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

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Abstract

Mitochondrial gene expression uses a non-universal genetic code in mammals. Besides reading the conventional AUG codon, mitochondrial (mt-)tRNA^{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^{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^{Met} to generate an f^{5}C34 modification. *In vitro* codon recognition studies with mitochondrial translation factors reveal preferential utilisation of m^{5}C34 mt-tRNA^{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^{Met} function. Together, our data reveal how modifications in mt-tRNA^{Met} are generated by the sequential action of NSUN3 and ABH1, allowing the single mitochondrial tRNA^{Met} to recognise the different codons encoding methionine.

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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 (Fedeles *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 rRNA modifications are already installed co-transcriptionally 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-sythetases 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

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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 (m^5C). m^5C 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 m^5C modification in position 38 of tRNA^{Asp} 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 (Cámara *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) and 5-azacytidine (5-AzaC) CRAC, we show that NSUN3 specifically interacts with the mitochondrial tRNA^{Met} where it is responsible for introducing a 5-methylcytosine (m^5C) modification at the "wobble position". In addition, we find that the m^5C modification can be further oxidised by the alpha-ketoglutarate and Fe(II)-dependent dioxygenase ALKBH1/ABH1, generating a 5-formylcytidine (f^5C) at this position. Analysis of mt-tRNA^{Met} synthesised with the different cytosine modifications in the wobble position revealed that codon recognition in an *in vitro* translation system utilising mitochondrial initiation and elongation factors depends on the modification state of C34 in mt-tRNA^{Met}. *In vivo*, knock-down of ABH1 abolishes f^5C34 formation, while depletion of NSUN3 leads to a decrease in mt-tRNA^{Met} modification. Furthermore, reducing the levels of either NSUN3 or ABH1 leads to a significant decrease in mitochondrial translation *in vivo*, suggesting important roles for the modifications installed by the two enzymes in mt-tRNA^{Met} function. Interestingly, our data also show that NSUN3 requires the anticodon stem loop for substrate recognition and a pathogenic mutation in the ASL abolishes C34 methylation, implying that lack of this modification can lead to disease.

Results

NSUN3 localises to the mitochondrial matrix

More than 10 years ago computational analysis identified NSUN3 as a member of the Nol1/Nop2/Sun domain (NSUN) family of putative m^5C RNA methyltransferases (Bujnicki *et al*, 2004). NSUN3 was suggested to localise to mitochondria (Rhee *et al*, 2013); however, the target spectrum and biological function of the protein have remained unknown. To confirm the mitochondrial localisation of NSUN3, we generated a HEK293 cell line stably expressing NSUN3-GFP from a tetracycline-inducible promoter. 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-HisPrcFLAG (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^{Met}

To identify NSUN3 target RNAs, we performed UV cross-linking and analysis of cDNA (CRAC; Bohnsack *et al*, 2012; Sloan *et al*, 2015)

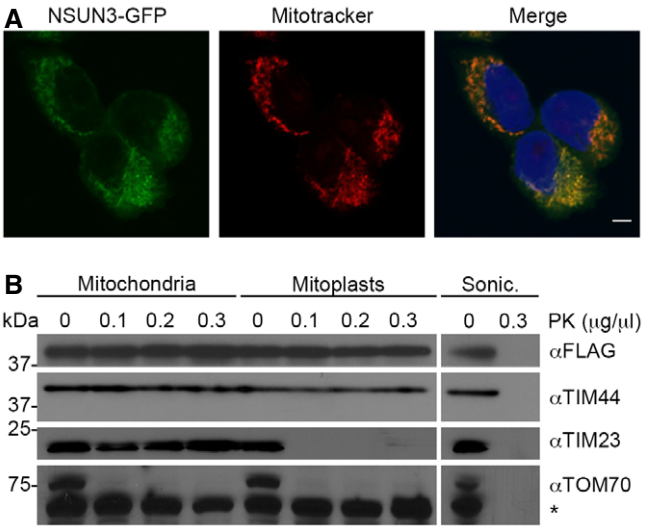

Figure 1. NSUN3 localises to the mitochondrial matrix.

A The localisation of NSUN3 was analysed in HEK293 cells stably expressing NSUN3-GFP. NSUN3-GFP (green) and staining with Mitotracker (red) are shown separately and in an overlay with DAPI to indicate nuclei. The scale bar represents 5 μm.

B To analyse submitochondrial localisation of NSUN3, human mitochondria were isolated and either left untreated, swollen in hypotonic buffer (Mitoplasts) or disrupted by sonication (Sonic.) before treatment with different amounts of proteinase K (PK) where indicated, followed by SDS–PAGE and Western blotting using antibodies against human TIM44, TIM23, TOM70 or FLAG-tagged NSUN3. Note that TIM44 extends into the matrix, while the N-terminus of TIM23 localises to the intermembrane space and TOM70 is largely exposed on the mitochondrial surface. The asterisk indicates a cross-reaction of the TOM70 antibody.

