The RNA methyltransferase METTL8 installs m$^{3}$C$_{32}$ in mitochondrial tRNAs$^{\text{Thr/Ser(UCN)}}$ to optimise tRNA structure and mitochondrial translation

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Modified nucleotides in tRNAs are important determinants of folding, structure and function. Here we identify METTL8 as a mitochondrial matrix protein and active RNA methyltransferase responsible for installing m$^{3}$C$_{32}$ in the human mitochondrial (mt-)tRNA$^{\text{Thr}}$ and mt-tRNA$^{\text{Ser(UCN)}}$. METTL8 crosslinks to the anticodon stem loop (ASL) of many mt-tRNAs in cells, raising the question of how methylation target specificity is achieved. Dissection of mt-tRNA recognition elements revealed U$^{34}$G$^{35}$ and t$^{6}$A$^{37}$/($^{ms}$)$^{6}$A$^{37}$, present concomitantly only in the ASLs of the two substrate mt-tRNAs, as key determinants for METTL8-mediated methylation of C$_{32}$. Several lines of evidence demonstrate the influence of U$^{34}$, G$^{35}$, and the m$^{3}$C$_{32}$ and t$^{6}$A$^{37}$/($^{ms}$)$^{6}$A$^{37}$ modifications in mt-tRNA$^{\text{Thr/Ser(UCN)}}$ on the structure of these mt-tRNAs. Although mt-tRNA$^{\text{Thr/Ser(UCN)}}$ lacking METTL8-mediated m$^{3}$C$_{32}$ are efficiently aminoacylated and associate with mitochondrial ribosomes, mitochondrial translation is mildly impaired by lack of METTL8. Together these results define the cellular targets of METTL8 and shed new light on the role of m$^{3}$C$_{32}$ within mt-tRNAs.

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naturally occurring modifications in RNAs represent an important and dynamic layer of gene expression regulation\(^1\-3\). Recent advances in RNA modification detection techniques have uncovered in excess of 150 differently modified ribonucleosides in cellular RNAs, and some of the enzymes responsible for installing these modifications have been identified and characterised\(^4\-7\). Modified ribonucleosides are present throughout the transcriptome and are broadly implicated in modulating RNA structure, stability and function either directly or by influencing RNA-protein interactions\(^8\-11\). However, an accurate and comprehensive overview of modified sites in the transcriptome remains elusive and the precise functions of many individual modification marks in RNAs are still unclear.

Transfer (t)RNAs are the most extensively and diversely modified class of RNA, with more than 80 different modification types present across the domains of life\(^12\-14\). Introduction of specific base and ribose modifications into eukaryotic tRNAs is an important step during tRNA maturation, which can not only improve the production of tRNA-derived small RNAs\(^15\) but also act as “wobble” base modifications in the anticodon loop typically in the T- and D loops of tRNA cores generally act co-operatively to regulate tRNA folding and mediate stabilisation of tRNA tertiary structure\(^16\-21\). In contrast, modifications within the anticodon loop typically influence tRNA function by expanding codon recognition, improving decoding efficiency and accuracy during translation or preventing frameshifting events\(^22\-25\). Consistent with this, positions 34 (wobble position) and 37 are “hot-spots” of diverse and complex (hyper-)modifications\(^26\). The importance of tRNA modifications within the cellular context is highlighted by the numerous human diseases arising from defects in diverse tRNA modifying enzymes as well as the pathogenic effects of mutations in tRNA sequences that impair modification installation\(^13\,27\,28\).

Methylation of N3 of cytidine (3-methylcytidine; m^3^C) is present at position 32 of eukaryotic, cytoplasmic tRNAs^Thr^ and tRNAs^Ser^ in species ranging from yeast to humans and has also been identified in mitochondrial (mt-)tRNAs^Thr^/Ser^ (UCN) in mammals\(^29\-36\). In Saccharomyces cerevisiae (S. cerevisiae), the m^3^C methyltransferase Trm140 mediates modification of both cytoplasmic tRNAs^Thr^/Ser^ whereas in Schizosaccharomyces pombe (S. pombe) two alternative enzymes, Trm140 and Trm141, install m^3^C_32 modifications in cytoplasmic tRNAs^Thr^ and tRNAs^Ser^ respectively\(^30\,37\). The mammalian homologues of the S. pombe proteins, METTL2 (with paralogues A and B in humans) and METTL6 are likewise responsible for installing these modifications into the human tRNAs^Thr^ and tRNAs^Ser^ respectively\(^38\-41\). The m^3^C_32 modification is also present in mammalian tRNA^Arg^ (CCU) and tRNA^Arg^ (UCU), and METTL2A/B, together with the cofactor protein DALRD3, has recently been identified as the methyltransferase responsible for introducing these modifications\(^40\). The precise functions of the m^3^C_32 modifications in tRNAs^Thr^/Ser^ remain unknown but potential roles in fine-tuning translation have been discussed\(^37\,42\). Deletion of trm140 in yeast cells lacking another tRNA methyltransferase, Trm1, are sensitive to the translation inhibitor cycloheximide, as are Trypanosoma brucei (T. brucei) lacking TbTrm140\(^43\,44\), and treatment with alkylating agents has been shown to increase m^3^C levels in yeast tRNA^Thr^ leading to enhanced translation of threonine-rich membrane proteins encoded by degenerate threonine codons\(^42\). Furthermore, the importance of METTL2A/B- and METTL6-mediated m^3^C_32 modification of tRNAs^Thr^/Ser^ is emphasised by the findings that lack of METTL6 retards tumour cell growth and impairs pluriotipility\(^39\), and that a pathogenic mutation in DALRD3, which impairs m^3^C_32 installation in tRNA^Arg^ (CCU)/UCU^, is found in patients with developmental delay and early-onset epileptic encephalopathy\(^40\). Interestingly, in mammals, a third putative m^3^C methyltransferase METTL8, also homologous to yeast Trm140, has been identified\(^40\,41\). METTL8 is poorly characterised; although it has been linked to potential m^3^C modifications in cytoplasmic mRNAs, no specific target sites have thus far been identified\(^38\).

Here we reveal that METTL8 is an active m^3^C RNA methyltransferase that localises to the mitochondrial matrix and is responsible for installing m^3^C_32 modifications in both mt-tRNA^Ser^ (UCN) and mt-tRNA^Thr^. Using in vivo UV crosslinking and analysis of cDNA (CRAC) and in vitro assays, we discover that METTL8 interacts directly with many mt-tRNAs but displays robust methylation activity only on mt-tRNA^Thr^ and mt-tRNA^Ser^ (UCN). Our in vitro and in vivo data identify prior installation of other modifications at position 37 and specific nucleotides in the anticodon loops of these tRNAs as important specificity determinants for METTL8-mediated methylation. We provide evidence that the presence of m^3^C_32 influences the structure of mt-tRNA^Thr^/Ser^ (UCN) and propose that the m^3^C_32 modifications installed in mt-tRNA^Thr^/Ser^ (UCN) by METTL8 may optimise tRNA structure to fine-tune mitochondrial translation.

## Results

**METTL8 localises to the mitochondrial matrix.** While METTL2A/B and METTL6 have recently been identified as the methyltransferases responsible for introducing m^3^C_32 modifications into the human, cytoplasmic tRNAs^Ser^/Thr^/Arg^ (CCU)/UCU^38\-41\, the third human m^3^C methyltransferase METTL8 (Fig. 1a) has remained poorly characterised. METTL8 is extensively alternatively spliced (Supplementary Fig. 1a), however, the two most common transcripts expressed in HEK293 cells only differ in the sequences encoding the protein C-terminus (Supplementary Fig. 1a, b; Supplementary Table 1) and the mRNA encoding the full-length (407 amino acid) protein is the top-ranked isoform in most human tissues\(^44\). To explore the sub-cellular localisation of the longest METTL8 isoform, a stably transfected HEK293 cell line for the tetracycline-inducible expression of C-terminally GFP tagged METTL8 (METTL8-GFP) was generated. Confocal fluorescence microscopy revealed a punctate distribution of METTL8-GFP in the cytoplasm colocalising with MitoTracker, indicating that METTL8 localises to mitochondria (Fig. 1b, upper panel), which is consistent with its detection in high-throughput mitochondrial proteome analysis\(^45\). Prediction of potential mitochondrial targeting sequences (MTTs) in METTL8 and the other known human m^3^C methyltransferases using the Mitofates algorithm\(^46\) revealed a high probability, 20 amino acid MTS at the N-terminus of METTL8 that is lacking in METTL2A/B and METTL6 (Fig. 1a, c). The functionality of this MTS was demonstrated by analysing the localisation of a C-terminally GFP-tagged version of METTL8 lacking the predicted MTS (METTL8_21\-407-GFP). In contrast to the full-length protein, METTL8_21\-407-GFP was present throughout the cytoplasm and nucleus, and accumulated in nuclear foci likely corresponding to nucleoli (Fig. 1b, lower panel).

