NSUN3 and ABH1 modify the wobble position of mt-tRNA$^{\text{Met}}$ to expand codon recognition in mitochondrial translation

Sara Haag$^{1,\dagger}$, Katherine E Sloan$^{1,\dagger}$, Namit Ranjan$^{2,\ddagger}$, Ahmed S Warda$^{1,\dagger}$, Jens Kretschmer$^{1}$, Charlotte Blessing$^{1}$, Benedikt Hübner$^{1}$, Jan Seikowski$^{3,4}$, Sven Dennerlein$^{5}$, Peter Rehling$^{4,5,6}$, Marina V Rodnina$^{2}$, Claudia Höbartner$^{3,**}$ & Markus T Bohnsack$^{1,6,*}$

Introduction

More than a hundred different chemical modifications of ribonucleosides have been identified in cellular RNAs (Czerwoniec et al., 2009; Motorin & Helm, 2011). Modifications regulate the biogenesis, structure and function of the corresponding RNAs and RNA–protein complexes (RNPs). Many modifications occur in RNAs involved in translation and are therefore likely to affect protein synthesis. Several modified ribonucleosides including 6-methyladenosine (m$^6$A), 5-methylcytidine (m$^5$C), 1-methyladenosine (m$^1$A) and pseudouridine have recently been shown to occur in messenger (m) RNAs and to affect their biogenesis, translation and stability (see e.g. Carlile et al., 2014; Liu & Jia, 2014; Dominissini et al., 2016). Methylated nucleosides can undergo further modification and proteins of the AlkB family of alpha-ketoglutarate and Fe(II)-dependent dioxygenases (ALKBH1-8 and FTO in human cells) can oxidise or even remove modifications in DNA and RNA (Fedele et al., 2015; Ougland et al., 2015), increasing the dynamics and regulation of RNA modifications and their roles in RNA metabolism. Compared to mRNAs and other cellular RNAs, transfer (t)RNAs and ribosomal (r)RNAs contain the highest proportion of modified nucleosides. The large majority of tRNA modifications are already installed co-translationally by small nucleolar (sno)RNPs, and only few base modifications require the action of lone-standing enzymes (Watkins & Bohnsack, 2012; Sharma & Lafontaine, 2015). tRNAs contain the largest variety of nucleoside modifications, and many of them are suggested to affect tRNA biogenesis and nuclear export, tRNA structure, interaction with aminoacyl-tRNA-synthetases or codon recognition during translation (Agris et al., 2007; Leisegang et al., 2012; Hori, 2014; Duechler et al., 2016; Ranjan & Rodnina, 2016). Many tRNAs contain base modifications of the nucleoside at

Abstract

Mitochondrial gene expression uses a non-universal genetic code in mammals. Besides reading the conventional AUG codon, mitochondrial (mt-)tRNA$^{\text{Met}}$ mediates incorporation of methionine on AUA and AUU codons during translation initiation and on AUA codons during elongation. We show that the RNA methyltransferase NSUN3 localises to mitochondria and interacts with mt-tRNA$^{\text{Met}}$ to methylate cytosine 34 (C34) at the wobble position. NSUN3 specifically recognises the anticodon stem loop (ASL) of the tRNA, explaining why a mutation that compromises ASL basepairing leads to disease. We further identify ALKBH1/ABH1 as the dioxygenase responsible for oxidising m$^5$C34 of mt-tRNA$^{\text{Met}}$ to generate an fC34 modification. In vitro codon recognition studies with mitochondrial translation factors reveal preferential utilisation of m$^5$C34 mt-tRNA$^{\text{Met}}$ in initiation. Depletion of either NSUN3 or ABH1 strongly affects mitochondrial translation in human cells, implying that modifications generated by both enzymes are necessary for mt-tRNA$^{\text{Met}}$ function. Together, our data reveal how modifications in mt-tRNA$^{\text{Met}}$ are generated by the sequential action of NSUN3 and ABH1, allowing the single mitochondrial tRNA$^{\text{Met}}$ to recognise the different codons encoding methionine.

Keywords ABH1; mitochondria; NSUN3; RNA modification; translation

Subject Categories Protein Biosynthesis & Quality Control; RNA Biology

DOI 10.15252/embj.201694885 | Received 26 May 2016 | Revised 19 July 2016 | Accepted 20 July 2016 | Published online 5 August 2016

The EMBO Journal (2016) 35: 2104–2119

See also: F Boos et al (October 2016)
position 34 of the tRNA anticodon (the “wobble position”). These modifications modulate codon–anticodon basepairing, often allowing one tRNA to recognise several different nucleosides in the third position of the codon. Mutations in enzymes responsible for introducing these “wobble base” modifications or genetic alterations in tRNA sequences that affect such modifications are often associated with disease, especially in mitochondrial tRNAs (Lott et al., 2013; Powell et al., 2015).

One ribonucleoside modification that has been identified in several tRNAs, in both cytoplasmic and mitochondrial rRNA, in other non-coding RNAs and in mRNAs is 5-methylcytosine (m5C). m5C modifications can be installed by any of the seven proteins of the Nol1/Nop2/Sun domain (NSUN) family and by an enzyme named DNA methyltransferase 2 (DNMT2). DNMT2 mainly catalyses the mC modification in position 38 of tRNA^Apo in human cells (Goll et al., 2006), while the so far characterised NSUN proteins show specificity for tRNAs (NSUN2, NSUN6; Schaefer et al., 2010; Tuorto et al., 2012; Blanco et al., 2014; Haag et al., 2015a) or rRNA (NSUN1/NOP2, NSUN5; Sloan et al., 2013; Tafforeau et al., 2013; Schosserer et al., 2015). NSUN2 can also modify vault RNAs and mRNAs (Hussain et al., 2013), and NSUN4 was described to localise to mitochondria where it was shown to methylate the mitochondrial 12S rRNA in mice (Camara et al., 2011; Metodiev et al., 2014).