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^{5}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 adaptors 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 cysteine of the TCT tripeptide that is conserved in motif IV in m^{5}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 cysteine and the efficient cross-linking of NSUN3 to 5-AzaC containing mt-tRNA^{Met} strongly suggest that NSUN3 is an active m^{5}C RNA methyltransferase that uses the conserved mechanism of the NSUN family to mediate m^{5}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-[^{3}H-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$_i^{Met}$) and elongation (tRNA$_e^{Met}$) 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$_i^{Met}$ and tRNA$_e^{Met}$ 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$_i^{Met}$ and tRNA$_e^{Met}$ transcripts, respectively (Fig EV1C). To analyse possible interactions between NSUN3 and tRNA$_i^{Met}$ or tRNA$_e^{Met}$ *in vivo*, 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$_i^{Met}$ or tRNA$_e^{Met}$ 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$_i^{Met}$ 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$_i^{Met}$ as a substrate *in vitro*, but that mt-tRNA^{Met}, rather than tRNA$_i^{Met}$, 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^{5}C34 was treated with NSUN3 in methylation assays (Fig 3E), supporting the finding that NSUN3 generates an m^{5}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

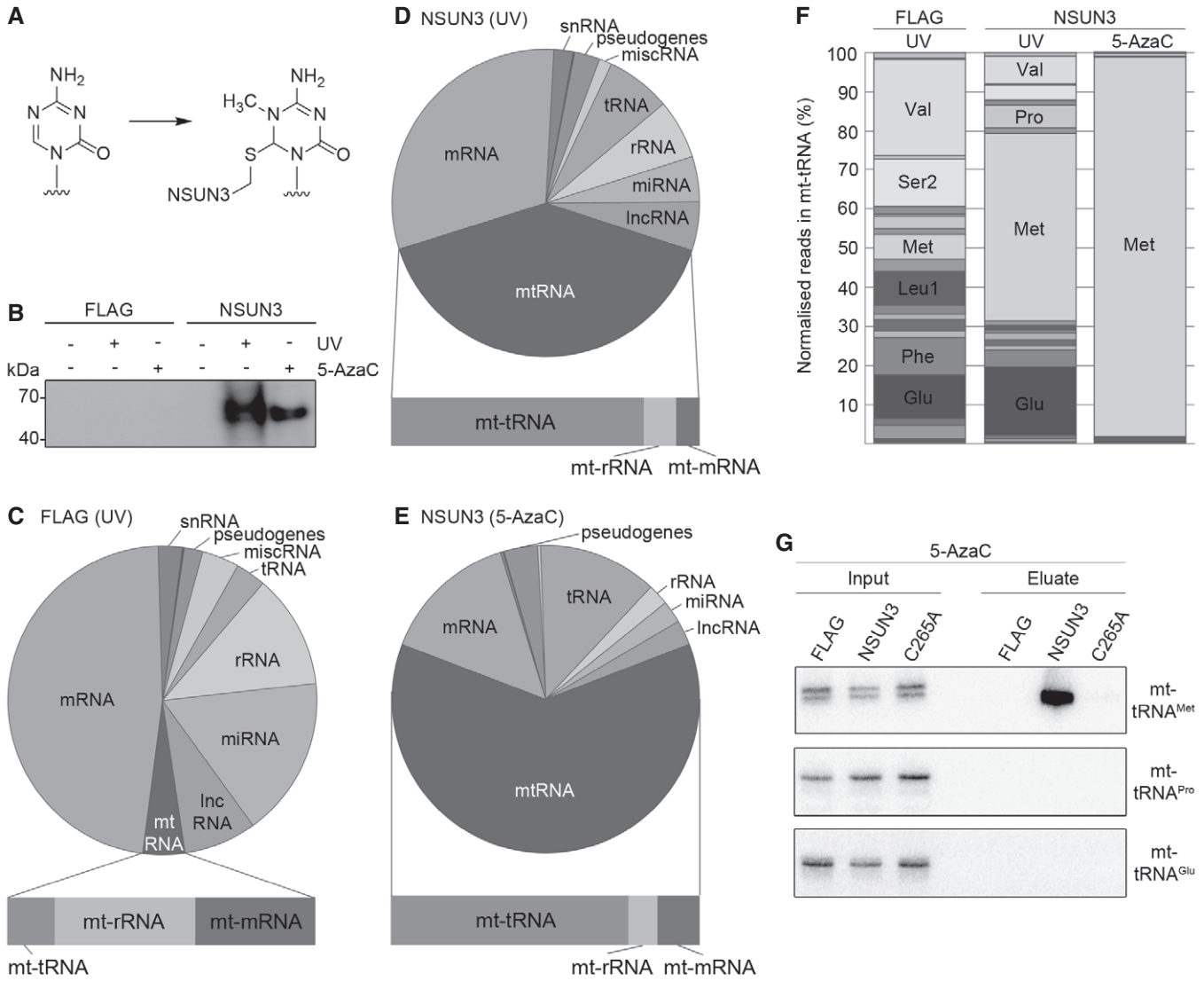

Figure 2. NSUN3 cross-links to the mitochondrial tRNA^{Met} *in vivo*.

A Structure of 5-azacytidine and formation of a covalent RNA methyltransferase adduct.

B HEK293 cells expressing NSUN3-HisPrcFLAG (NSUN3) or the HisPrcFLAG tag alone (FLAG) were either not cross-linked (–), UV cross-linked (UV) or treated with 5-azacytidine (5-AzaC). The protein–RNA complexes were affinity purified and the bound RNA was trimmed, end-labelled with ^{32}P phosphate and ligated to linkers. Protein–RNA complexes were separated by SDS–PAGE, transferred to nitrocellulose and exposed to an X-ray film.

C–E The UV or 5-AzaC cross-linking and analysis of cDNA (CRAC) experiments with NSUN3-HisPrcFLAG (D, E) or the FLAG control (C) samples were treated as described in (B). The RNA was isolated from the nitrocellulose membrane-bound protein–RNA complexes and converted into cDNA for sequence library production and Illumina deep sequencing. Pie charts present different RNA classes and the relative distribution of Illumina sequence reads that were obtained after mapping of the reads on the human genome. Bar graphs below indicate the distribution of mitochondrial (mt-)tRNA, mt-rRNA and mt-mRNA sequence reads among the reads mapped to the mitochondrial genome. Abbreviations: tRNA, transfer RNA; snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; rRNA, ribosomal RNA; mtRNA, mitochondrial-encoded RNA; miscRNA, miscellaneous RNA; miRNA, microRNA; lncRNA, long non-coding RNA.