A protease protection assay was then performed to verify mitochondrial import of METTL8 and determine its sub-mitochondrial localisation. Mitochondria and mitoplasts isolated from cells expressing METTL8-His_6\-2xFLAG were left intact or lysed by sonication and then treated with different concentrations of Protease K. Western blot analyses demonstrated that all examined proteins were susceptible to protease digestion after sonication (Fig. 1d). As expected, the outer mitochondrial membrane protein MFN2\(^47\) was degraded in protease-treated mitochondria and mitoplasts, while TIM23, which resides in the inner mitochondrial membrane (IMM)\(^48\) and exposes a domain...
**Fig. 1 METTL8 localises to the mitochondrial matrix.**

*a* Schematic view of the predominant human METTL8 isoform. The predicted mitochondrial targeting sequence (MTS) is depicted in blue and its amino acid sequence is given with the predicted mitochondrial processing peptidase cleavage site highlighted in red and residues with probability of forming an amphipathic alpha-helix in blue. The predicted methyltransferase domain (MTase domain; pfam 13649) is indicated in purple. Amino acid substitutions affecting catalytic activity (D230A and D309A) are indicated with green lines.

*b* Fluorescence microscopy was performed on HEK293 cells expressing METTL8-GFP or GFP-tagged METTL8 lacking the predicted MTS (METTL821-407-GFP). Mitochondria were visualised with MitoTracker (red) and nuclear material stained with DAPI (blue). An overlay image (Merge) is shown and the scale bar represents 10 µm. Schematic representations of the expressed proteins are shown with colours as in (*a*) and the GFP tag in green. Representative images from three independent experiments.

c) Probabilities of the indicated human methyltransferase-like (METTL) proteins containing a mitochondrial targeting signal were calculated. Range 0–1.

d, e Mitochondria isolated from HEK293 cells expressing METTL8-His6-2xFLAG (**d**) or METTL821-407-His6-2xFLAG (**e**) were converted to mitoplasts or sonicated (Sonic.), then treated with different concentrations of Proteinase K (PK). Proteins were analysed by western blotting using the indicated antibodies. MFN2, TIM23 and uS14m are proteins of the outer mitochondrial membrane, inner mitochondrial membrane and mitochondrial matrix respectively. Schematic representations of the tagged proteins are given below using colours as in (**d, e**). For **d**, three independent experiments were performed and representative data are shown, and for **e** and **f**, a single experiment was performed. Source data are provided as a Source Data file.
towards the intermembrane space, was protected in mitochondria but not mitoplasts. Similar to the mitochondrial ribosomal protein uS14m present in the mitochondrial matrix\(^5\), METTL8-His\(_6\)-2xFLAG, but not METTL8-His\(_6\)-2xFLAG, was resistant to digestion in both protease-treated mitochondria and mitoplasts, indicating that METTL8 is present within the mitochondrial matrix (Fig. 1d, e).

To further refine the localisation of METTL8 within the mitochondrial matrix by determining if it is associated with the IMM, mitochondria isolated from METTL8-His\(_6\)-2xFLAG cells were treated with sodium carbonate at different alkaline pH values before centrifugation (Fig. 1f). As the pH increases, only IMM or soluble in the matrix, such as TIM44\(^5\) and uL3m\(^5\), are integral membrane proteins, such as TIM23, are retained and partially released into the supernatant. Analogous to TIM44, METTL8 was found predominantly in the pellet after sodium carbonate treatment at pH 10.5 and centrifugation, but was partially extracted into the soluble fraction at higher pH values. Together, these data demonstrate that METTL8 is a mitochondrial matrix protein peripherally associated with the IMM.

METTL8 interacts with mt-tRNA ASLs in vivo and in vitro.

Based on its close homology to METTL2A/B and METTL6, METTL8 is a predicted m\(^3\)C RNA methyltransferase but no targets have yet been defined. Therefore, a UV cross-linking and analysis of cDNA (CRAC) experiment\(^52-54\) was performed to identify cellular RNAs directly contacted by METTL8. Cells expressing METTL8-His\(_6\)-2xFLAG, or just the His\(_6\)-2xFLAG tag as a control, were UV crosslinked and protein-RNA complexes were purified. Bound RNAs were trimmed, radiolabelled with \(^{32}\)P and ligated to adaptors. Protein-RNA complexes were separated by denaturing polyacrylamide gel electrophoresis (PAGE), transferred to a membrane and radiolabelled RNAs were detected by autoradiography (Fig. 2a). No radioactive signal was detected in the sample derived from the cells expressing the His\(_6\)-2xFLAG tag while the strong signal at and above the migration position of METTL8-His\(_6\)-2xFLAG indicated association of METTL8 with cellular RNAs (Fig. 2a). The region of the membrane containing the radiolabelled RNAs, and a corresponding area of the membrane from the control lane, were excised and extracted RNAs were copied into cDNA libraries that were subjected to Illumina deep sequencing. Consistent with the protein localisation analyses (Fig. 1), mapping of the obtained sequencing reads on the human genome revealed a strong enrichment of mitochondrial-encoded RNA species (mitoRNA) in the METTL8-His\(_6\)-2xFLAG sample compared to the control (Fig. 2b). The numbers of sequencing reads and nucleotide substitutions mapping to each nucleotide of the two most enriched mt-tRNAs (mt-tRNA\(^{3\text{Thr}}\) and mt-tRNA\(^{3\text{Ser(U CN)}}\)) were plotted to generate profiles of METTL8-His\(_6\)-2xFLAG crosslinking (Fig. 2e; Supplementary Tables 4 and 5). Nucleotide substitutions can arise due to reverse transcriptase errors when nucleotides crosslinked to amino acids are encountered and can therefore indicate sites of protein-RNA crosslinking. These profiles indicate that METTL8-His\(_6\)-2xFLAG predominantly contacts the ASL region of mt-tRNA\(^{3\text{Thr}}\) (Fig. 2e).

To further explore interactions between METTL8 and RNA, fluorescence anisotropy experiments were performed using fluorescein-labelled model substrates together with recombinantly expressed His\(_{14}\)-MBP-METTL8 purified from E. coli. Consistent with the CRAC data, His\(_{14}\)-MBP-METTL8 bound both the mt-tRNA\(^{3\text{Ser(U CN)}}\) and mt-tRNA\(^{3\text{Met}}\) ASLs with high affinity (Fig. 2f). To assess the specificity of these interactions and determine whether METTL8 preferentially binds the ASL structure, further experiments were performed using two unstructured RNA substrates of comparable length with diverse nucleotide sequences and also a DNA oligonucleotide (Fig. 2g). His\(_{14}\)-MBP-METTL8 did not bind the DNA oligonucleotide, demonstrating its specificity for RNA binding (Fig. 2g). However, both unstructured RNAs were bound with similar affinities to the mt-tRNA ASLs (Fig. 2f, g), implying that the robust RNA binding of METTL8 is not strongly influenced by sequence or structure.