NSUN3 was one of the last uncharacterised members of the family, and we show here that this RNA methyltransferase localises to the mitochondrial matrix in human cells. Using in vivo UV cross-linking and analysis of cDNA (CRAC; Bohnsack et al., 2012) and 5-azacytidine (5-AzaC) analysis of cDNA (CRAC; Bohnsack et al., 2012; Sloan et al., 2015) we show that NSUN3 specifically interacts with the mitochondrial matrix in human cells. Using confocal fluorescence microscopy revealed that NSUN3-GFP localises to distinct cytoplasmic foci that showed co-localisation with a mitotracker (Fig 1A), indicating a mitochondrial localisation of NSUN3. To determine whether NSUN3 is imported into mitochondria, we performed protease protection assays using a tetracycline-inducible NSUN3-HisPreFLAG (Hexahistidine-PreScission protease cleavage site-2×FLAG tagged NSUN3) cell line. We isolated mitochondria that were then either left intact, subjected to swelling to rupture the outer mitochondrial membrane and generate mitoplasts or were disrupted using sonication before treatment with different concentrations of proteinase K. While treatment of intact mitochondria led to the degradation of the outer membrane protein TOM70, the intermembrane space domain of TIM23 was digested in mitoplasts. Similar to the matrix-localised domain of TIM44, NSUN3 only became susceptible to proteinase K digestion upon rupture of mitochondria by sonication (Fig 1B), indicating that NSUN3 is localised in the mitochondrial matrix in human cells.

**NSUN3 associates with mitochondrial tRNA\textsuperscript{Met}**

To identify NSUN3 target RNAs, we performed UV cross-linking and analysis of cDNA (CRAC; Bohnsack et al., 2012; Sloan et al., 2015)
experiments using the NSUN3-HisPrcFLAG cell line; a HEK293 cell line expressing only the HisPrcFLAG tag was used as a control. In addition, cells expressing NSUN3-HisPrcFLAG were treated with the cytidine derivative 5-azacytidine (5-AzaC) as a cross-linking reagent, which is incorporated into nascent RNA and specifically traps m^3^C RNA methyltransferases on their target nucleotides in a covalent protein–RNA intermediate during the methylation reaction (Fig 2A; Khoddami & Cairns, 2013). Without cross-linking or after UV or 5-AzaC cross-linking in vivo, protein–RNA complexes were purified followed by RNA trimming, radiolabelling and ligation of adapters to the bound RNA. Protein–RNA complexes were separated by SDS–PAGE, transferred to a membrane and exposed to an X-ray film. Both UV and 5-AzaC cross-linking of NSUN3-HisPrcFLAG resulted in a strong specific signal not observed for the controls (Fig 2B), indicating association of NSUN3 with cellular RNAs. Interacting RNAs were then extracted from the membrane and subjected to RT–PCR to generate a cDNA library for Illumina deep sequencing. Mapping of the obtained sequence reads on the human genome resulted in a strong over-representation of mitochondrial-encoded RNA (mt-RNA). mt-RNA represented 40% and 62% of total reads obtained upon UV or 5-AzaC cross-linking of NSUN3, respectively, compared to only 4% of sequence reads from the HisPrcFLAG control (Figs 2C–E and EV1A), suggesting a specific association of NSUN3 with mitochondrial RNA. As sequences from mitochondrial tRNAs were strongly enriched in the NSUN3-cross-linked fractions (Fig 2D and E, lower panels) compared to the control (Fig 2C, lower panel), we analysed the distribution of reads between the 22 mitochondrial tRNAs. Strikingly, 50 and 95% of the reads mapped to mt-tRNA^Met^ in the NSUN3 UV and 5-AzaC cross-linking experiments, respectively (Fig 2F). In contrast, the data obtained for the HisPrcFLAG control contained only 5% sequencing reads that mapped to mt-tRNA^Met^, indicating that NSUN3 specifically interacts with this tRNA (Fig 2F). To confirm the specificity of this interaction, we performed 5-AzaC cross-linking using cells expressing the HisPrcFLAG control, NSUN3-HisPrcFLAG and the catalytically inactive NSUN3(C265A)-HisPrcFLAG mutant, in which the catalytic cisteine of the TCT tripeptide that is conserved in motif IV in m^3^C methyltransferases of the NSUN family is replaced by alanine (C265A). After cross-linking and isolation of complexes via the FLAG-tagged proteins, interacting RNAs were analysed by Northern blotting using probes for the detection of the mitochondrial tRNAs mt-tRNA^Pro^, mt-tRNA^Glu^ and mt-tRNA^Met^ (Fig 2G). While mt-tRNA^Pro^ and mt-tRNA^Glu^ could not be detected in any of the eluates, mt-tRNA^Met^ was strongly enriched in the eluate from the NSUN3 wild-type sample, but was not detected in any of the controls, further supporting that mt-tRNA^Met^ specifically interacts with NSUN3. The specific requirement for the conserved catalytic cisteine and the efficient cross-linking of NSUN3 to 5-AzaC containing mt-tRNA^Met^ strongly suggest that NSUN3 is an active m^3^C RNA methyltransferase that uses the conserved mechanism of the NSUN family to mediate m^3^C methylation of its substrate mt-tRNA^Met^ in human mitochondria.

**NSUN3 specifically methylates cytosine 34 in mt-tRNA^Met^**

To gain further insight into the catalytic activity of NSUN3, we prepared recombinant NSUN3 protein and the catalytically inactive mutant (NSUN3-C265A) and performed in vitro methylation experiments using in vitro T7 RNA-polymerase transcripts of mt-tRNA^Met^, mt-tRNA^Pro^ and mt-tRNA^Glu^ in the presence of S-[3H-methyl] adenosylmethionine (SAM) as a methyl group donor. NSUN3 efficiently methylated mt-tRNA^Met^, but not the other transcripts, and the catalytic activity of NSUN3 was abolished by mutation of the catalytic cysteine (Fig 3A).

Besides the strong enrichment of reads from mt-tRNA^Met^ in the CRAC data sets, we had observed that reads mapping to the cytoplasmic tRNAs that mediate incorporation of methionine during translation initiation (tRNA^Met^) and elongation (tRNA^Met^) were over-represented in the NSUN3 cross-linking data (8% of reads mapped to cytoplasmic tRNA were tRNA^Met^ reads in FLAG control; 18% after UV and 79% after 5-AzaC cross-linking; Fig EV1B). We therefore tested whether NSUN3 could methylate transcripts of tRNA^Met^ and tRNA^Met^ in in vitro methyltransferase assays. While mt-tRNA^Met^ was methylated very efficiently by NSUN3, only very weak or no methylation was observed for the tRNA^Met^ and tRNA^Met^ transcripts, respectively (Fig EV1C). To analyse possible interactions between NSUN3 and tRNA^Met^ or tRNA^Met^ in vitro, we performed 5-AzaC cross-linking and immunoprecipitation experiments using HEK293 cells expressing the HisPrcFLAG tag alone, wild-type or mutant (C265A) NSUN3-HisPrcFLAG and analysed the co-precipitation of tRNAs by Northern blotting. While mt-tRNA^Met^ was strongly enriched with wild-type NSUN3, no association of the cytoplasmic tRNA^Met^ or tRNA^Met^ could be detected (Fig EV1D), indicating that NSUN3 does not specifically bind cytoplasmic tRNAs in vivo and that the interactions observed in the 5-AzaC CRAC likely occurred after cell lysis due to similar sequences of the anticodon stem loop of tRNA^Met^ and mt-tRNA^Met^ (Fig EV1E). Together with the mitochondrial localisation of NSUN3 (Fig 1), these data indicate that NSUN3 can weakly recognise the tRNA^Met^ as a substrate in vitro, but that mt-tRNA^Met^, rather than tRNA^Met^, represents its genuine methylation substrate in vivo.

