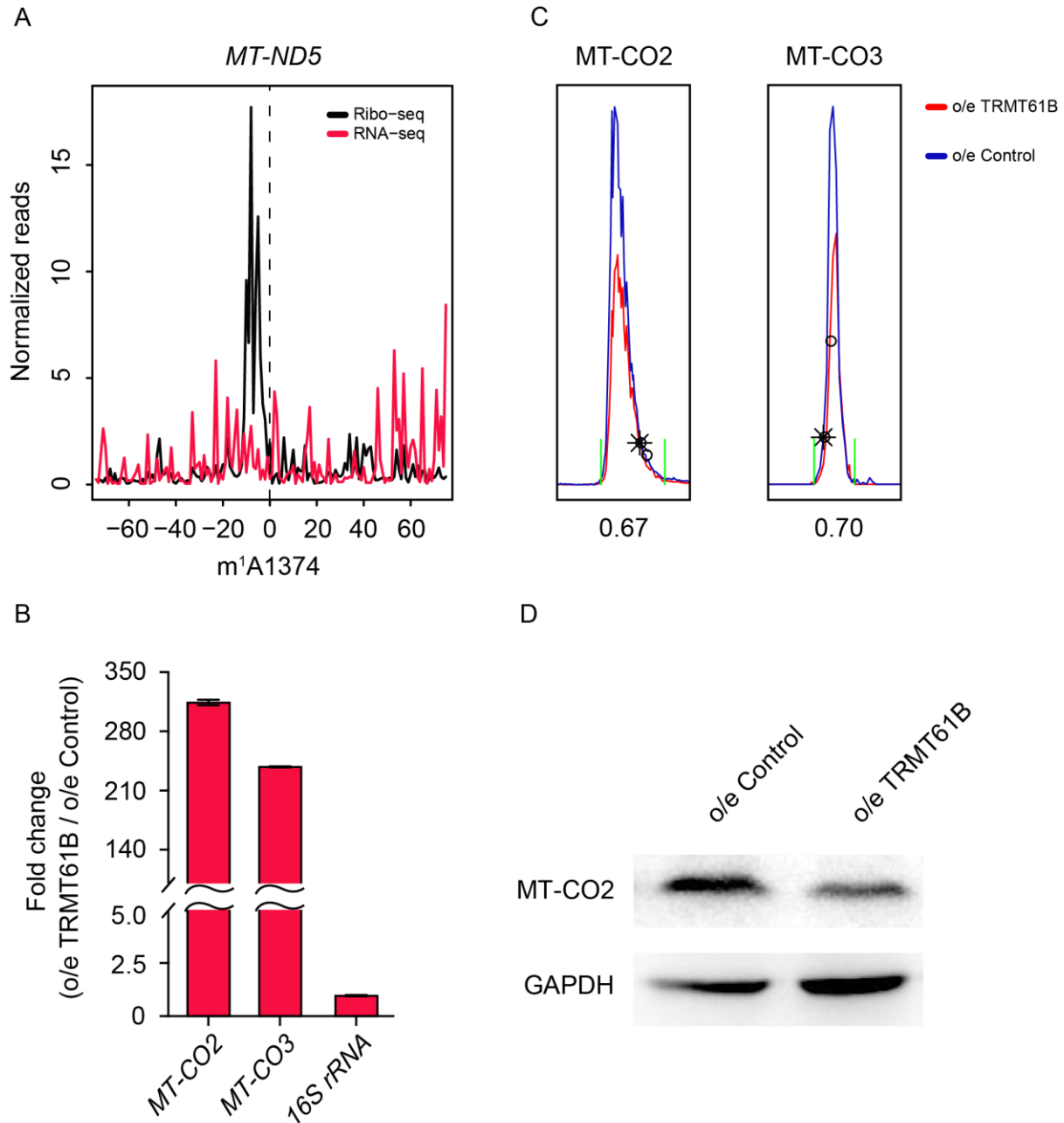
4 and mRNA, respectively. The red line in the inner circle represents the difference of mismatch

5 rate of individual nucleotide while each red dot represents an identified m¹A site.

6 (B) m¹A methylome of the light strand.

7 See also Figure S5.

8



1

2 **Figure 5. m¹A in the mitochondrial mRNA interferes with translation.**

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

6 (B) TRMT61B overexpression led to increased m¹A level in *MT-CO2* and *MT-CO3* mRNA, as
7 measured by the qPCR-based assay. Data are mean ± SD; n = 2.

- 1 (C) Extracted ion chromatograms of MT-CO2 and MT-CO3, showing decreased protein level.
- 2 (D) Western blot of MT-CO2 upon TRMT61B overexpression.
- 3 See also Figure S5.
- 4