METTL8 installs m\(^3\)C\(_{32}\) modifications on mt-tRNA\(^{3\text{Thr}}\) and mt-tRNA\(^{3\text{Ser(U CN)}}\). METTL8 is a putative m\(^3\)C methyltransferase\(^38\) and among all mt-tRNAs, only mt-tRNA\(^{3\text{Thr}}\) and mt-tRNA\(^{3\text{Ser(U CN)}}\) are reported to contain m\(^3\)C\(_{32}\)\(^,33,36,55\). As these two mt-tRNAs were the most enriched RNAs with METTL8-His\(_6\)-2xFLAG in our crosslinking experiments, we explored if they are substrates of METTL8 methylation activity. Using the CRISPR/Cas9 genome editing system, two HEK293 cell lines (KO1 and KO2) carrying 15 and 1 nucleotide (nt) deletions within exon 3 of METTL8 that do not express functional full-length METTL8 were generated (Fig. 3a, b). Small RNAs recovered from wild-type (WT) HEK293 cells and those lacking METTL8 (KO1 and KO2) were analysed by primer extension to detect m\(^3\)C\(_{32}\) in mt-tRNA\(^{3\text{Thr}}\) and mt-tRNA\(^{3\text{Ser(U CN)}}\) primers. Primer extension on small RNAs derived from WT cells showed strong reverse transcription stalling at position 32 and minimal read-through for both mt-tRNA\(^{3\text{Thr}}\) and mt-tRNA\(^{3\text{Ser(U CN)}}\) (Fig. 3d). In contrast, primer extension on small RNAs derived from the cells lacking METTL8 showed minimal reverse transcriptase stalling at C\(_{32}\) and clear signals corresponding to the m\(^2\)G\(_{10}\) and/or m\(^1\)A\(_{9}\) modifications in mt-tRNA\(^{3\text{Thr}}\) or the 5’ end of the tRNA in the case of mt-tRNA\(^{3\text{Ser(U CN)}}\), which does not contain any modifications 5’ of...
the m$^3$C$_{32}$ site (Fig. 3d). In parallel, m$^3$C$_{32}$ levels in mt-tRNA$^{Thr}$/mt-tRNA$^{Ser{(UCN)}}$ in WT, KO1 and KO2 cells were monitored using deoxyribozymes that either preferentially cleave adjacent to m$^3$C nucleotides compared to unmodified cytosines (AL112) or whose cleavage activity is impaired by the presence of methylated cytosines (AK104)56. Under the conditions used, approximately 60 and 38% of mt-tRNA$^{Thr}$ and mt-tRNA$^{Ser{(UCN)}}$ respectively, from wild-type cells were cleaved by the AL112 deoxyribozymes.
Fig. 2 METTL8 crosslinks to mt-tRNAs in cells. a HEK293 cells expressing METTL8-His6-2xFLAG or the His6-2xFLAG tag were UV crosslinked. Protein-RNA complexes were retrieved and co-purified RNAs trimmed, radioactively labelled and ligated to adaptors. Protein-RNA complexes were separated by denaturing PAGE, transferred to a nitrocellulose membrane and detected by autoradiography. Areas of the membrane excised are indicated with red boxes. Data presented in a-c and e derive from a single experiment, b RNAs eluted from the membrane areas indicated in (a) were reverse transcribed and the cDNA library deep sequenced. Doughnut charts show the relative distribution of reads derived from different classes of RNA in the His6-2xFLAG and METTL8-His6-2xFLAG samples. Abbreviations: mRNA - messenger RNA, tRNA - transfer RNA, lncRNA - long non-coding RNA, mt-tRNA - mitochondrial tRNA, mt-rRNA - mitochondrial rRNA, mt-mRNA - mitochondrial mRNA. c The normalised numbers of sequencing reads mapping to each mt-tRNA gene in the METTL8-His6-2xFLAG and His6-2xFLAG datasets are depicted as a heatmap in logarithmic scale (left panel). The fold-enrichment of reads derived from mt-tRNAs in the METTL8-His6-2xFLAG compared to His6-2xFLAG control is depicted in the right panel. d Lysates from crosslinked cells expressing METTL8-His6-2xFLAG or the His6-2xFLAG tag were used for immunoprecipitation experiments. Proteins and RNAs in input and eluate samples were analysed by western and northern blotting respectively. Three biologically independent experiments were performed and representative blots are shown. e The numbers of sequencing reads and nucleotide substitutions (Sub.) mapping to each nucleotide of the mt-tRNAThr and mt-tRNASer(UCN) genes in the METTL8-His6-2xFLAG and control sample (His6-2xFLAG) are shown. The nucleotide sequence of each mt-tRNA is given with nucleotides of the anticodon stemloop (ASL) indicated in red. RPM - reads per million mapped reads. f, g Fluorescence anisotropy measurements were taken to determine the affinity of recombinant His64-MBP-METTL8 for different fluorescent-labelled oligonucleotides: ASLs of mt-tRNA Thr and mt-tRNA Ser(UCN) and mt-tRNA Met, unstructured RNAs and DNA oligonucleotide (g). Data from three independent experiments are shown as mean ± standard deviation and dissociation constants (Kd) are given. Source data are provided as a Source Data file.

supporting the presence of m3C32 in these mt-tRNAs (Supplementary Fig. 2b). These results are consistent with the cleavage yields obtained for synthetic mt-tRNAThr36 and mt-tRNASer(UCN) containing m3C32 (Supplementary Fig. 2c). Consistent with the primer extension assays, cleavage of mt-tRNAThr/Ser(UCN) derived from the METTL8 KO cells was significantly reduced compared to the wild-type (Supplementary Fig. 2b). Similarly, while mt-tRNAThr/Ser(UCN) were barely affected by treatment with the AKI04 deoxyribozymes, cleavage of the RNAs derived from METTL8 KO cells was readily detected. Together, these results further support reduced C32 methylation of mt-tRNAThr/Ser(UCN) in the absence of METTL8.

To verify that METTL8 is directly responsible for methylation of C32 in these tRNAs, small RNAs extracted from WT, KO1 and KO2 cells were subjected to in vitro methylation using S-adenosylmethionine (SAM) as a methyl group donor and recombinant His14-MBP-METTL8 or METTL8-derivatives carrying amino acid substitutions in the catalytic domain predicted to impair methylation activity (His14-MBP-METTL8D230A and His14-MBP-METTL8D309A; Fig. 1a)29. Primer extension performed with primers specific for mt-tRNAThr and mt-tRNASer(UCN) showed that treatment of RNAs from WT cells with recombinant His14-MBP-METTL8 lead to slightly decreased read-through signals, consistent with the methylation of endogenously unmethylated mt-tRNAThr/Ser(UCN) (m3C modification level: mt-tRNAThr 85 - 94%,56,57) (Fig. 3e, f). In the presence of SAM, addition of His14-MBP-METTL8 to small RNAs derived from the METTL8 KO cells allowed methylation of C32 of both mt-tRNAThr and mt-tRNASer(UCN) to be fully restored (Fig. 3e, f). In contrast, primer extension of RNAs incubated with His14-MBP-METTL8D230A showed C32 methylation similar to untreated RNAs from METTL8 KO cells and only a low level of methylation of C32 on RNAs from METTL8 KO cells treated with His14-MBP-METTL8D309A (Fig. 3e, f). Together these results demonstrate that the methylation activity of METTL8 targets C32 of mt-tRNAThr/Ser(UCN) and that amino acid substitutions in the methyltransferase domain impair its catalytic activity.

As these methylation assays were performed on RNAs isolated from human cells, to investigate whether METTL8 was able to methylate nascent transcripts or whether other elements installed within the cellular context are required for C32 methylation, the activity of His14-MBP-METTL8D230A/METTL8D309A on in vitro transcribed mt-tRNAThr and mt-tRNASer(UCN) was examined using [3H]-SAM as the methyl group donor. His14-MBP-METTL8 was readily able to methylate mt-tRNAThr, but only minimal methylation of mt-tRNASer(UCN) was observed (Fig. 3g), despite similar levels of C32 methylation of these two mt-tRNAs being observed in cellular RNAs (Fig. 3e, f). Specificity of the observed methylation activity of His14-MBP-METTL8 on mt-tRNAThr was confirmed as neither His14-MBP-METTL8D230A nor His14-MBP-METTL8D309A was able to substantially methylate the in vitro transcript that, in contrast to the endogenous mt-tRNAThr (Fig. 3e, f), was present at an equimolar level to the recombinant protein (Fig. 3h).

Modifications at position 37 promote installation of m3C32 in mt-tRNAThr/Ser(UCN) by METTL8. The finding that METTL8 efficiently methylates cellular mt-tRNAThr but not in vitro transcribed mt-tRNAThr56,57 suggests the requirement of other RNA modifications for efficient METTL8-mediated methylation of C32. In yeast, interdependence of m3C32 and modifications at position 37 has been observed30,31,58, raising the possibility that (ms2)6A37 in mt-tRNASer(UCN) and 6A37 in mt-tRNAThr may stimulate C32 methylation by METTL8. Unmodified ASLs or those containing appropriate combinations of the modified nucleotides m3C, 6A, ms2A and 6A were prepared by solid phase synthesis using ribonucleoside phosphoramidite building blocks, which were chemically synthesised from canonical nucleosides in five or six linear steps (Fig. 4a and Supplementary Fig. 3). Methylation assays with recombinant His14-MBP-METTL8 and [3H]-SAM showed that for mt-tRNAThr, the unmodified ASL was sufficient for METTL8-mediated methylation and that the presence of m3C32 prevented methylation, confirming this as the target nucleotide also in vitro (Fig. 4b). Strikingly, the presence of the 6A37 modification increased significantly the level of METTL8-mediated methylation and that the presence of m3C32 prevented methylation, confirming this as the target nucleotide also in vitro (Fig. 4b). For mt-tRNASer(UCN), very little methylation of the unmodified ASL was observed, as previously seen for the unmodified in vitro transcript (Fig. 3g), but the presence of either 6A37 or ms2A37 facilitated methylation by METTL8 (Fig. 4c). Interestingly, the ms2A37 modification, which is predominantly present in cellular mt-tRNASer(UCN)36, stimulated methylation significantly more than 6A37, an intermediate formed during ms2A hypermethylation. ASLs containing m3C32 as well as 6A37 or ms2A37 were not methylated, consistent with C32 of mt-tRNASer(UCN) being the METTL8 target nucleotide (Fig. 4c).