In order to identify which region of mt-tRNA^Met^ interacts with NSUN3, we analysed the distribution of reads obtained by NSUN3 cross-linking to mt-tRNA^Met^. Analysis of both UV and 5-AzaC cross-linking experiments showed that the highest read density was obtained with sequences corresponding to the anticodon stem loop (ASL) of mt-tRNA^Met^ (Fig 3B) suggesting that the NSUN3 target residue lies within this region. As NSUN3 is a member of the cytosine methyltransferase family of NSUN proteins, we generated in vitro transcripts of mt-tRNA^Met^ in which each cytosine present in the ASL was individually mutated to an adenosine (ASL loop cytosines) or uracil (cytosines in the stem of the ASL; Fig 3C). Although mutation of several cytosines affected NSUN3-mediated methylation in in vitro methylation assays, only mutation of cytosine 34 abolished the modification (Fig 3D), suggesting that the C34 wobble nucleotide is the NSUN3 target in mt-tRNA^Met^. This conclusion was confirmed by a lack of methylation when chemically synthesised mt-tRNA^Met^ containing an m^3^C34 was treated with NSUN3 in methylation assays (Fig 3E), supporting the finding that NSUN3 generates an m^3^C moiety at position 34 in mt-tRNA^Met^.

Among the mt-tRNA^Met^ mutants (Fig 3D), the C39U mutant, which has previously been identified in patients with mitochondrial dysfunction (Lott et al., 2013; Tang et al., 2013), was a particularly poor substrate for NSUN3, suggesting that this residue might be critical for methylation or that a stable stem in the ASL could be required for NSUN3 recognition. To distinguish between these possibilities, we generated a series of ASL mutants where individual
cytosines in the stem were either replaced by uracil allowing for less stable G:U basepairing or mutants in which guanosine and cytosine in G:C basepairs were swapped between the strands of the stem, resulting in identical stability of the stem but a change in the sequence (Fig 4A). While no reduction in NSUN3 methylation was observed for the mutants generated by swapping the G:C basepairs in the stem, mutations to G:U basepairs reduced methylation efficiency and again almost abolished it for the C39U mutant (Fig 4B). These data indicate that a stable stem in the ASL is required for NSUN3 substrate recognition and methylation of C34 in mt-tRNAMet.
Incubation of synthesised mt-tRNA\textsuperscript{Met} ASL with NSUN3 in a methylation assay further revealed that the ASL is sufficient for recognition and methylation (Fig 4C).

Taken together, we have identified the mitochondrial tRNA\textsuperscript{Met} as the methylation substrate of the RNA methyltransferase NSUN3.

NSUN3 recognises the ASL of mt-tRNA\textsuperscript{Met} and requires a stable stem structure for substrate recognition and generation of m\textsuperscript{5}C34. Furthermore, a pathogenic mutation in the stem loop abolishes NSUN3-mediated modification, indicating that lack of modification of C34 can lead to disease.

**ALK8H1/ABH1 localises in mitochondria and specifically interacts with mt-tRNA\textsuperscript{Met}**

Previous reports suggested that mt-tRNA\textsuperscript{Met} can be modified at position 34 to contain a 5-formylcytosine (f\textsuperscript{5}C; Moriya et al, 1994; Suzuki & Suzuki, 2014). We hypothesised that a specific oxygenase might oxidise the m\textsuperscript{5}C34 moiety established by NSUN3 to generate an f\textsuperscript{5}C34 modification in mt-tRNA\textsuperscript{Met}. While the Ten-Eleven Translocation (TET) protein family of dioxygenases primarily mediates oxidation of m\textsuperscript{5}C in nuclear DNA and has also been implicated in
The interaction of ABH1 with mt-tRNA\textsuperscript{Met} suggests that it might mediate oxidation of m\textsuperscript{5}C34 in mt-tRNA\textsuperscript{Met}. We therefore radioactively methylated mt-tRNA\textsuperscript{Met} using \textsuperscript{3}H-methyl-labelled S-adenosylmethionine and recombinant NSUN3 and generated recombinant ABH1 and FTO for in vitro oxidation assays. The oxidation assays were performed in the presence of alpha-ketoglutarate and Fe\textsuperscript{2+} either without enzyme, with maltose binding protein (MBP), wild-type ABH1, the ABH1 alpha-ketoglutarate/Fe\textsuperscript{2+}-binding mutants R338A or D233A (Westbye et al, 2008), or FTO, and oxidation was monitored by measuring tritium release from the methyl group. Only wild-type ABH1 could oxidise m\textsuperscript{5}C34 in mt-tRNA\textsuperscript{Met} and the reaction required the presence of alpha-ketoglutarate and Fe\textsuperscript{2+} (Fig 6A), which further supports the notion that mt-tRNA\textsuperscript{Met} is a genuine substrate of ABH1 and that ABH1 utilises the conserved mechanism of the ALKBH family.

We next analysed whether the mt-tRNA\textsuperscript{Met} ASL alone is sufficient for in vitro recognition by ABH1 and oxidation of m\textsuperscript{5}C34. Indeed, m\textsuperscript{5}C34-containing mt-tRNA\textsuperscript{Met} ASL was efficiently oxidised by ABH1 (Fig 6B), allowing further characterisation of the oxidation product by HPLC. Treatment of chemically synthesised m\textsuperscript{5}C34-containing ASL with ABH1 resulted in almost quantitative oxidation of m\textsuperscript{5}C to 5-formylcytosine (\textit{f}5C). The presence of \textit{f}5C was confirmed by mass spectrometry and by the efficient conversion in a 5-formylpyrimidine-specific reaction with the trimethylindol derivative TMI (Fig 6C; Samanta et al, 2016). Upon treatment of the oxidation product with NaBH\textsubscript{4}, \textit{f}5C was chemically reduced to 5-hydroxymethyl-cytosine (hm\textsuperscript{5}C), which did not react with TMI (Fig 6C). Coinjection of mt-tRNA\textsuperscript{Met} ASL derivatives further confirmed that the different oxidation states can be distinguished by HPLC (Fig 6D).