F Relative distribution of mitochondrial tRNA sequence reads obtained from the CRAC experiments using UV or 5-AzaC cross-linking with cells expressing the NSUN3-HisPrcFLAG (NSUN3) protein or control cells (FLAG). Only mt-tRNAs that were represented by more than 5% of all mt-tRNAs reads are labelled.

G 5-AzaC cross-linking was performed and RNA associated with wild-type NSUN3, the catalytically inactive NSUN3 mutant (C265A) or the FLAG tag alone was isolated as described in (B). The RNA was analysed by Northern blot using probes against the mt-tRNA^{Met}, mt-tRNA^{Pro} and mt-tRNA^{Glu}. Inputs (0.1%) are shown on the left and eluates (50%) on the right.

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-tRNA^{Met}.

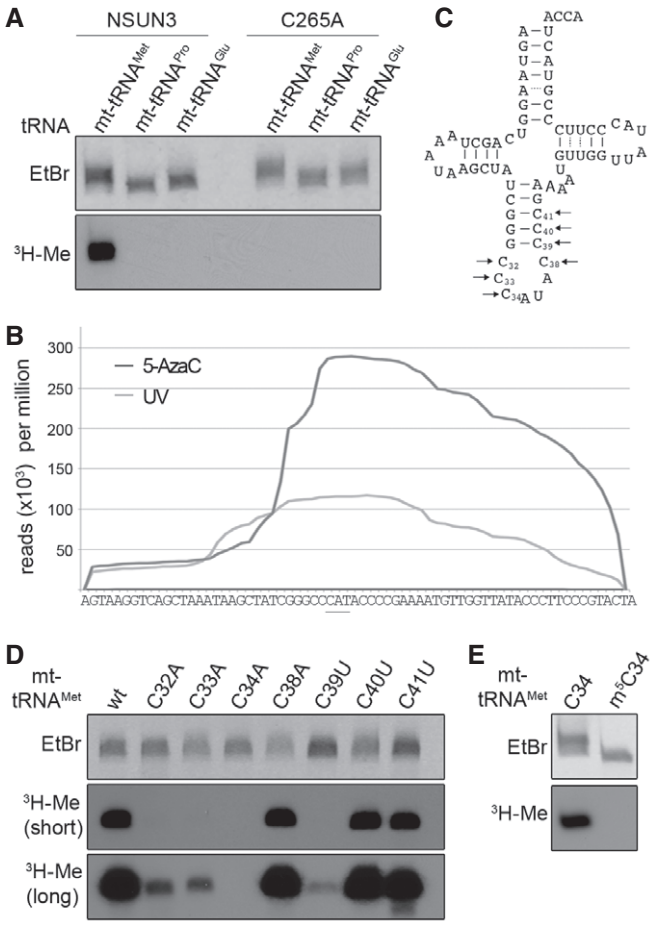

Figure 3. NSUN3 modifies the wobble position of mt-tRNA^{Met}.

A *In vitro* methylation reactions were performed using recombinant His$_{14}$-MBP-NSUN3 (NSUN3) or the catalytically inactive mutant His$_{14}$-MBP-NSUN3-C265A (C265A), ^{3}H-labelled S-adenosylmethionine as a methyl group donor and *in vitro*-transcribed mt-tRNA^{Met}, mt-tRNA^{Pro} and mt-tRNA^{Glu}. The RNA was then separated on a denaturing polyacrylamide gel, stained with ethidium bromide (EtBr) to indicate inputs and exposed to an X-ray film to analyse methylation (^{3}H-Me).

B The distribution of Illumina sequence reads along the mt-tRNA^{Met} sequence obtained from CRAC experiments with NSUN3 after UV (light grey) or 5-AzaC cross-linking (dark grey) is given as reads per million mapped reads. The position of the anticodon is indicated by a bar.

C Cloverleaf scheme of the mt-tRNA^{Met} sequence. Nucleosides that were exchanged in the mutational analysis shown in the following panels are marked with arrows, and the nucleotide positions in the tRNA are given.

D *In vitro* methylation assays were performed as described in (A) with His$_{14}$-MBP-NSUN3 and *in vitro*-transcribed wild-type mt-tRNA^{Met} and cytidine mutants of the anticodon stem and loop region indicated in (C). Two exposure times of X-ray films are shown 16 h (short) and 3 days (long).

E *In vitro* methylation assay of *in vitro*-transcribed mt-tRNA^{Met} and chemically synthesised mt-tRNA^{Met} containing an m^{5}C modification at the wobble position. The experiment and analysis were performed as described in (A).

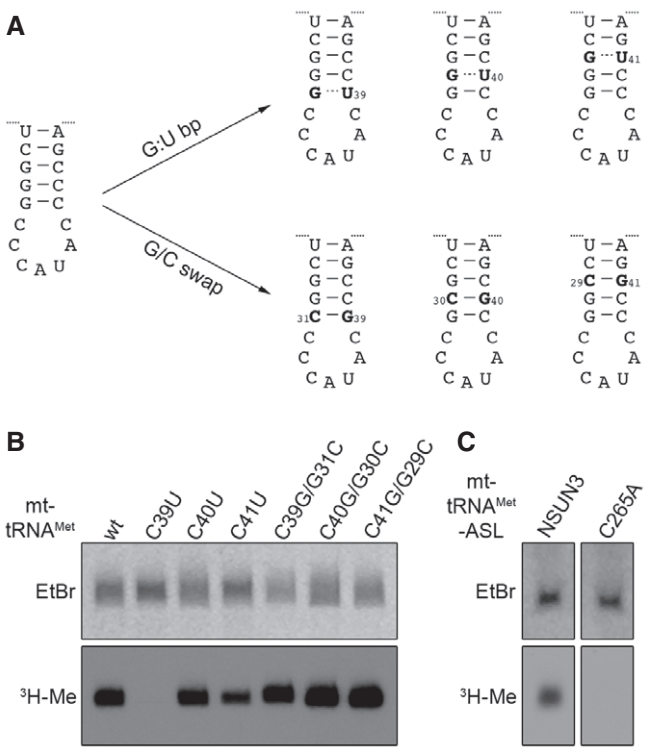

Figure 4. NSUN3 requires a stable anticodon stem loop of mt-tRNA^{Met} for methylation of cytosine 34.