As prior modification of A37 of mt-tRNAThr/Ser(UCN) strongly enhances C32 methylation in vitro, the effect of depleting enzymes responsible for modifying A37 on the levels of m3C32 in cellular mt-tRNAThr/Ser(UCN) was examined. RNAi-mediated depletion of the N6-threonylcarbamoyltransferase...
OSGEPL1 and the \(N^6\)-isopentenyltransferase TRIT1, which target mt-tRNA\(^{\text{Thr}}\) and mt-tRNA\(^{\text{Ser(UCN)}}\), respectively, was established\(^{57,59}\). Treatment of cells with two independent siRNAs against OSGEPL1 and TRIT1 decreased protein levels to approximately 11% and 33–38% respectively, of those observed in cells treated with non-target siRNAs (Fig. 4d and Supplementary Figs. 4a, b). Analysis of m\(^3\)C\(_{32}\) levels in mt-tRNA\(^{\text{Thr/Ser(UCN)}}\) by primer extension showed that the
percentage of read-through of m$C_{32}$ was mildly, but significantly, increased on RNAs obtained from cells treated with siRNAs against OSGEP1L or TRIT1 compared to those treated with the non-target siRNA (Fig. 4e). Hypomodification of tRNAs has, in some cases, been shown to destabilise tRNAs thus increasing turnover.\(^{20,21}\) To determine whether the mild effects of these knockdowns on m$C_{32}$ levels in cellular mt-tRNA$^{Thr}$/Ser$(UCN)$ compared to the effects in vitro arise due to degradation of hypomethylated mt-tRNAs in the knockdown cells, the levels of mt-tRNA$^{Thr}$/Ser$(UCN)$ in these samples were determined by northern blotting. No significant differences were observed (Supplementary Fig. 4c, d), implying that the mild effects, especially of the TRIT1 depletion, may rather be caused by residual protein remaining in the siRNA-treated cells. Furthermore, although the presence of $t^A_{37}/(ms^2)t^A_{37}$ stimulates METTL8-mediated methylation in vitro, some methylation activity, especially on mt-tRNA$^{Thr}$, is observed in the absence of these modifications. Thus it is also possible that some m$C_{32}$ installation may take place even when OSGEP1L/TRIT1 are lacking.

Our earlier finding that METTL8 binds not only the ASLs of its modification substrate mt-tRNAs, but also other RNAs (Fig. 2c, d), together with the discovery that modification of $A_{37}$ in mt-tRNA$^{Thr}$/Ser$(UCN)$ stimulates C$32$ methylation by METTL8, raised the possibility that the affinity of METTL8 for its methylation substrates is enhanced by the presence of the modified nucleotides at position 37. Fluorescence anisotropy experiments were therefore performed using recombinant His$_{14}$-MBP-METTL8 and fluorescent-labelled mt-tRNA$^{Ser}(UCN)$ ASLs without modified nucleotides, or with the $t^A_{37}$ or $t^A_{32}$ and m$C_{32}$ modifications present (Fig. 4f). The highest affinity, with a $K_d$ of 0.2 $\mu$M, was observed for His$_{14}$-MBP-METTL8 interacting with the $t^A_{37}$-containing ASL. The presence of both $t^A_{37}$ and m$C_{32}$ lead to approximately 2.5-fold weaker binding of His$_{14}$-MBP-METTL8 (Fig. 4f). Although the effects are small, these data may indicate that the presence of $t^A_{37}$ is more important for the methylation activity of METTL8 than for binding of the methyltransferase, but that upon installation of m$C_{32}$, METTL8 has lower affinity for its substrate, potentially promoting its dissociation.

Among the mt-tRNAs, eight have a cytidine in position 32 and a modified adenosine at position 37, yet only mt-tRNA$^{Thr}$ and mt-tRNA$^{Ser}(UCN)$ are reported to have m$C_{32}$ (Supplementary Fig. 5). To verify that other mt-tRNAs containing the features thus far identified as important for C$32$ methylation are not targets of METTL8, in vitro methylation assays were performed using $[\text{H}]$-SAM, His$_{14}$-MBP-METTL8, and the ASLs of mt-tRNA$^{Ser}(AGY)$ containing $t^A_{37}$ and mt-tRNA$^{Phe}$ containing $ms^2t^A_{37}$. Neither of these ASLs were methylated by His$_{14}$-MBP-METTL8 (Fig. 4g), suggesting that METTL8 also relies on other elements within mt-tRNA$^{Thr}$ and mt-tRNA$^{Ser}(UCN)$ for substrate recognition.

U$34$ and G$35$ in the anticodon loop are recognition elements for METTL8. It has previously been shown that a G$_{35}$U$34$A$_{37}$ motif in tRNA$^{Thr}$ is required for substrate recognition by the yeast m$C_{32}$ methyltransferase Trm140, whereas a distinctive variable loop and the seryl-tRNA synthetase are specificity determinants important for m$C_{32}$ installation in tRNA$^{Ser}(CGA/UGA)$. As the mitochondrial seryl-tRNA synthetase (SARS2) has been recovered with METTL8,\(^{60}\), a potential role in methylation of C$_{32}$ of mt-tRNA$^{Ser}(UCN)$ by METTL8 was explored. However, RNAi-mediated depletion of SARS2 lead to only a very mild decrease in the amount of m$C_{32}$ detected in mt-tRNA$^{Ser}(UCN)$ and the addition of His$_{10}$-SARS2 to in vitro methylation assays did not significantly stimulate methylation by METTL8, suggesting that SARS2 is not strictly required for C$_{32}$ methylation of mt-tRNA$^{Ser}(UCN)$ (Supplementary Note 1 and Supplementary Fig. 6b, d, e).

We therefore focused instead on analysing the influence of individual nucleotide substitutions within the anticodon loop on METTL8-mediated installation of m$C_{32}$ (Fig. 5a, b). In vitro methylation assays performed using His$_{14}$-MBP-METTL8 and $[\text{H}]$-SAM and various mt-tRNA$^{Thr}$ transcripts revealed that nucleotide substitutions at positions 32, 34, 35, 36 and 38 abolished methylation by METTL8 (Fig. 5a, right), indicating that the U$_{34}$G$_{35}$U$36$A$_{37}$A$_{38}$ motif is important for substrate recognition. Only exchange of U$_{34}$ for A did not significantly affect methylation of C$_{32}$ by METTL8. For mt-tRNA$^{Ser}(UCN)$, as the mitochondrial seryl-tRNA synthetase are specificity determinants important for m$C_{32}$ installation in tRNA$^{Ser}(CGA/UGA)$, as the mitochondrial seryl-tRNA synthetase (SARS2) has been recovered with METTL8, a potential role in methylation of C$_{32}$ of mt-tRNA$^{Ser}(UCN)$ by METTL8 was explored. However, RNAi-mediated depletion of SARS2 lead to only a very mild decrease in the amount of m$C_{32}$ detected in mt-tRNA$^{Ser}(UCN)$ and the addition of His$_{10}$-SARS2 to in vitro methylation assays did not significantly stimulate methylation by METTL8, suggesting that SARS2 is not strictly required for C$_{32}$ methylation of mt-tRNA$^{Ser}(UCN)$ (Supplementary Note 1 and Supplementary Fig. 6b, d, e).

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Fig. 4 A37 modifications promote m^3C32 incorporation in mt-tRNA^{Thr} and mt-tRNA^{Ser(UCN)}. a Schematic views of synthetic anticodon stem loops (ASL) of mt-tRNA^{Thr} (upper) and mt-tRNA^{Ser(UCN)} (lower) containing the indicated modified nucleotides. Orange U indicates a uridine substituted for pseudouridine. b, c In vitro methylation assays were performed using variants of the mt-tRNA^{Thr} ASL (b) or mt-tRNA^{Ser(UCN)} (ASL) (c), with His_{44}-MBP-METTL8 and [³H]-SAM. Tritium incorporated into the RNA was measured by scintillation counting. Bar plots show mean counts per minute (CPM) of ³H-Me. d Schematic views of synthetic anticodon stem loops (ASL) of mt-tRNA^{Thr} (left panel) and mt-tRNA^{Ser(UCN)} (right panel). Products were separated by denaturing PAGE alongside [³2P]-labelled DNA oligonucleotides of the indicated lengths. Signal intensities of the extension products were quantified and significance calculated using Tukey’s multiple comparisons test. e Protein extracted from cells transfected with non-target siRNAs (NT) or two different siRNAs against OSGEPL1 or TRIT1 (KD1, KD2) was analysed by western blotting. Representative image of three independent experiments. Asterisk indicates a non-specific cross-reaction of the TRIT1 antibody. f Fluorescence anisotropy measurements were taken to determine the affinity of recombinant His_{44}-MBP-METTL8 for fluorescence-labelled mt-tRNA^{Ser(UCN)} ASLs containing different modified nucleotides depicted in (a lower panel). Data from n = 3 independent experiments is show as mean ± standard deviation and dissociation constants (K_d) are given. g In vitro methylation assays were performed as in (b, c) using recombinant His_{44}-MBP-METTL8, [³H]-SAM and the ASLs shown in (a left panel). p-values for data in b, c, e are given in source data; *p < 0.05, **p < 0.01, ***p < 0.001. Source data and p values are provided as a Source Data file.
Fig. 5 Methylation substrate recognition by METTL8. a, b In vitro transcribed mt-tRNAThr (a) or synthesised mt-tRNASer(UCN) ASL containing ms2i6A37 (b) or derivatives containing individual nucleotide substitutions within the anticodon loop were subjected to methylation assays with His14-MBP-METTL8 and [3H]-SAM. Tritium incorporated into the RNA was measured by scintillation counting. Bar plots show mean counts per minute (CPM) of experiments ± standard deviation. Statistical analysis was performed using one-way ANOVA (F = 214.6; p < 0.0001 (a) and F = 277.1, p < 0.0001 (b)) and significance calculated using Tukey’s multiple comparisons test. RNA was also separated by denaturing PAGE, stained with ethidium bromide (EtBr) and labelled RNAs (3H-Me) were detected by autoradiography (lower panel). Representative images from three independent experiments.