To understand how the modification state of C34 in mt-tRNA\textsuperscript{Met} affects its function in translation, we studied codon recognition by mt-tRNA\textsuperscript{Met} variants on the ribosome. As a reconstituted mitochondrial in vitro translation system is not readily available, we tested binding of different modification states of mt-tRNA\textsuperscript{Met} in the presence of purified recombinant human mitochondrial translation factors on ribosomes from \textit{Escherichia coli}. Even though the structure of bacterial and mitochondrial ribosomes is significantly different, the structure of the decoding centre of the ribosome is highly conserved (reviewed in Greber & Ban, 2016), allowing mitochondrial translation factors to bind to the conserved sites of bacterial ribosomes. To mimic codon recognition during translation initiation, we used recombinant human mitochondrial initiation factor 2

Figure 5. ABH1 localises to mitochondria in HEK293 cells and specifically interacts with mt-tRNA\textsuperscript{Met}.

A The localisation of ABH1 was analysed by immunofluorescence in HEK293 cells. ABH1 (green) localisation and mitochondria stained with Mitotracker (red) are shown separately and in an overlay with DAPI to indicate nuclei. The scale bar represents 5 μm.

B HEK293 cells expressing ABH1-HisPrcFLAG (ABH1) or the HisPrcFLAG tag alone (FLAG) were UV cross-linked (UV), and protein–RNA complexes were affinity purified. Co-precipitated RNA was isolated and analysed by Northern blot using probes against mt-tRNA\textsuperscript{Met} and mt-tRNA\textsuperscript{Glu}. Inputs (0.1%) are shown on the left and eluates (50%) on the right.
MTIF2, which recruits mt-tRNA\textsubscript{Met} to the P site of the ribosome. Codon recognition during the elongation phase was studied with the human mitochondrial translation elongation factor TUFM, which delivers the tRNA to the A site. We used chemically synthesised mt-tRNA\textsubscript{Met} containing either unmodified C34, m5C34, hm5C34 or f5C34 aminoacylated with \([14C]\)Met. Ribosomes programmed with mRNAs presenting AUG, AUA or AUU codons in the P or A site were mixed with MTIF2–GTP or TUFM–GTP and one of the mt-tRNA\textsubscript{Met} variants. mt-tRNA\textsubscript{Met}–ribosome complexes were retrieved by nitrocellulose filtration and quantified by scintillation counting. The universal AUG codon or the AUA codon in the P site was preferentially recognised by the m5C34-modified mt-tRNA\textsubscript{Met} (Fig 7A). Binding to the ribosomes containing an AUU codon in the P site was generally lower and less specific with respect to mt-tRNA\textsubscript{Met} modification.

**Figure 6.** ABH1 can oxidise m\textsuperscript{C}34 in mt-tRNA\textsubscript{Met} in vitro.

A In vitro-transcribed mt-tRNA\textsubscript{Met} was methylated at C34 using recombinant NSUN3 and \([3H]\)-labelled S-adenosylmethionine as a methyl group donor. Radiolabelled mt-tRNA\textsubscript{Met} was re-extracted and then subjected to oxidation assays without protein (−), with maltose binding protein (MBP), with the dioxygenase FTO or using wild-type (ABH1) or mutant (R338A, D233A) His\textsubscript{14}–MBP–ABH1. Besides ABH1 controls lacking \(\alpha\)-ketoglutarate (\(\alpha\)KG) or Fe\textsuperscript{2+} ions, all samples contained \(\alpha\)-ketoglutarate and Fe\textsuperscript{2+} ions. After oxidation, RNA was precipitated and the tritium released upon oxidation of radiolabelled mt-tRNA\textsubscript{Met} was quantified in the supernatant. Counts per minute (CPM) are shown for experiments performed in triplicate with error bars indicating ± SD (upper panel). Pelleted RNA was separated on a denaturing polyacrylamide gel and exposed to an X-ray film to analyse the tritium retained ([3H]-Me).

B Synthetic anticodon stem loop (ASL) was radioactively labelled and subjected to oxidation assays that were performed and analysed as described in (A) using no protein (−), MBP or wild-type His\textsubscript{14}–MBP–ABH1 (ABH1). Experiments were performed in triplicate with error bars indicating ± SD.

C Anion exchange HPLC analysis was performed on synthetic m\textsuperscript{C}34-containing ASL (20 nt) before and after oxidation by ABH1. The small shift in retention time indicates formation of f\textsuperscript{C}-modified RNA. The ABH1 oxidation product was then treated with NaBH\textsubscript{4} to generate hm\textsuperscript{C}34-modified RNA. All three samples were analysed by ESI-MS, and the molecular weight (m.w.) is indicated on the HPLC trace. Only the f\textsuperscript{C}-containing RNA was labelled efficiently with 1-ethyl-2,3,3-trimethylindoleninium-5-sulphonate (TMI).

D The different retention times of m\textsuperscript{C}, hm\textsuperscript{C} and f\textsuperscript{C}-modified ASL RNA were confirmed by co-injection of samples shown in (C). HPLC was performed as in (C).
Controls showed only weak binding of mt-tRNAMet in the absence of ribosomes or mRNA independent of the modification status of the tRNA, indicating that the observed differences in the P site binding are due to specific recognition of these codons by mt-tRNAMet in complex with MTIF2. Also the TUFM-dependent decoding in the A site was generally more efficient on AUG than on AUA codons (Fig 7B). The modified and unmodified mt-tRNAMet variants were capable of reading the AUG codon. Notably the mC34-modified mt-tRNAMet was less efficient than other variants in AUG decoding, while AUA was read with similar efficiencies by all variants of mt-tRNAMet. Together these data indicate that the modification state of C34 in mt-tRNAMet influences codon recognition by the tRNA in the P and A site, with mC acting as a predominant decoder during initiation at AUG and all tRNAs capable of decoding during elongation. We note that the kinetics of decoding may be different depending on the modification and thus some mt-tRNAMet variants may be kinetically preferred over the others. However, a kinetic analysis of decoding upon initiation and elongation is beyond the scope of the present work.