A Scheme showing the mutations introduced in the anticodon stem loop (ASL) of mt-tRNA^{Met} for analysing tRNA substrate recognition by NSUN3. ASL mutants included G:U basepairs (G:U bp) to affect the stability of basepairing and sequence of the stem or mutants were generated by swapping G:C basepairs (G/C swap), leading to changes in sequence without affecting basepairing stability.

B *In vitro* methylation assays were performed using [^{3}H-methyl]-labelled S-adenosylmethionine, the *in vitro* transcripts of the mt-tRNA^{Met} mutants described in (A) and recombinant His$_{14}$-MBP-NSUN3. RNA was then separated on a denaturing polyacrylamide gel, stained with ethidium bromide (EtBr), dried and exposed to an X-ray film to detect methylated transcripts (^{3}H-Me).

C *In vitro* methylation assay using chemically synthesised ASL. The experiment and analysis were performed as described in (B).

Incubation of synthesised mt-tRNA^{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^{Met} as the methylation substrate of the RNA methyltransferase NSUN3. NSUN3 recognises the ASL of mt-tRNA^{Met} and requires a stable stem structure for substrate recognition and generation of m^{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.

ALKBH1/ABH1 localises in mitochondria and specifically interacts with mt-tRNA^{Met}

Previous reports suggested that mt-tRNA^{Met} can be modified at position 34 to contain a 5-formylcytosine (f^{5}C; Moriya *et al*, 1994; Suzuki & Suzuki, 2014). We hypothesised that a specific oxygenase might oxidise the m^{5}C34 moiety established by NSUN3 to generate an f^{5}C34 modification in mt-tRNA^{Met}. While the Ten-Eleven Translocation (TET) protein family of dioxygenases primarily mediates oxidation of m^{5}C in nuclear DNA and has also been implicated in

histone modification, most of the members of the AlkB-like Fe(II)/alpha-ketoglutarate-dependent dioxygenases (ALKBH) have been shown to act on RNA (reviewed in Shen *et al*, 2014; Fedeles *et al*, 2015; Li *et al*, 2015; Ougland *et al*, 2015). These include FTO (ALKBH9) that is implicated together with ALKBH5 in the oxidative removal of several modifications including 6-methyladenosine (m^6A) from RNA and ALKBH8 that is involved in the generation of 5-methoxycarbonylmethyluridine (mcm^5U) in cytoplasmic tRNAs (Fu *et al*, 2010a,b; Songe-Møller *et al*, 2010; Jia *et al*, 2011; Thalhammer *et al*, 2011; Berulava *et al*, 2013; Zheng *et al*, 2013). So far, only ALKBH7, which was suggested to act on protein substrates during necrosis (Fu *et al*, 2013; Solberg *et al*, 2013; Wang *et al*, 2014), and ALKBH1/ABH1 have been reported to localise to mitochondria; however, the cellular localisation of ABH1 has been a matter of debate (Pan *et al*, 2008; Westbye *et al*, 2008; Ougland *et al*, 2012). We therefore analysed the cellular localisation of ABH1 in HEK293 cells by immunofluorescence analysis and co-staining with a mitotracker (Fig 5A). The ABH1 antibody showed a cytoplasmic localisation with enrichment in foci that were also stained by the mitotracker, indicating that ABH1 is largely present in mitochondria in HEK293 cells, which could allow it to act on mt-tRNA^{Met}. Partial localisation of ABH1 to the mitochondrial matrix was further supported by proteinase K protection assays, in which ABH1 remained intact in mitoplasts and was only degraded upon mitochondrial lysis that allowed access of the protease to the matrix (Fig EV2). To test whether ABH1 specifically interacts with mt-tRNA^{Met}, we generated a HEK293 cell line expressing ABH1-HisPrcFLAG and performed UV cross-linking and pull-down experiments followed by Northern blotting to analyse for ABH1-associated RNAs. mt-tRNA^{Met} (and not mt-tRNA^{Glu}) was retrieved with ABH1-HisPrcFlag, but not with the HisPrcFLAG control (Fig 5B), indicating that ABH1 interacts specifically and directly with mt-tRNA^{Met} in mitochondria.

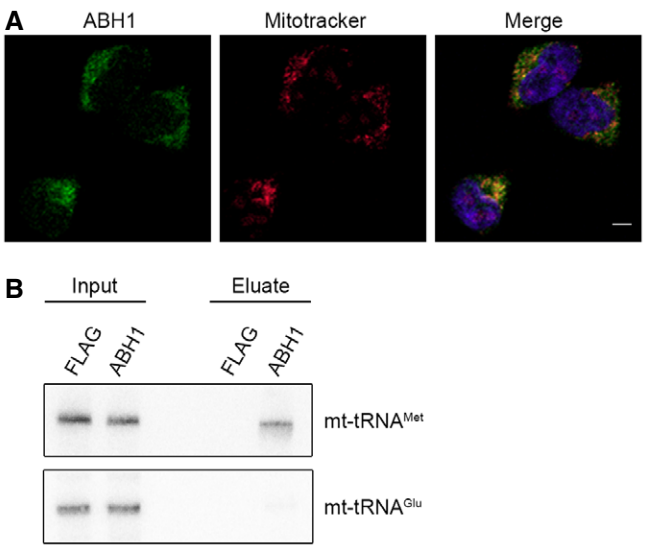

Figure 5. ABH1 localises to mitochondria in HEK293 cells and specifically interacts with mt-tRNA^{Met}.