c Schematic view of chimeric tRNA/ASLs used for in vitro methylation assays. Upper panel shows the anticodon loop of mt-tRNAThr (unmodified; green) or also with the anticodon stem (green shading) within mt-tRNAAsn. Lower panel shows a hybrid of the anticodon loop of mt-tRNASer(UCN) with the anticodon stem of mt-tRNAThr. d, e The chimeric RNAs represented in (c) were used for in vitro methylation assays with recombinant His14-MBP-METTL8 and [3H]-SAM. Tritium incorporated into the RNA was measured by scintillation counting. Bar plots show mean counts per minute (CPM) of n = 3 independent experiments ± standard deviation. Statistical analysis was performed using one-way ANOVA (F = 190.5; p < 0.0001) and significance calculated using Tukey’s multiple comparisons test (ns non-significant) (e). RNA was also separated by denaturing PAGE, stained with ethidium bromide (EtBr) and labelled RNAs (3H-Me) were detected by autoradiography. Representative images from three independent experiments. f CD spectra of unmodified mt-tRNAThr or mt-tRNASer(UCN) ASL (black) (top), its ms2i6A37 modified analogue (red) (bottom), and the analogous ASL containing A38C (green) (bottom) and G35A (blue) (top) substitutions were recorded at 5 μM in 10 mM Na-phosphate buffer pH 7.0, 1 mM MgCl2. CD spectra were recorded at least twice for each sample. p values for data in a, b, d, e are given in source data; ***p < 0.001, ****p < 0.0001, ns not significant. Source data and p values are provided as a Source Data file.
the A38C substitution in mt-tRNA<sub>Ser(UCN)</sub> lead to increased METTL8-mediated methylation, similar to the recently observed higher methylation activity of METTL6 on cytoplasmic tRNA-Ser(GCU) carrying an A38C substitution. These data suggest that METTL8 relies less on the sequence context for recognition of mt-tRNA<sub>Ser(UCN)</sub> than mt-tRNA<sub>Thr</sub>. However, in both cases G<sub>15</sub> and U<sub>36</sub>, which are notably absent from the other t<sub>6</sub>A<sup>37</sup>- and ms<sup>2</sup>i6A<sup>37</sup>-containing mt-tRNAs (Supplementary Fig. 5a), play important roles in the recognition of both substrate mt-tRNAs.

To determine if the identified sequence motif is sufficient to induce methylation of non-substrate mt-tRNAs that have a C<sub>32</sub> residue, chimeric mt-tRNA<sub>Ser</sub> transcripts containing the anticodon loop or anticodon stem loop of mt-tRNA<sub>Thr</sub> were generated (Fig. 5c, top). In vitro methylation assays demonstrated that, in contrast to mt-tRNA<sub>Ser</sub> (Supplementary Fig. 6f), the chimeric ondon loop or anticodon stem loop of mt-tRNA<sub>Thr</sub> were generated similarly, whereas the ASL containing the G<sub>35</sub>A mutation either the wild-type sequence or the A<sub>38</sub>C substitution migrated Fig. 5c).

Interestingly, a mutation identified in patients with mitochondrial disorders and myoclonic epilepsy with ragged red fibres (MERRF) syndrome (m.15923A>G) leads to exchange of A<sub>38</sub> for G in mt-tRNA<sub>Thr</sub>. It has previously been shown that this nucleotide substitution impairs N<sub>6</sub>-threonylcarbamoylation of A<sub>37</sub>, implying that loss of t<sub>6</sub>A<sup>37</sup> in mt-tRNA<sub>Thr</sub> might have pathological consequences. As m<sup>3</sup>C<sub>32</sub> can be installed in mt-tRNA<sub>Thr</sub> in the absence of t<sub>6</sub>A<sup>37</sup>, we explored how this nucleotide substitution affects METTL8-mediated methylation of m<sup>3</sup>C<sub>32</sub>. Both in the context of an unmethylated full-length mt-tRNA<sub>Thr</sub> transcript and a synthetic mt-tRNA<sub>Thr</sub> ASL containing t<sub>6</sub>A<sup>37</sup>, exchange of A<sub>38</sub> for G strongly reduced METTL8 methylation (Fig. 5a and Supplementary Fig. 5b), implying that lack of m<sup>3</sup>C<sub>32</sub> in mt-tRNA<sub>Thr</sub> may also contribute to the disease phenotype observed in patients carrying this mutation.

The sensitivity of the anticodon loop to point mutations was also reflected in the circular dichroism (CD) spectra of mt-tRNA<sub>Ser(UCN)</sub> ASLs (Fig. 5f), thus supporting the functional findings by structural aspects. Strikingly, introducing the G35A point mutation that prevents METTL8-mediated methylation into the ms<sup>2</sup>i6A<sup>37</sup>-modified ASL resulted in a CD signature reminiscent of the unmodified ASL, which is also a poor substrate for METTL8 (Fig. 5f, upper panel). In contrast, the A38C substitution that does not impede m<sup>3</sup>C<sub>32</sub> installation, maintained the overall shape of the ms<sup>2</sup>i6A<sup>37</sup>-containing wild-type sequence, which is also efficiently methylated (Fig. 5f, lower panel). Furthermore, separation of these ASLs by native gel electrophoresis revealed that the ms<sup>2</sup>i6A<sup>37</sup>-containing ASLs with either the wild-type sequence or the A38C substitution migrated similarly, whereas the ASL containing the G35A mutation and the unmodified ASL both migrated faster (Supplementary Fig. 5c).

m<sup>3</sup>C<sub>32</sub> affects the structure of mt-tRNA<sub>Thr</sub>Ser(UCN). Characterisation of METTL8 as the m<sup>3</sup>C methyltransferase responsible for methylating C<sub>32</sub> of mt-tRNA<sub>Ser(UCN)</sub> enables the functional relevance of these RNA modifications to be explored. The presence of modified nucleotides in tRNAs can contribute to their stability and achieving correct folding. Therefore, the influence of m<sup>3</sup>C<sub>32</sub> on the stability of mt-tRNA<sub>Thr</sub>Ser(UCN) was analysed by northern blotting.
**Fig. 6 Requirement of m^3C32 in mt-tRNA^{Thr/Ser(U CN)} for tRNA stability and structure.**

(a) Total RNAs from WT, KO1 and KO2 cells were separated by denaturing PAGE, and mt-tRNA^{Thr/Ser(U CN)} and the U6 snRNA were detected using northern blotting. Representative images of three independent experiments (left). Hybridisation signals detected by northern blotting were quantified and the normalised signal intensity from n = 3 independent experiments is shown as mean ± standard deviation (right). Statistical analysis was performed using one-way ANOVA (F = 0.568, ns for mt-tRNA^{Thr}; F = 0.5221, ns for mt-tRNA^{Ser(U CN)}) and significance calculated using Tukey’s multiple comparisons test. 

(b) Total RNAs from WT, KO1 and KO2 cells (b) or synthetic mt-tRNA^{Thr} ASL and mt-tRNA^{Ser(U CN)} ASL with the modifications indicated (c) were separated by native PAG, and mt-tRNA^{Thr/Ser(U CN)} and the U6 snRNA were detected using northern blotting (b) or sybr gold staining (c). Representative images of three independent experiments. 

(c) H^1 NMR spectra of unmodified mt-tRNA^{Ser(U CN)} ASL (black, bottom), its i^6A_{37} modified analogue (green, middle), and the analogous ASL containing both i^6A_{37} and m^3C_{32} modified nucleotides (blue, top) were recorded at 600 MHz. Conditions: 200 µM RNA, 10 mM Na-phosphate buffer pH 7.0, 100 mM NaCl, H_2O/D_2O 9/1, 10 °C. 

(d) CD spectra of unmodified mt-tRNA^{Ser(U CN)} ASL (black), its ms^2i^6A_{37} modified analogue (red), and the analogous ASL containing both ms^2i^6A_{37} and m^3C_{32} modified nucleotides (purple) were recorded at 5 µM in 10 mM Na-phosphate buffer pH 7.0, 1 mM MgCl_2. 

(e) Relative signal intensity for ms^2i^6A_{37} + m^3C_{32}, + m^3C_{32} + ms^2i^6A_{37} from WT and KO1 KO2 samples. 

**mt-tRNA^{Thr/Ser(U CN)} aminoacylation and mitoribosome association are not affected by lack of METTL8 but mitochondrial translation is impaired.** The finding that mt-tRNA^{Thr/Ser(U CN)} lacking m^3C_{32} are structurally impaired suggests that their functional capacity may be compromised. The importance of m^3C_{32} of mt-tRNA^{Thr/Ser(U CN)} on the cellular level was first investigated by comparing the growth rate of WT cells and those lacking METTL8. Equal numbers of cells were seeded and counted every 24 h for 72 h during exponential growth. Compared to WT, both KO1 and KO2 cells grew slower (Fig. 7a), indicating a physiological relevance of METTL8-mediated m^3C_{32} of mt-tRNA^{Thr/Ser(U CN)}.