Different modification states of cytosine 34 occur in mt-tRNAMet in vivo

The cross-linking and in vitro modification data show that cytosine 34 in mt-tRNAMet can be methylated by NSUN3 to generate mC and then further oxidised by the dioxygenase ABH1 to fC. In addition, these different modifications in mt-tRNAMet may influence codon recognition. To gain insight into the occurrence of the mt-tRNAMet modification states in vivo, we first established RNAi-mediated depletion of NSUN3 and ABH1 (Fig 8A). After siRNA treatment, analysis of mRNA levels showed an ~80% decrease in NSUN3 or ABH1 mRNA levels. Equal amounts of RNA extracted from knockdown cells were then treated with the 5-formylpyrimidine-specific TMI to convert fC into a hemicyanine derivative, which blocks primer extension by reverse transcriptase at the site of modification, thereby allowing to analyse the presence of fC in the RNA (Samanta et al., 2016). Primer extension analysis revealed that the fraction of fC34-containing mt-tRNAMet decreased by more than three-fold (NSUN3) and more than four-fold (ABH1) when NSUN3 and ABH1 were depleted (Fig 8B and C), confirming the roles of these enzymes in establishing the modification in vivo. To identify the presence of other modification states at C34, RNA from wild-type cells or cells transfected with non-target siRNAs or those targeting NSUN3 or ABH1 was first subjected to DNase digest and then treated with bisulphite. Alternatively, the DNase digest was followed by chemical reduction of the RNA with NaBH4 to convert fC to mC and bisulphite treatment. In both cases, after deamination and desulphonation, mt-tRNAMet was specifically amplified by reverse transcription and PCR and then cloned. Analysis of sequences derived from wild-type RNA after reduction indicated that although the majority of these modifications are fC, a portion of cytosines at this position are not converted by the bisulphite treatment, indicating that they carry the mC34 modification installed by NSUN3 (Fig 8D). Consistent with these data, depletion of NSUN3 resulted in a decrease in the mt-tRNAMet fraction carrying a modification on C34 and a decrease in the portion of mC, while upon depletion of ABH1,

© 2016 The Authors

Published online: August 5, 2016

Sara Haag et al

mt-tRNAMet wobble base modification

The EMBO Journal

Vol 35 | No 19 | 2016
position 34 was almost exclusively read as cytosine independent of whether the RNA had been reduced. These results confirm methylation of C34 by NSUN3 and further show that the ABH1 knock-down abolishes the formation of f5C34 in mt-tRNAMet in vivo. We note that the bisulphite data do not rule out the presence of hm5C, which is also resistant to bisulphite conversion. Oxidative bisulphite sequencing, which can distinguish m5C and hm5C in DNA (Booth et al., 2013), resulted in degradation of the RNA. However, hm5C was not observed upon ABH1 oxidation in vitro and had no beneficial effect in ribosome binding assays, suggesting that this modification might not play a major role for mt-tRNAMet.

**Modifications of cytosine 34 in mt-tRNAMet are required for mitochondrial translation in vivo**

Our finding that the mt-tRNAMet C39U mutation, which has previously been identified in patients with mitochondrial dysfunction (Lott et al., 2013; Tang et al., 2013), largely abolishes mC34 formation by NSUN3, suggests that the C34 modification is required for mt-tRNAMet function in vivo and that mt-tRNAMet malfunction might cause the disease in these patients. To analyse the requirement for the modifications installed by NSUN3 and ABH1 for translation in mitochondria, we measured the amount of 35S-methionine incorporated into proteins during mitochondrial translation in vivo after depletion of NSUN3 or ABH1. Indeed, depletion of either NSUN3 or ABH1 resulted in reduced 35S-incorporation, suggesting that the modifications installed by these proteins are required for mt-tRNAMet function. Close inspection of the individual synthesis rates of the mitochondrial proteins revealed that the translation of all mitochondrial proteins was affected by NSUN3 or ABH1 depletion, which is in line with the presence of both AUG and non-canonical codons encoding methionine (AUA, AUU) in all of these mRNAs. Moreover, we observed that cell growth was affected by knock-down of either NSUN3 or ABH1 (Fig EV3), further supporting the important roles of the modifications installed by these enzymes for mitochondrial function and the cellular metabolism.

**Discussion**

Expression of the mitochondrial genome is fundamental in eukaryotes for maintaining the cellular energy metabolism and various
metabolic pathways. The human mitochondrial DNA encodes 13 mRNAs that are translated on mitochondrial ribosomes to generate proteins of the respiratory chain complexes, which are essential for oxidative phosphorylation. The mitochondrial protein synthesis machinery employs a minimalistic set of 22 mitochondrial tRNAs and, even though they contain a reduced number of modified residues compared to their cytoplasmic counterparts, mitochondrial tRNAs possess multiple RNA modifications that require import of the corresponding modification enzymes from the cytoplasm (Watanabe & Yokobori, 2011; Suzuki & Suzuki, 2014; Powell et al., 2015). The largest diversity of modifications in these tRNAs occurs in and around the anticodon, especially at the wobble position. This coincides with the extreme reduction in isoacceptors, requiring most tRNAs to act in decoding of several different codons, and with specific mitochondrial changes in the universal genetic code. Despite the importance of the RNA modifications for mitochondrial translation and physiology, many of the modification pathways, the enzymes involved and the roles of these modifications in mitochondrial translation have remained unknown so far.

Here, we describe the biosynthetic pathway that introduces modifications at the wobble position of the mitochondrial tRNAMet. We show that the RNA methyltransferase NSUN3 efficiently methylates C34 of mt-tRNAMet to produce m5C, which can then be oxidised by the alpha-ketoglutarate and Fe(II)-dependent dioxygenase ALKBH1/ABH1. Mammalian mt-tRNAMet can be modified to f5C at the wobble position, and our data demonstrate that this modification is introduced in vivo by the consecutive action of NSUN3 and ABH1 (Fig 9C). Interestingly, the bisulphite sequencing data further suggest that after methylation by NSUN3 only a part of the mitochondrial pool of mt-tRNA Met is oxidised by ABH1, indicating the presence of m5C34-containing mt-tRNA Met in vivo.