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^{Met} and mt-tRNA^{Glu}. Inputs (0.1%) are shown on the left and eluates (50%) on the right.

ABH1 mediates oxidation of m^5C34 in mt-tRNA^{Met}

The interaction of ABH1 with mt-tRNA^{Met} suggests that it might mediate oxidation of m^5C34 in mt-tRNA^{Met}. We therefore radioactively methylated mt-tRNA^{Met} using [^{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^{2+} either without enzyme, with maltose binding protein (MBP), wild-type ABH1, the ABH1 alpha-ketoglutarate/Fe^{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^5C34 in mt-tRNA^{Met} and the reaction required the presence of alpha-ketoglutarate and Fe^{2+} (Fig 6A), which further supports the notion that mt-tRNA^{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^{Met} ASL alone is sufficient for *in vitro* recognition by ABH1 and oxidation of m^5C34. Indeed, m^5C34-containing mt-tRNA^{Met} ASL was efficiently oxidised by ABH1 (Fig 6B), allowing further characterisation of the oxidation product by HPLC. Treatment of chemically synthesised m^5C34-containing ASL with ABH1 resulted in almost quantitative oxidation of m^5C to 5-formylcytosine (f^5C). The presence of 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 $NaBH_4$, f^5C was chemically reduced to 5-hydroxymethyl-cytosine (hm^5C), which did not react with TMI (Fig 6C). Co-injection of mt-tRNA^{Met} ASL derivatives further confirmed that the different oxidation states can be distinguished by HPLC (Fig 6D).

Together, these data show that ABH1 is present in mitochondria of HEK293 cells where the enzyme can mediate oxidation of the m^5C34-containing mt-tRNA^{Met} generated by NSUN3 to provide f^5C-containing mt-tRNA^{Met} for mitochondrial translation.

Modifications of C34 modulate codon recognition by mt-tRNA^{Met}

To understand how the modification state of C34 in mt-tRNA^{Met} affects its function in translation, we studied codon recognition by mt-tRNA^{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^{Met} in the presence of purified recombinant human mitochondrial translation factors on ribosomes from *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 at the conserved sites of bacterial ribosomes. To mimic codon recognition during translation initiation, we used recombinant human mitochondrial initiation factor 2