Functionally, tRNAs serve as adaptors between mRNA codons and amino acids. Thus, aminoacylation is a key step in achieving functional competence. As some tRNA modifications influence the association and/or activity of aminoacyl-tRNA synthetases, the aminoacylation status of mt-tRNA^{Ser(Ser(U CN))} in the presence or absence of METTL8 were determined. RNAs purified from WT and METTL8 KO cells under acidic conditions to retain covalently bound amino acids were either subjected to alkaline conditions to drive deacylation or left untreated. Separation by acidic PAGE in which deacylated tRNAs migrate faster than their aminoacylated counterparts revealed that mt-tRNA^{Ser(U CN)} was almost constitutively aminoacylated in WT cells and only a very minor fraction of mt-tRNA^{Thr} is non-acylated (Fig. 7b). As expected, mt-tRNA^{Met} from METTL8 KO cells migrated as the one obtained from WT cells, both with and without alkaline treatment, consistent with no effect on aminoacylation. In contrast, the case of mt-tRNA^{Thr/Ser(U CN)}, both the aminoacylated and deacylated mt-tRNAs from METTL8 KO cells migrated differently to those from WT cells (Fig. 7b), reminiscent of the altered migration patterns of these mt-tRNAs observed by native gel electrophoresis (Fig. 6b). However, under acidic conditions both mt-tRNA^{Thr/Ser(U CN)} from METTL8 KO cells were (almost) fully aminoacylated demonstrating that lack of m^3C_{32} and the consequent structural alterations do not impede charging of these mt-tRNAs with the respective amino acids.

Aminoacylated (mt-)tRNAs are recruited to ribosomes during translation so we investigated the influence of lack of m^3C_{32} on the mitoribosome-association of mt-tRNA^{Thr/Ser(U CN)}. Extracts from mitochondria isolated from WT or METTL8 KO cells were separated by sucrose density centrifugation and western blotting was used to identify fractions containing mitoribosomal small subunits (28S), large subunits (39S) and monosomes (55S) (Fig. 7c). Fractions containing either non-mitoribosome-associated mt-tRNAs (1–2) or mitoribosome-associated...
mt-tRNAs (11–12) were pooled and analysed by northern blotting (Fig. 7d). Similar to the non-m3C32-containing mt-tRNA<sub>Met</sub>, mt-tRNA<sub>Thr/Ser(UCN)</sub> from both WT and METTL8 KO cells were readily detectable in fractions containing 55S monosomes, demonstrating that the lack of m3C32 and the associated conformational changes do not prevent their recruitment to mitoribosomes. Interestingly, compared to mt-tRNA<sub>Met</sub>, a mild accumulation of mt-tRNA<sub>Thr/Ser(UCN)</sub> on ribosomes was observed in the METTL8 KO cells (Fig. 7e).

The mitochondrial translation machinery is responsible for synthesising 13 mitochondrial-encoded proteins. Production of nascent mitochondrial proteins was therefore monitored in WT, KO1 and KO2 cells using [35S]-methionine incorporation (Fig. 7f). Despite the normal aminoaacylation and mitoribosome-association of mt-tRNA<sub>Thr/Ser(UCN)</sub> in cells lacking METTL8, compared to WT, METTL8 KO cells showed decreases in the levels of the some de novo synthesised mitochondrial proteins under these conditions (Fig. 7g). ND5, COX1, ND2, ND3 and ND4L/ATP8 were most affected by lack of METTL8, while production of CYTB, ND1, COX2/COX3, ATP6 and ND6 was less reduced (Fig. 7g). Overall, these data are in line with the model that m3C32 fine-tunes mt-tRNA<sub>Thr/Ser(UCN)</sub> structure to support optimal mitochondrial translation.

**Discussion**

The extensive landscape of modified nucleotides within cellular RNAs expands the functionality of the four basic nucleosides and allows gene expression to be delicately fine-tuned at many levels for optimal accuracy and efficiency. The development of novel RNA modification detection methods has rapidly expanded our
knowledge on sites of RNA modification throughout the transcriptome, and advances in the identification and characterisation of cognate RNA modifying enzymes have emerged in parallel.

The family of human mC methyltransferases composed of METTLL2A/B, METTLL6 and METTLL8 has been a focus of much recent attention with METTLL2A/B and METTLL6 being identified as the enzymes responsible for methylating C32 of cytoplasmic tRNA\(_{Thr}^{UCU}\) and tRNA\(_{Ser}^{UCU}\) (MTS) in the cytoplasmic tRNA\(_{Thr}^{UCU}\) and tRNA\(_{Ser}^{UCU}\) methyltransferase domain present in the arginyl-tRNA synthetase40. Although a number of other mC methyltransferases have been identified, METTLL8 is the only member of this family that is known to methylate mt-tRNA\(_{Ser}^{UCU}\) and mt-tRNA\(_{Thr}^{UCU}\) specifically in the mitochondrial ribosome. In contrast, other mC methyltransferases have been shown to methylate mRNAs encoding tRNA\(_{Thr}^{UCU}\) and tRNA\(_{Ser}^{UCU}\) in different contexts, including in the nucleus and in the cytoplasm.

Recent studies have suggested that METTLL8 may play a role in the regulation of mitochondrial translation. In particular, it has been shown that METTLL8 can methylate C32 in mt-tRNA\(_{Ser}^{UCU}\) and mt-tRNA\(_{Thr}^{UCU}\), leading to an increase in the efficiency of mitochondrial translation. This suggests that METTLL8 may have a role in the regulation of mitochondrial protein synthesis.

Furthermore, the methyltransferase activity of METTLL8 has been shown to be dependent on the presence of specific mC32 modification sites within the tRNA. This suggests that METTLL8 may use mC32 modification sites to regulate the translation of specific mitochondrial proteins, potentially allowing for the fine-tuning of mitochondrial protein synthesis to the needs of the cell.

In conclusion, METTLL8 is a member of the human mC methyltransferase family that is specifically involved in the regulation of mitochondrial translation. Its role in the regulation of mitochondrial protein synthesis is likely to be important in the overall function of the mitochondrial translation machinery.
mitochondrial seryl-tRNA synthetases interact with their substrate tRNAs; the cytoplasmic seryl-tRNA synthetases interacts with tRNA^Ser in E. coli via the large variable loop, which is absent in the mitochondrial counterparts and SARS2 instead requires the YTC and D loops to associate with mt-tRNA^Ser(UCN)69. Importantly, we observe that aminocarboxylation of mt-tRNA^Ser(UCN) is unaffected by lack of METTL8-mediated m^3C^32 implying that functional relevance of the association between METTL8 and SARS2 may be independent of SARS2 catalytic activity. It is possible that the recovery of SARS2 with METTL8, and the very mild decrease in m^3C^32 observed in SARS2-depleted cells, reflect an indirect role for the synthetase in optimising mt-tRNA^Ser(UCN) structure for METTL8-mediated methylation.

While m^3C^32 modifications are conserved features of eukaryotic (mt-)tRNAs, the precise function of this modification has remained elusive. Strikingly, the migration behaviours of mt-tRNA^Thr^Ser(UCN) ASLs on native polyacrylamide gels and thermal melting temperatures vary slightly depending on the modifications at positions A^37 and C^23, while additional evidence from imino proton NMR and CD spectra further support m^3C^32-dependent alterations in ASL structure. The chemical shift changes of imino protons and ellipticity signatures demonstrated that the modified nucleotides act together to cause distinct alterations in base stacking and electrostatic interactions, which in turn modulate the accessibility of hydrogen bond donors and acceptors to the environment. The migration patterns of full-length mt-tRNA^Thr^Ser(UCN) lacking m^3C^32 on native and acidic polyacrylamide gels also differ from those of the wild-type mt-tRNAs. As the extent of the migrational shifts observed is different for the two mt-tRNAs, this implies that regions outside the ASL are also structurally affected by the presence or absence of m^3C^32, and that the exact composition of nucleotides outside the anticodon loop may influence how these structural changes manifest. A notable difference between mt-tRNA^Ser(UCN) and mt-tRNA^Thr is the modifications they carry outside the anticodon loop. As modifications that influence RNA folding/stability often function as a co-operative network, it may therefore be that m^3C^32 differentially affects the structures of these tRNAs due to their different modification content. It is also possible that m^3C^32 is a pre-requisite for installation of another modification present in mt-tRNA^Ser(UCN) but not mt-tRNA^Thr, which would be similar to the previous observation that yeast lacking both Trm140 and the guanine dimethyltransferase Trm1 are sensitive to cycloheximide.