The ability of ABH1 to oxidise m5C to f5C is striking with respect to the previously described substrate specificity of this oxygenase enzyme. ABH1 can demethylate single-stranded DNA and RNA in vitro with low efficiency, with a preference for oxidation of N3-methylcytosine (m3C) (Westbye et al., 2008), and has been suggested to act as histone demethylase and abasic site lyase (Müller et al., 2010; Ougland et al., 2012). While the homologous E. coli AlkB cannot oxidise m5C in vitro (Li et al., 2010) and human ALKBH2 and ALKBH3 preferentially repair alkylations at nucleobase heteroatoms such as m3C and 1-methyladenosine (m1A) (Aas et al., 2003; Falnes et al., 2004), the oxidation of m5C involves transformation of a pseudobenzylcylic methyl group. In DNA, this reaction is catalysed by related Fe(II)/α-ketoglutarate-dependent oxygenases of the TET enzyme family, and the oxidation products play a significant role in epigenetic regulation in mammals (Tahiliani et al., 2009; Breiling & Lyko, 2015; Li et al., 2015). The TET enzymes produce hm5C as primary stable oxidation product, which can be further oxidised to f5C and 5-carboxycytosine (caC), although these higher oxidation products are 10- to 100-fold less abundant than hm5C in DNA and are mainly linked to active demethylation (Ito et al., 2011; Pfaffender et al., 2011; Wagner et al., 2013). In RNA, the analogous oxidation of m5C to hm5C has been reported by catalytic domains of mammalian TET enzymes (Fu et al., 2014) and the homologous Drosophila protein dTET (Delatte et al., 2016). f5C was detected as minor oxidation product in total cellular RNA by mass spectrometry-based isotope tracing (Huber et al., 2015), but the enzymes generating this modification have remained unknown. The observation that oxidation products of m5C have been detected in RNA from all domains of life, including organisms that do not contain homologous TET enzymes, suggests that m5C can be metabolically oxidised by enzymes other than those of the TET family. We have identified ABH1 as the first such enzyme that produces f5C in human mitochondria. Under the conditions tested, f5C was the only oxidation product detected in vitro; hm5C did not accumulate as intermediate and no further oxidation to caC was detected. In the absence of a three-dimensional structure of ABH1, the molecular reasons for the apparent specificity of ABH1 for the biosynthesis of f5C remain unknown. With the broad target spectrum reported for ABH1, it will also be interesting to understand on the structural level how this enzyme can accommodate interactions with diverse protein and RNA substrates and modulate their modification state.

In mitochondria, NSUN3 and ABH1 act on mt-tRNA Met, which represents the only tRNA Met that acts both in translation initiation and elongation, in contrast to bacterial and eukaryotic cytoplasmic translation systems. Besides reading the universal AUG codons, mt-tRNA Met is employed for decoding of AUA codons during initiation and elongation, as well as an AUU initiation codon in the case of the NADH dehydrogenase 2 (ND2) mRNA. Our data obtained with synthetic aminoacylated mt-tRNA Met containing unmodified C34, m3C34, hm5C34 or f5C34 and the human mitochondrial translation initiation factor MITF2 reveal that the presence of the m5C modification in the wobble position enhances codon reading of the AUG and, to a lesser extent, AUU initiation codons in the P site of the ribosome, suggesting a specific role of m5C34 modification during translation initiation. The AUU initiation codon, which is only present in the ND2 mRNA, is recognised, albeit poorly, by non-modified or f5C-modified mt-tRNA Met.

The recognition efficiency of the AUU and AUU initiation codons is low, consistent with the previous results obtained with mt-tRNA Met anticodon stem loop (Bilbille et al., 2011). However, given that translation in mitochondria is generally slow and the mRNA recruitment for translation often relies on protein factors specific for each mRNA (Kuzmenko et al., 2014), it is conceivable that even weak codon-anticodon interaction with mt-tRNA Met may be sufficient to start translation. While m5C-modified mt-tRNA Met preferentially acts in translation initiation, results from A site binding studies in the presence of the mitochondrial elongation factor TUFM suggest that mt-tRNA Met variants other than m5C34 are more efficient in decoding of the internal AUG codons during translation elongation. In combination with the generally lower efficiency of the alternative codons in the in vitro binding assays, only small differences between the binding of the unmodified, m5C- or f5C-modified mt-tRNA Met to AUU codons in the ribosomal A site were observed, while the binding of hm5C-containing mt-tRNA Met was less efficient. Previous reports with the unmodified or f5C34-containing ASL of mt-tRNA Met suggested that the formyl group might stabilise the non-conventional basepairing of f5C34 with an adenosine in the third position of an AUG codon (Bidbille et al., 2011; Cantara et al., 2013). These studies also observed that binding of mt-tRNA Met to alternative codons was weaker than to AUG and it is likely that ribosome interactions with mt-tRNA Met outside of the ASL, that is with the tRNA body, further influence mt-tRNA Met binding.
Together, our data indicate that the different modification states of cytosine 34 in mt-tRNAMet can expand the ability of the single tRNAMet to read the different codons encoding methionine in mitochondrial translation initiation and elongation.

The modification state of C34 in mt-tRNAMet is controversially discussed in the literature and two reports that were published while this manuscript was under consideration find different levels of NSUN3-dependent m\textsuperscript{5}C34 and f\textsuperscript{5}C34 in human mt-tRNAMet (Nakano et al., 2016; Van Haute et al., 2016). Our findings imply that in vivo a large fraction of the m\textsuperscript{5}C34-containing mt-tRNAMet is oxidised by ABH1, which is in line with previous reports that found the f\textsuperscript{5}C34 modification in mt-tRNAMet (Moriya et al., 1994; Takemoto et al., 2009; Suzuki & Suzuki, 2014). However, we also observed that mt-tRNAMet carrying m\textsuperscript{5}C34 is present in vivo, which is supported by findings of Van Haute et al. (2016), and that this modification state of mt-tRNAMet is efficiently recruited to the P site of the ribosome in vitro. Importantly, mutations in mt-tRNAMet itself have been shown to cause severe mitochondrial disorders (Lott et al., 2013; Tang et al., 2013) and we found that one such mutation (C39U), which leads to destabilisation of the anticodon stem structure, largely abolishes mt-tRNAMet methylation by NSUN3. These results indicate that NSUN3 malfunction and a lack in mt-tRNAMet modification might represent the molecular cause of such diseases. An important role of the modifications installed in mt-tRNAMet by NSUN3 and ABH1 is further supported by our findings that knock-down of either NSUN3 or ABH1 affects mitochondrial translation and leads to reduced cell survival. While mt-tRNAMet likely represents the only substrate of NSUN3, ABH1 has a broader target spectrum and its depletion might also influence other molecules affecting mitochondrial translation. Interestingly, previous reports have suggested a differential localisation of ABH1 in different cell types. While the dioxygenase is mainly localised in mitochondria and the cytoplasm in HEK293 and HeLa cells (Fig 5; Westbye et al., 2008), it has been reported to be nuclear in embryonic stem cells (Ougland et al., 2012, 2016). Together, these findings suggest that the methylation of cytosine 34 in mt-tRNAMet by NSUN3 represents an important modification present in many, if not all cell types, while the different localisation of ABH1 might result in differential modification of mt-tRNAMet on cytosine 34 in different cell types, tissues and developmental stages and might thereby fine tune mitochondrial translation in vivo.
Materials and Methods