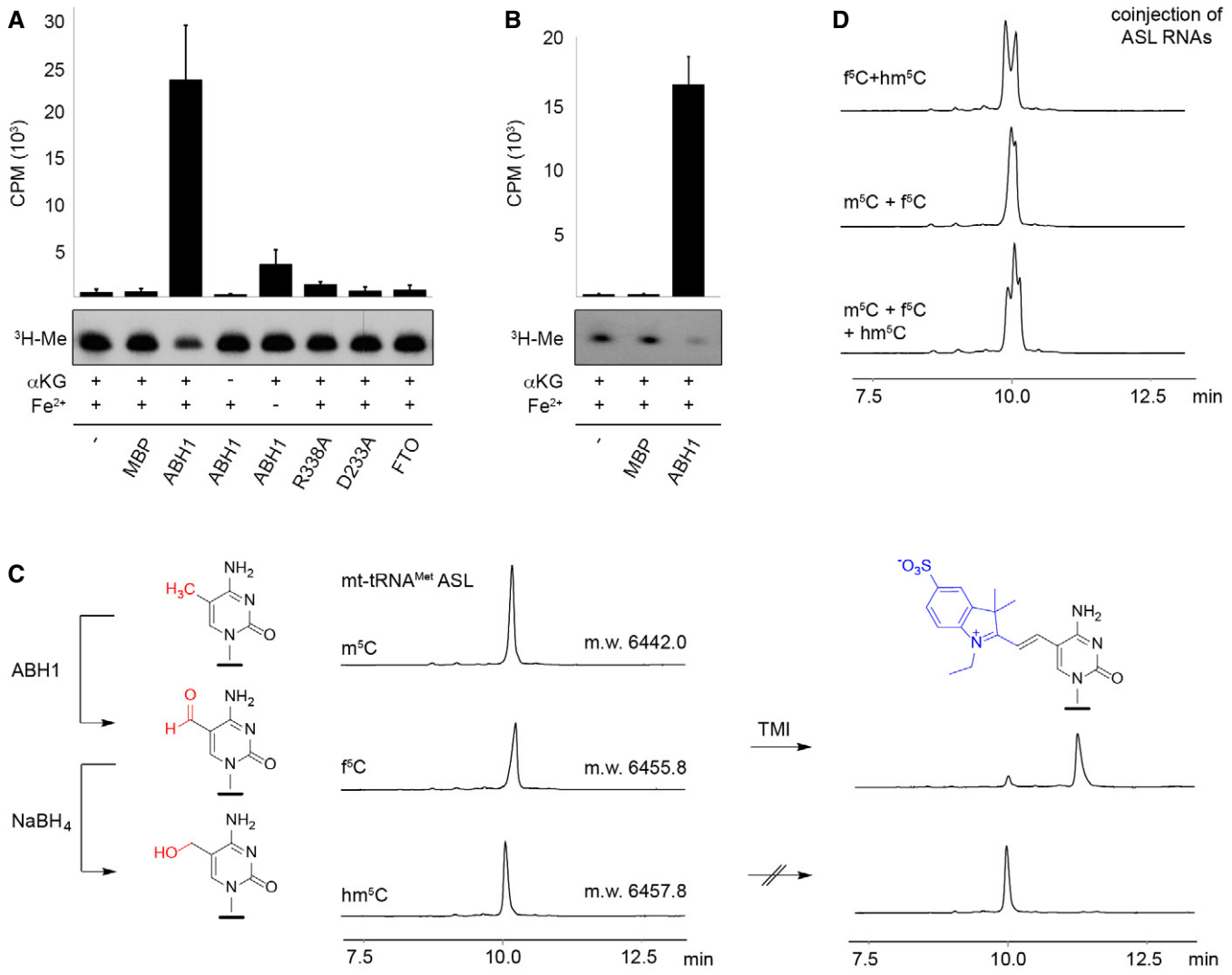

Figure 6. ABH1 can oxidise m^{5}C34 in mt-tRNA^{Met} *in vitro*.

A *In vitro*-transcribed mt-tRNA^{Met} was methylated at C34 using recombinant NSUN3 and ^{3}H-labelled S-adenosylmethionine as a methyl group donor. Radiolabelled mt-tRNA^{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$_{14}$-MBP-ABH1. Besides ABH1 controls lacking α-ketoglutarate (αKG) or Fe^{2+} ions, all samples contained α-ketoglutarate and Fe^{2+} ions. After oxidation, RNA was precipitated and the tritium released upon oxidation of radiolabelled mt-tRNA^{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 (^{3}H-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$_{14}$-MBP-ABH1 (ABH1). Experiments were performed in triplicate with error bars indicating ± SD.

C Anion exchange HPLC analysis was performed on synthetic m^{5}C-containing ASL (20 nt) before and after oxidation by ABH1. The small shift in retention time indicates formation of f^{5}C-modified RNA. The ABH1 oxidation product was then treated with $NaBH_4$ to generate hm^{5}C-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^{5}C-containing RNA was labelled efficiently with 1-ethyl-2,3,3-trimethylindoleninium-5-sulphonate (TMI).

D The different retention times of m^{5}C-, hm^{5}C- and f^{5}C-modified ASL RNA were confirmed by co-injection of samples shown in (C). HPLC was performed as in (C).

(MTIF2), which recruits mt-tRNA^{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^{Met} containing either unmodified C34, m^{5}C34, hm^{5}C34 or f^{5}C34 aminoacylated with [^{14}C]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^{Met} variants. mt-tRNA^{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 m^{5}C34-modified mt-tRNA^{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^{Met} modification.

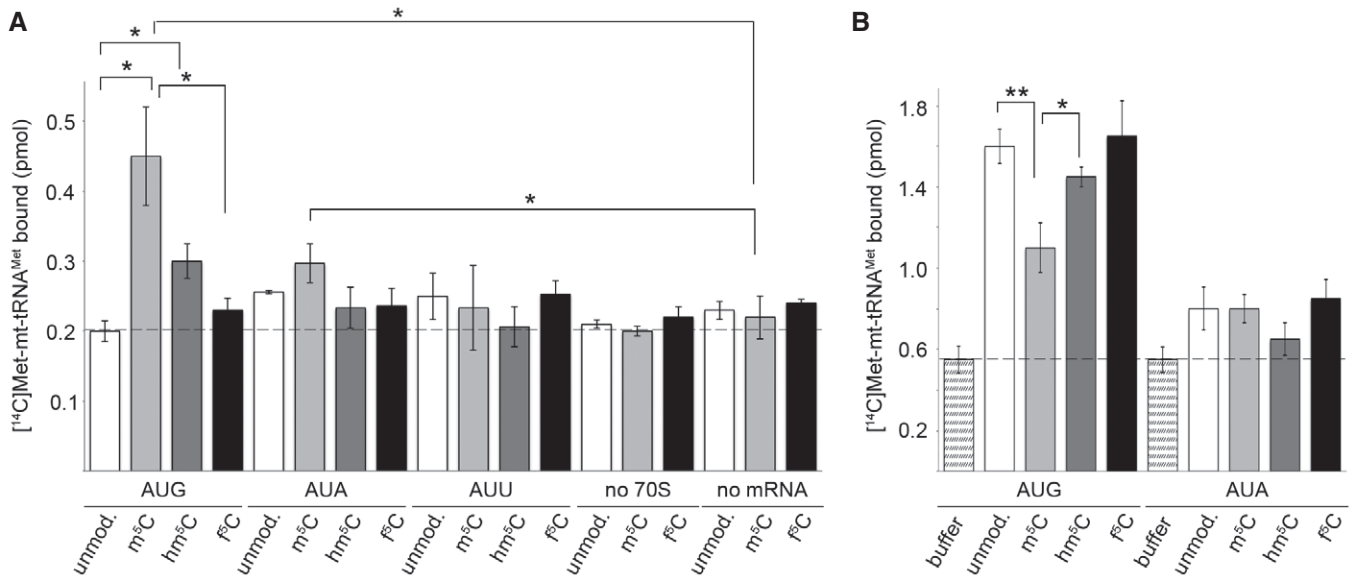

Figure 7. Modification of cytosine 34 modulates codon recognition by mt-tRNA^{Met} *in vitro*.

A MTIF2-dependent reading of initiation codons AUG, AUA or AUU in the P site of the ribosome by unmodified (unmod.) or C34-modified [^{14}C]Met-tRNA^{Met}. Binding was determined by nitrocellulose filtration, and [^{14}C]Met-tRNA^{Met} retrieved on the membrane was quantified by scintillation counting. Binding in the absence of ribosomes (no 70S) or mRNA (no mRNA) served as controls. Data from three independent experiments are presented with error bars indicating ± SEM. The statistical significance of the results was analysed by *t*-test and is indicated by the asterisks in the graph (*$P < 0.05$).