On the cellular level, the functional relevance of m^3C^32 modifications has remained challenging to address. Lack of METTL2A/B or METTL6 does not influence polysome formation and while ribosome profiling revealed changes in ribosome occupancy in cells lacking METTL6, these alterations mostly correlated with changes in transcript level, suggesting they arise due to an altered metabolic state of METTL6 KO cells rather than a direct effect of m^3C^32 on translation. Likewise, Ribo-tRNA-seq results indicate no significant differences in translation between wild-type yeast and a Δtrm140 strain. We observe that neither mt-tRNA^Thr^Ser(UCN) nor mt-tRNA^Thr^Ser(UCN) charging nor recruitment to the ribosome is impaired by lack of METTL8-mediated m^3C^32. However, the levels of most nascent mitochondrial-encoded proteins are mildly affected by loss of METTL8, implying a role for m^3C^32 in mt-tRNA^Thr^Ser(UCN) during mitochondrial translation. Consistent with this, and the finding that mt-tRNA^Thr^Ser(UCN) mildly accumulate on mitorbosomes in the absence of METTL8, a parallel study revealed the enrichment of codons recognised by mt-tRNA^Ser(UCN) in ribosome profiling data from METTL8 KO cells. Together, these data suggest that the presence of m^3C^32 fine-tunes mt-tRNA^Thr^Ser(UCN) structure to optimise its functionality.

As mitochondria are the hubs of many metabolic processes and responsible for most cellular energy production, even very mild effects on mt-tRNA structure and mitochondrial translation can have profound effects on the cellular level. Impaired t^6A^37 and m^3C^32 modification of mt-tRNA^Thr in cells caused by exchange of A^38 of mt-tRNA^Thr for G is associated with mitochondrial myopathy. Interestingly, METTL8 expression is upregulated in cancer2, when the demand for mitochondrial function is increased. Quantitative analysis of m^3C^32 levels in mt-tRNA^Thr^Ser(UCN) and our reconstitution of METTL8 methylation on cellular RNAs indicate that these modifications are not fully stoichiometric, raising the possibility that mitochondrial translation could be regulated by differentially modified mt-tRNA^Thr^Ser(UCN). m^3C-containing tRNAs can be actively demethylated by ALKBH3 or ALKBH1. However, ALKBH3 is not present in mitochondria and no changes in m^3C^32 levels in mt-tRNA^Thr^Ser(UCN) were observed in in vitro demethylase assays using purified ALKBH1 and m^3C-containing synthetic anticodon stem loops (Supplementary Fig. 4). Therefore, if the levels of m^3C^32 vary in different conditions to dynamically fine-tune mitochondrial translation, regulation likely occurs via differential expression and/or methylation by METTL8.

In summary, this work firmly establishes the role of the mitochondrial methyltransferase METTL8 in the biosynthesis of fully functional mt-tRNA^Thr^Ser(UCN). Several independent lines of evidence reveal the molecular requirements for METTL8 targets and identify a role of m^3C^32 in fine-tuning tRNA structure and function in mitochondrial translation.

**Methods**

**Molecular cloning**

The METTL8 coding sequence (CDS: NM_024770.5) and a truncated version lacking the 5’ 60 nucleotides were amplified using primers listed in Supplementary Table 6, and cloned into pcDNA5 vectors for inducible expression of proteins with a C-terminal GFP or His^6-2xFLAG (Hexahistidine-PreScission protease cleavage site-2x FLAG) tag in HEK293 T-Rex cells (Supplementary Table 7). For CRISPR/Cas9 constructs, guide sequences (Supplementary Table 6) were cloned into the PX459 plasmid pSpCas9(BB)-2A-Puro (Addgene #62989) using the BbsI restriction sites as previously described. For recombinant expression of His^6-2xFLAG-METTL8 in E. coli, the CDS was cloned into a pQE80-derivative vector and site-directed mutagenesis (Supplementary Table 6) was employed to introduce mutations to induce substitution of arginines 230 and 309 with alanine. Plasmids containing tRNA sequences including a 3’ CCA tail were generated by cloning a recursive PCR product generated using four overlapping oligonucleotides (Supplementary Table 6) into a pQE-derivative vector (Supplementary Table 7). All constructs were verified by Sanger sequencing (Eurofins Genomics).

**Human cell culture**

HEK293 Flp-In T-Rex cells (ThermoFisher Scientific) were cultured at 37 °C in 5% CO^2 in DMEM (Gibco) supplemented with 10% foetal calf serum (Merck) and 1% penicillin/streptomycin (Gibco) according to standard protocols. Cells lines for expression of C-terminally His^6-2xFLAG or GFP tagged METTL8 or METTL8^ΔC81 were generated by transfection of the pcDNA5-base constructs (Supplementary Table 7) into HEK293 Flp-In T-Rex cells using T-xenogene 9 DNA Transfection Reagent (Roche) according to the manufacturer’s instructions. Stably transfected cells were selected using hygromycin (100 µg/ml) and blasticidin (10 µg/ml) and expression of the transgene was induced by addition of 1 µg/ml tetracycline for 24 h before harvesting. Human METTL8 knock-out cell lines were generated by CRISPR/Cas9-mediated mutagenesis. HEK293 Flp-In T-Rex cells were transfected with 1 µg of PX459-based plasmid (Supplementary Table 7) and transfectants were selected with puromycin (1 µg/ml). Genomic cleavage efficiency was assessed using the GeneArt Genomic Cleavage Detection Kit (ThermoFisher Scientific) according to the manufacturer’s instructions. Following selections, genomic DNA was harvested from cell density in 6-well plates and cultured to generate a clonal population. Genomic DNA was extracted using PureLink Genomic DNA kit (ThermoFisher Scientific) according to the manufacturer’s instructions. The target region of the genome in each clone was PCR-amplified using primers listed in Supplementary Table 6 and genomic mutations were detected by sequencing. Lack of the METTL8 protein was analysed by western blotting using antibodies listed in Supplementary Table 8.

**RNN**

Cells were seeded at a density of 3 x 10^6 per 10 cm dish and transfected on the following day with siRNAs (50 nM; Supplementary Table 9) using Lipofectamine RNAimax reagent (ThermoFisher Scientific) according to the
manufacturer’s instructions. Cells transfected with siRNAs against TRIT1 were harvested 72 h after transfection whereas those transfected with siRNAs against OGG1/G2D were harvested twice using 72 h and used for western blotting and extracted RNAs were analysed by primer extension.

**Cell counting by analytical flow cytometry.** Viable cells were counted by trypan blue staining in a hemocytometer chamber. Increasing cell concentrations were measured for 10 x 10^6 at medium flow using a FACScanCanto II to generate a standard growth curve. Cells were seeded at a density of 7.5 x 10^5 cells in a 12 well dish and, at 24 h intervals over a 72 h period, cells were harvested by trypsinisation, transferred to flow cytometry tubes, washed with PBS and counted. Data acquisition and gating were performed using FACS Diva software (version 6.1.1) and data were exported using FlowCore (version 2.6.0). Total numbers of cells per well were extrapolated from the average from three technical measurements of three biological replicates (see Supplementary methods for further details).

**Fluorescence microscopy.** HEK293 cells for expression of METTL8-GFP or METTL11-407-GFP were treated with 1 µg/ml of tetracycline for 24 h. Cells were treated with MitoTracker® Deep Red FM (ThermoFisher Scientific) in media without FCS for 30 min at 37 °C, washed twice in PBS and fixed with 4% formaldehyde in PBS for 10 min at room temperature. Cells were mounted on coverslips using Vectashield® (Vector labs) and fluorescent images were visualised using a Nikon Ti-Eclipse inverted/UltraView VoX spinning disc confocal microscope.

**Isolation of mitochondria and protease protection assays.** HEK293 cells for expression of METTL8-His6, 2xFLAG and METTL11-407-His6, 2xFLAG were treated for 24 h with 1 µg/ml of tetracycline to induce protein expression and mitochondrial RNA were isolated from cells or isolated mitochondria using TRIzol® (Sigma-Aldrich) according to the manufacturer’s instructions. Cells were resuspended in 100 mM trehalose, 10 mM KCl, 10 mM HEPES-KOH pH 7.4, 1 mM PMSF and 0.2% BSA and homogenised using a Homogenplus Homogeniser (Schuett-Biotec). Differential centrifugation was performed and mitochondria were pelleted by centrifugation at 11,000 g for 10 min. After washing with homogenisation buffer, mitochondria were either resuspended in homogenisation buffer or in swelling buffer (10 mM PMSF-HEPES-KOH pH 7.4, 1 mM EDTA) with a final protein concentration of 1 mg/ml. After 15 min incubation on ice, samples were treated with Proteinase K for 15 min. Proteinase digestion was arrested by addition of 2 mM PMSF and samples were analysed by western blotting using antibodies listed in Supplementary Table 8. To determine whether proteins are integrated into, or associated with, the IMM, mitochondria isolated from cells expressing METTL8-His6, 2xFLAG were resuspended in 0.1 M Na2CO3 at pH 10.5, 11.5 and 12.5, then incubated on ice for 20 min. Samples were centrifuged at 100,000 x g for 60 min at 4 °C and supernatant and pellet fractions were analysed by western blotting.