Human cell culture, stable cell lines and in vivo cross-linking

HeLa CCL2 and HEK293 Flp-In T-Rex cells (Life Technologies) were cultured with 5% CO2 at 37°C in DMEM supplemented with 10% FCS and 2 mM glutamine. For generation of tetracycline-inducible stable cell lines the NSUN3 or ABH1 CDS were cloned into the pcDNAs vector with C-terminal GFP or His-PreScission protease cleavage site-2×FLAG (HisPreFlag) tag. The catalytically inactive NSUN3 C265A mutant was generated by site-directed mutagenesis (Haag et al., 2015b). The constructs were transfected into HEK293 Flp-In T-Rex cells according to the manufacturer’s instructions and as described (Sloan et al., 2015). UV and 5-AzaC cross-linking and analysis of cDNA (CRAC) experiments were carried out as previously described (Bohnsack et al., 2012; Haag et al., 2015a; see also Appendix Supplementary Methods). Detection of co-immunoprecipitated tRNA by Northern blot was performed as previously described (Haag et al., 2015a). In brief, after cross-linking and immunoprecipitation of protein–RNA complexes the RNA was eluted by protease K digestion for 16 h, precipitated and resuspended in loading dye (95% formamide, 5 mM EDTA, bromophenol blue). The RNA was separated on a denaturing 12% polyacrylamide gel (7M urea), transferred to a nylon membrane and selected tRNAs were detected by Northern blotting using specific 32P-5’ end-labelled probes (anti-mt-tRNAMet, anti-mt-tRNAeGlu, anti-mt-tRNAePro, anti-tRNAiMet, anti-tRNAeMet; Appendix Table S1) on a phosphorimager.

Microscopy, isolation of mitochondria and protease protection assays

HEK293 cells expressing NSUN3-GFP under the control of a tetracycline-inducible promoter were selected and NSUN3-GFP expression was induced by 1 µg/mL doxycycline treatment for 24 h. Cells were treated with MitoTracker® Orange CMTMRos (Life Technologies) in PBS for 20 min at 37°C, washed in PBS and fixed with 4% formaldehyde in PBS for 10 min at room temperature. After washing with PBS, cells were mounted on coverslips using Vectashield® (Vector labs) for confocal microscopy and localisation analysis. Alternatively, immunofluorescence using an antibody against ABH1 (see Appendix Table S2) was performed as previously described (Haag et al., 2015a). Isolation of mitochondria, analysis of submitochondrial localisation and protease protection assays were performed as described using the antibodies listed in Appendix Table S2 (Dennerlein et al., 2015).

RNA interference, RNA isolation and qRT–PCR

HeLa CCL2 cells were transfected with siRNAs (40 nM) targeting NSUN3 (siNSUN3_1, siNSUN3_2) or ABH1 (siABH1_1, siABH1_2) or a non-target siRNA (siNT) using Lipofectamine RNAiMax (Life Technologies) according to the manufacturer’s instructions. Cells were harvested 96 h after siRNA transfection and total RNA was isolated using TRI reagent (Sigma-Aldrich). The knock-down efficiency was determined by qRT–PCR and relative quantification was performed using primers for NSUN3 (NSUN3_qPCR_fwd, NSUN3_qPCR_rev), ABH1 (ABH1_qPCR_fwd, ABH1_qPCR_rev) and GAPDH (GAPDH_qPCR_fwd, GAPDH_qPCR_rev; for siRNA and primer sequences see Appendix Table S1 and S3).

NaBH4 treatment and bisulphite reaction

To analyse the cytosome modification status of mt-tRNAMet, DNase I treated total RNA from wild-type, NSUN3 or ABH1 knock-down cells was either directly subjected to bisulphite sequencing (Schafer et al., 2009) or treated with 0.25 M NaBH4 in 200 mM Tris–HCl pH 7.5, 20 mM MgCl2, 200 mM KCl for 30 min on ice and precipitated prior to the bisulphite reaction. Reduced or untreated RNA was bisulphite treated using the QiaGen bisulphite kit according to the manufacturer’s instructions. The deamination reaction was carried out in a thermocycler with 5 min at 70°C, 60 min at 60°C (3 times). Samples were desalted using 6×SSC Micro bio spin chromatography columns and subsequently desulphonated by incubation in Tris pH 9 for 30 min at 37°C. The RNA was precipitated and reverse transcribed using the mt-tRNAMet RT primer and Superscript III reverse transcriptase (Thermo) according to the manufacturer’s instructions. PCR products were then cloned using a TOPO-TA kit (Thermo) and sequenced. At least 50 sequences were analysed per sample and only sequences in which all other cytosines besides C34 in mt-tRNAMet were converted were used for the analysis presented.

Cloning and recombinant expression of proteins and in vitro transcription of tRNAs

The coding sequences of human NSUN3, ABH1 or FTO were cloned into a pQE80 derivative encoding an N-terminal His14-MBP-tag (Weis et al., 2014) and the CDS of MTIF2 or TUFM into a pQE80 derivative encoding a C-terminal His10-tag (Mingot et al., 2004). The ABH1 D233A and R233A, and NSUN3 C265A mutants were generated by site-directed mutagenesis (Haag et al., 2015b). Recombinant proteins were expressed in Escherichia coli (DE3) Rosetta pLysS (NSUN3, ABH1) or (BL21) Codon Plus (MTIF2, TUFM) cells and details of protein purification are given in the Appendix Supplementary Methods. Mt-tRNAMet, mt-tRNAeGlu, mt-tRNAePro, tRNAiMet and tRNAeMet sequences were generated by recursive PCR as described (Müller et al., 2013) using four overlapping oligonucleotides each and cloned into a pQE vector derivative lacking an internal T7 promoter. The CCA tail and a BsII restriction site were added at the 3’ end of the tRNA gene and the forward primer contained the sequence of the T7 promoter. Point mutations were introduced by site-directed mutagenesis. For in vitro transcription, 500 ng of BsI-linearised plasmid were incubated with 1 mM NTPs, T7-RNA polymerase, 1× transcription buffer (Thermo) and RiboLock (Thermo) for 1 h at 37°C. After transcription, samples were treated with DNase I for 15 min and purified over a Sephadex G-25 spin column (Roche).