B TUFM-dependent recognition of A site codons during elongation. Data from three independent experiments are presented with error bars indicating ± SEM and statistical analysis as in (A) (*$P < 0.05$, **$P < 0.01$).

Controls showed only weak binding of mt-tRNA^{Met} 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-tRNA^{Met} 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-tRNA^{Met} variants were capable of reading the AUG codon. Notably the m^{5}C-modified mt-tRNA^{Met} was less efficient than other variants in AUG decoding, while AUA was read with similar efficiencies by all variants of mt-tRNA^{Met}. Together these data indicate that the modification state of C34 in mt-tRNA^{Met} influences codon recognition by the tRNA in the P and A site, with m^{5}C 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-tRNA^{Met} 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-tRNA^{Met} *in vivo*

The cross-linking and *in vitro* modification data show that cytosine 34 in mt-tRNA^{Met} can be methylated by NSUN3 to generate m^{5}C and then further oxidised by the dioxygenase ABH1 to f^{5}C. In addition, these different modifications in mt-tRNA^{Met} may influence codon recognition. To gain insight into the occurrence of the mt-tRNA^{Met} 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 f^{5}C into a hemicyanine derivative, which blocks primer extension by reverse transcriptase at the site of modification, thereby allowing to analyse the presence of f^{5}C in the RNA (Samanta *et al*, 2016). Primer extension analysis revealed that the fraction of f^{5}C34-containing mt-tRNA^{Met} 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 $NaBH_4$ to convert f^{5}C to hm^{5}C and bisulphite treatment. In both cases, after deamination and desulphonation, mt-tRNA^{Met} was specifically amplified by reverse transcription and PCR and then cloned. Analysis of sequences derived from wild-type RNA after reduction indicated that mt-tRNA^{Met} is fully modified at position C34. Comparison to the non-reduced sample suggested that although the majority of these modifications are f^{5}C, a portion of cytosines at this position are not converted by the bisulphite treatment, indicating that they carry the m^{5}C34 modification installed by NSUN3 (Fig 8D). Consistent with these data, depletion of NSUN3 resulted in a decrease in the mt-tRNA^{Met} fraction carrying a modification on C34 and a decrease in the portion of m^{5}C, while upon depletion of ABH1,

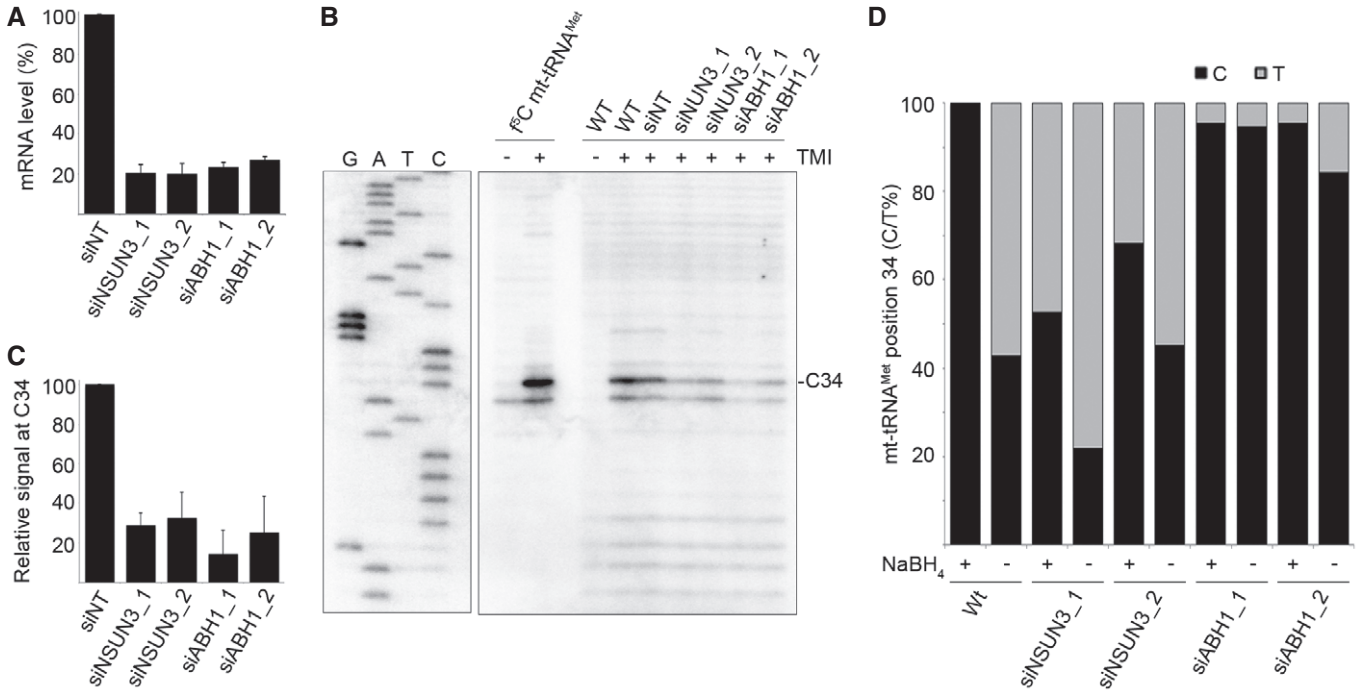

Figure 8. Knock-down of NSUN3 or ABH1 leads to a reduction in the modification of cytosine 34 in mt-tRNA^{Met} *in vivo*.