**Analysis of mitoribosomal association of mt-tRNAs by sucrose density gradient centrifugation.** Isolated mitochondria from WT and METTL8 KO cells were lysed in a buffer containing 3% sucrose, 100 mM KCl, 10 mM MgCl2, 20 mM HEPES-KOH pH 7.4, 1% digitonin, 1% protease inhibitor cocktail (300 mM trehalose, 10 mM KCl, 10 mM HEPES-KOH pH 7.4, 1 mM PMSF and 0.2% BSA) and homogenised using a Homogenplus Homogeniser (Schuett-Biotec). Differential centrifugation was performed and mitochondria were pelleted by centrifugation at 11,000 x g for 10 min. After washing with homogenisation buffer, mitochondria were either resuspended in homogenisation buffer or in swelling buffer (10 mM PMSF-HEPES-KOH pH 7.4, 1 mM EDTA) with a final protein concentration of 1 mg/ml. After 15 min incubation on ice, samples were treated with Proteinase K for 15 min. Proteinase digestion was arrested by addition of 2 mM PMSF and samples were analysed by western blotting using antibodies listed in Supplementary Table 8. To determine whether proteins are integrated into, or associated with, the IMM, mitochondria isolated from cells expressing METTL8-His6, 2xFLAG were resuspended in 0.1 M Na2CO3 at pH 10.5, 11.5 and 12.5, then incubated on ice for 20 min. Samples were centrifuged at 100,000 x g for 60 min at 4 °C and supernatant and pellet fractions were analysed by western blotting.

**Total RNA extraction and small RNA enrichment.** Total RNA and total mitochondrial RNA were isolated from cells or isolated mitochondria using TRI Reagent® (Sigma-Aldrich) according to the manufacturer’s instructions. Small RNAs (<200 nt) were enriched from 100 µg of total RNA using the mirVana™ miRNA isolation kit (ThermoFisher Scientific) following the manufacturer’s instructions. RNA concentrations and purity were measured using a Nanodrop One® (ThermoFisher Scientific) and fold changes in the numbers of reads mapping per transcript were calculated from sequencing reads obtained from the same RNA template during bioinformatic analyses. After elution, protein-RNA complexes were separated by Superase-12 (ThermoFisher Scientific), transferred to a nitrocellulose membrane and exposed to an X-ray film. Relevant areas of the membrane were excised and RNA eluates were purified using Proteinase K (Roche) treatment. Individual small RNAs were reverse transcribed with Superscript II (Invitrogen), and cDNA libraries were amplified by PCR and subjected to Illumina sequencing.
over Quick Spin RNA columns (Roche), according to the manufacturer’s instructions. The RNA concentration was determined using a Nanodrop One© (ThermoFisher Scientific).

Chemical synthesis of RNAs containing modified nucleotides. RNA oligonucleotides were prepared by solid-phase synthesis using appropriately protected building blocks that were synthesised from 6-chloropurine riboside and uridine, respectively, as previously described.82 The syntheses of 5′-O-DMT-2′-O-TBDMS m5A³′-CEP and of 5′-O-DMT-2′-O-TBDMS m1A³′-CEP are described in Supplementary methods (Supplementary Figs. 9 and 10). Briefly, TBDMS-protected threonine p-nitrophenoxyethylyester was coupled to the N⁶-amino group of persilylated adenine via activation with 1-N-methyl-3-phenoxyboronimidoazolium chloride, in analogy to previous reports.83,84 The nucleobase modifications in m5A³′A were introduced by sequential substitutions on peracetylated 6-chloro-2′-aminopurine riboside. Diazotation of the amino group and displacement with methylsulphide85 was followed by substitution of chloride by isopentenyl amine83. The final phosphoramidite building blocks were used as 100 mM solutions in dry acetonitrile together with 0.25 M ethylthiotetrazole as activator and a coupling time of 12 min. Dethiolation, capping and oxidation were performed under standard conditions. Cleavage from solid support and alkaline deprotection was specific for each type of modified nucleotide: ASLs containing m⁵C₃₂ were treated with 25% NH₄OH/EtOH 3/1 at 55 °C for 6 h; ASLs containing m⁶A₂₃ were treated with 25% NH₄OH/MεN₄₃(H₂O) 1/1 at 37 °C for 3 h and 55 °C for 2 h; ASLs containing m⁵A₃₄ were treated with MeNH₂(H₂O/EOH) 1/1 at 37 °C for 6 h, and ASLs containing m⁶A₂₃ were treated with 10% DBU in THF at ambient temperature for 2 h, and then cleaved from the washed solid support by incubation with 25% NH₄OH/(MeH₄N)₃(H₂O) 1/1 at ambient temperature for 1.5 h. Deprotection of silyl groups was performed with 1 M TBAF in THF for 12 h. Oligonucleotides were purified by denaturing PAGE (20%) and analysed by anion-exchange HPLC (Dionex DNA Pac PA200, 2 × 250 mm). Solvent A: 25 mM Tris-HCl (pH 8.0), 6 M urea. Solvent B: 25 mM Tris-HCl (pH 8.0), 6 M urea, 0.5 M NaClO₄. Gradient: linear, 0→100% solvent B in 1 min, 100% solvent B for 5 min. Oligonucleotide concentration was determined using a Nanodrop One© (ThermoFisher Scientific) by heating at 95 °C for 3 min and allowing the mixture to cool slowly to 55 °C. Extension mix (10 U/µl SuperScript III (ThermoFisher Scientific), 1 U/µl Ribolock (ThermoFisher Scientific)) and 6 ng/dNTPs in 1x First Strand buffer) was added to each reaction and samples were incubated at 55 °C for 30 min. Reactions were stopped by adding 2x formamide dye (95% formamide, 0.5 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol, 0.025% SDS) and samples were heated at 85 °C for 5 min. Samples were resolved in 15% denaturing (7 M urea) polyacrylamide gels, which were dried and exposed to a phosphorimager screen (cya®) overnight. Radiolabelled cDNA fragments were detected using a phosphorimager Typhoon FLA 9500 (cya®) and quantified using the Image Studio 5.2.5 software (LI-COR). For primer extension analyses of RNAs derived from cells treated with siRNAs against OSF GEP1 or TRIT1, 2 U/µl AMV reverse transcriptase (Promega) was used in 1x RT buffer (Promega) and extension was performed at 58°C for 1 h.

De novo synthesis of [35S]–methionine labelled mitochondrial-encoded proteins. To analyse nascent protein synthesis, cells were depleted of methionine and cytoplasmic translation was inhibited in WT and METTL8 KO cells by treatment with 100 µg/ml emetine for 10 min. Cells were then incubated in media supplemented with 0.2 µCi/ml [35S]–methionine (Hartmann Analytics) for 1 h. Proteins were extracted, separated by electrophoresis PAGE and [35S]–labelled proteins were detected using a phosphorimager.

Imino proton NMR spectroscopy. Samples for NMR spectroscopy were prepared from 36 nmol of RNA oligonucleotides (concentrated and purified by precipitation from acetone/LiClO₄, 2% v/v), in a final volume of 180 µl containing 10 mM sodium cacodylate, 50 mM HEPES/KOH, pH 7.0, 100 mM NaCl, 1 mM MgCl₂, 1 mM DTT. The solution was heated to 90°C for 2 min and annealed for 15 min on ice and transferred to a 3 mm NMR tube. As internal standard, 0.4 µl DSS (sodium trimethylpropanesulfonate) was added. 1H NMR spectra with water suppression by excitation sculpting were recorded on a Bruker Avance III HD 600 MHz spectrometer at 283, 290, 298, and 306 K.

Circular dichroism spectroscopy. CD spectra were recorded on a JASCO Spectropolarimeter J-715 equipped with a Xenon lamp and power supply PS-150J, in quartz cuvettes with 1 cm path length at ambient temperature, with a bandwidth of 10 nm and a scanning speed of 500 nm/min. The ASL RNA samples (5 µm) were annealed in 10 mM sodium phosphate buffer (pH 7.0) and supplemented with 1 mM MgCl₂. All data were baseline corrected using a control containing buffer only. Spectra are shown as the average of duplicate data collection.

Data normalisation and statistical analyses. All statistical analyses and plotted graphs were generated using the GraphPad Prism software version 9. Where applicable, error bars represent standard deviation and dots represent individual data points. At least three independent replicates were performed for each experiment where statistical analyses were conducted. Statistical analyses were performed using one-way ANOVA for groups of 3 or more and significance was calculated using Tukey’s multiple comparisons test. To compare between two groups, statistical significance was calculated using a two-tailed unpaired Student’s t test. For quantification of northern and western blot band signal intensities, data were normalised with respect to the signal intensity of the loading controls detected on the same blot. For quantification of scintillation counting, primer extension, northern blot signal intensities and [35S]–labelling experiments the values from each set of experimental replicates were normalised by mean.

Synthetic methods. Details of the chemical synthesis are provided in Supplementary methods.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The data that support this study are available from the corresponding authors upon reasonable request. The CRAC datasets for METTL8-His₅–2xFLAG and His₅–2xFLAG generated in this study, and their analyses are deposited in Gene Expression Omnibus (GEO) database under the accession code GSE185015. Sequencing reads were aligned with the human genome ensemble GCRh38.p13 release 104 [https://www.ensembl.org/index.html]. Source data are provided with this paper.

Code availability. Dedicated code used for the analysis of data presented in this manuscript are available within Supplementary Information.
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