Preparation of synthetic tRNAs and ribosome binding assays

RNA oligonucleotides were prepared by solid-phase synthesis using 2’-O-TOM-protected ribonucleotide phosphoramidites, chemically phosphorylated on solid support, deprotected in two steps with methylamine in water/ethanol, followed by 1 M tetrabutyrammonium fluoride in tetrahydrofuran, purified by denaturing PAGE, and analysed by analytical anion exchange chromatography under
denaturing conditions (6M urea, 80°C) and ESI-MS. Synthetic tRNAs were prepared by enzymatic ligation of chemically synthesised RNA fragments using T4 DNA ligase and DNA splint oligonucleotides (2–5 nmol scale, incubation at 30°C for 12 h), analogous to previously reported procedures (Rieder et al., 2009). The full-length tRNAs were isolated by denaturing PAGE, extracted into Tris–NaCl buffer, precipitated with ethanol and re-dissolved in water. To generate 5F34 or hm5C-containing mt-tRNA\textsubscript{Met} ligation was performed with m\textsuperscript{5}C34-ASL RNA oligonucleotides that were treated with recombinant ABH1 on preparative scale (5–10 nmol) (see oxidation assays for conditions), or treated with ABH1 and then reduced with NaBH\textsubscript{4} (see NaBH\textsubscript{4} treatment). The modified ASLs oxidation assays for conditions), or treated with ABH1 and then reduced with NaBH\textsubscript{4} (see NaBH4 treatment). The modified ASLs were PAGE purified and their homogeneity and identity were confirmed by anion exchange HPLC and ESI-MS. Labelling of 5F34-RNA with 1-ethyl-2,3,3-trimethylindoleninium-5-sulphonate (TMI) and analysis of primer extension stops on sequencing gels were performed as described (van Nues et al., 2011; Samanta et al., 2016). Ribosome binding assays were performed as described (Rezgui et al., 2013; see also Appendix Supplementary Methods).

In vitro methylation and oxidation assays

Methylation of RNAs was carried out essentially as described (Jurkowski et al., 2008; Müller et al., 2013). Reactions containing 1 μM recombinant NSUN3 and 1 μM of RNA or 10 μg of total RNA in 1× methylation buffer (50 mM Tris–HCl pH 7.0, 5 mM NaCl, 5 mM MgCl\textsubscript{2}, 1 mM DTT) and 1.7 μM 5F34-SAM (Hartmann), 1 unit/ml RiboLock (Thermo) were incubated at 22°C for 2 h. After addition of protease K for 30 min to stop the reaction, RNAs were separated on a 12% denaturing (7 M urea) polyacrylamide gel, stained with ethidium bromide, fixed and immersed in amplification solution (Amersham) for 1 h. After drying, the gel was exposed to a X-ray film for 16 h to 2 weeks at –80°C. For in vitro oxidation reactions mt-tRNA\textsubscript{Met} or mt-tRNA\textsubscript{Met}\textsuperscript{5mC} ASL were labelled with [3H]-containing methyl group by in vitro methylation with NSUN3. The methylated RNA was precipitated and incubated with 1 μM recombinant wild-type or mutant His\textsubscript{14}\textsubscript{G}-MBP-ABH1, MBP or His\textsubscript{14}\textsubscript{G}-MBP-FTO in the presence of 50 mM HEPES pH 6.9, 5 mM MgCl\textsubscript{2}, 4 mM ascorbic acid and 100 μM Fe(NH\textsubscript{4})\textsubscript{2}(SO\textsubscript{4})\textsubscript{2} and 100 μM α-ketoglutarate for 1 h at 22°C. The reaction was stopped by addition of proteinase K and the RNA was precipitated. The supernatant containing released [3H] was analysed by scintillation counting, and the corresponding RNA pellets were separated by denaturing gel electrophoresis and analysed as described for the methylation assay. Preparative scale oxidation of synthetic m\textsuperscript{5}C34 ASL for preparation of mt-tRNA\textsubscript{Met}\textsuperscript{5mC} by ligation was performed under analogous conditions, followed by PCI extraction and PAGE purification.

In vivo analysis of mitochondrial translation

In vivo labelling was performed as previously described (Chomyn, 1996). HeLa cells were transfected with siRNAs (non-target, NSUN3 or ABH1) and cultivated for 72 h. Before labelling, cells were starved in medium lacking serum and methionine. Cytosolic translation was inhibited by treating cells with 100 μg/ml emetine (Sigma-Aldrich) for 10 min. Translation of mitochondrial proteins was pulsed with 0.2 mCi/ml \textsuperscript{13}S methionine for 30 min. Cells were harvested, and proteins were separated on a 10–18% Trisc–SDS–PAGE followed by transfer onto a PVDF membrane and exposure to a phosphor screen. Autoradiography signals were measured by a phosphorimager (Typhoon FLA 9500) and quantified by Imagequant TL software (GE Healthcare). Equivalent amounts of samples were run on SDS–PAGE for fluorescent Western blot analysis for normalisation.

Data availability

The primary high-throughput sequencing data of the UV and 5-azacytidine cross-linking and analysis of cDNA (CRAC) experiments have been submitted to the GEO SRA database and assigned the identifier GSE84664.

Expanded View for this article is available online.

Acknowledgements

We thank Philipp Hackert for technical assistance and Sebastian Leidel for helpful discussions. This work was supported by the Deutsche Forschungsgemeinschaft (SP 1784: BO3442/2-1 to M.T.B., HO4436/2-1 to C.H.; SF81190 to M.T.B., M.V.R. and P.R.), the Alexander von Humboldt Foundation (postdoctoral fellowship to K.E.S.), European Research Council (AdG No. 339580) to P.R., the Faculty of Medicine, Georg-August-University Göttingen (M.T.B., P.R. and "Startförderung" to S.H.) and the Max Planck Society (M.V.R., C.H and P.R.).

Author contributions

SH, KES, CB, ASW, BH and MTB purified proteins and performed methyltransferase and oxidation assays; SH and KES performed and analysed cross-linking experiments; SH, KES and CB did bisulphite treatment and analysis; KES, JK and MTB performed bioinformatics analysis; ASW, SD and PR analysed NSUN3 and ABH1 localisation; CH, SH, JS and KES synthesised and analysed RNAs; NR and MVR designed and performed ribosome binding assays; ASW, SD, KES, SH and PR performed RNAi and mitochondrial translation assays; PR, MVR, CH and MTB designed the study and analysed data; MTB, CH and KES wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References


Hori H (2014) Methylated nucleosides in tRNA and tRNA methyltransferases. *Front Genet* 5: 144


Potentially interesting information from the given text:

- Ranjan N, Rodnina MV (2016) tRNA wobble modification and protein homeostasis. Translation 4: e1143076

License: This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs 4.0 License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.