A HeLa cells were transfected with two different siRNAs against NSUN3 (siNSUN3_1, siNSUN3_2), ABH1 (siABH1_1, siABH1_2) or with non-target (siNT) siRNA, and the knock-down efficiency was analysed by quantitative PCR. The relative abundance of the NSUN3 or ABH1 mRNA was normalised to GAPDH levels. Data are presented as mean ± SD.

B Chemically synthesised f^{5}C modified mt-tRNA^{Met} and total RNA from wild-type (WT) cells or those transfected with siRNAs as in (A) were treated with TMI to specifically label f^{5}C residues. Primer extension, using a radiolabelled antisense primer, was performed under limited dNTP conditions. Products were separated on a denaturing polyacrylamide gel alongside a sequencing ladder, and RNAs were detected using a phosphorimager.

C Primer extension reactions were performed on total RNA from cells transfected with siRNAs as described in (B). Stops corresponding to position C34 in mt-tRNA^{Met} were quantified in three independent experiments, and results are shown graphically as mean ± SD.

D RNA from wild-type HeLa cells and cells treated with siRNAs against NSUN3 or ABH1 (as in A) was either first reduced with $NaBH_4$ or directly treated with bisulphite. After deamination and desulphonation, mt-tRNA^{Met} RNAs were reverse transcribed, amplified, cloned and sequenced. The proportions of thymine (grey) generated by bisulphite conversion or non-converted cytosine (black) at position 34 of mt-tRNA^{Met} are shown. Note that for sequences from non-reduced samples, thymine can also originate from unmodified or f^{5}C-containing mt-tRNA^{Met}, while in reduced samples, it originates from unmodified cytosine.

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 f^{5}C34 in mt-tRNA^{Met} *in vivo*. We note that the bisulphite data do not rule out the presence of hm^{5}C, which is also resistant to bisulphite conversion. Oxidative bisulphite sequencing, which can distinguish m^{5}C and hm^{5}C in DNA (Booth *et al*, 2013), resulted in degradation of the RNA. However, hm^{5}C 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-tRNA^{Met}.

Modifications of cytosine 34 in mt-tRNA^{Met} are required for mitochondrial translation *in vivo*

Our finding that the mt-tRNA^{Met} C39U mutation, which has previously been identified in patients with mitochondrial dysfunction (Lott *et al*, 2013; Tang *et al*, 2013), largely abolishes m^{5}C34 formation by NSUN3, suggests that the C34 modification is required for mt-tRNA^{Met} function *in vivo* and that mt-tRNA^{Met} 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 ^{35}S-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 ^{35}S-incorporation, suggesting that the modifications installed by these proteins are required for mt-tRNA^{Met} 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 tRNA 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 tRNA^{Met}. We show that the RNA methyltransferase NSUN3 efficiently methylates C34 of mt-tRNA^{Met} to produce m^{5}C, which can then be oxidised by the alpha-ketoglutarate and Fe(II)-dependent dioxygenase ALKBH1/ABH1. Mammalian mt-tRNA^{Met} can be modified to f^{5}C 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 m^{5}C34-containing mt-tRNA^{Met} *in vivo*.

The ability of ABH1 to oxidise m^{5}C to f^{5}C 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 (m^{3}C) (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 m^{5}C *in vitro* (Li *et al*, 2010) and human ALKBH2 and ALKBH3 preferentially repair alkylation at nucleobase heteroatoms such as m^{3}C and 1-methyladenosine (m^{1}A) (Aas *et al*, 2003; Falnes *et al*, 2004), the oxidation of m^{5}C involves transformation of a pseudobenzylic 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 hm^{5}C as primary stable oxidation product, which can be further oxidised to f^{5}C and 5-carboxycytosine (ca^{5}C), although these higher oxidation products are 10- to 100-fold less abundant than hm^{5}C in DNA and are mainly linked to active demethylation (Ito *et al*, 2011; Pfaffeneder *et al*, 2011; Wagner *et al*, 2015). In RNA, the analogous oxidation of m^{5}C to hm^{5}C has been reported by catalytic domains of mammalian TET enzymes (Fu *et al*, 2014) and the homologous *Drosophila* protein dTET (Delatte *et al*, 2016). f^{5}C 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 m^{5}C have been detected in RNA from all domains of life, including organisms that do not contain homologous TET enzymes, suggests that m^{5}C can be metabolically oxidised by enzymes other than those of the TET family. We have identified ABH1 as the first such enzyme that produces f^{5}C in human mitochondria. Under the conditions tested, f^{5}C was the only oxidation product detected *in vitro*; hm^{5}C did not accumulate as intermediate and no further oxidation to ca^{5}C 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 f^{5}C 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, m^{5}C34, hm^{5}C34 or f^{5}C34 and the human mitochondrial translation initiation factor MTIF2 reveal that the presence of the m^{5}C modification in the wobble position enhances codon reading of the AUG and, to a lesser extent, AUA initiation codons in the P site of the ribosome, suggesting a specific role of m^{5}C34 modification during translation initiation. The AUU initiation codon, which is only present in the ND2 mRNA, is recognised, albeit poorly, by non-modified or f^{5}C-modified mt-tRNA^{Met}. The recognition efficiency of the AUA 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 m^{5}C-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 m^{5}C34 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, m^{5}C- or f^{5}C-modified mt-tRNA^{Met} to AUA codons in the ribosomal A site were observed, while the binding of hm^{5}C-containing mt-tRNA^{Met} was less efficient. Previous reports with the unmodified or f^{5}C34-containing ASL of mt-tRNA^{Met} suggested that the formyl group might stabilise the non-conventional basepairing of f^{5}C34 with an adenosine in the third position of an AUA codon (Bilbille *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.

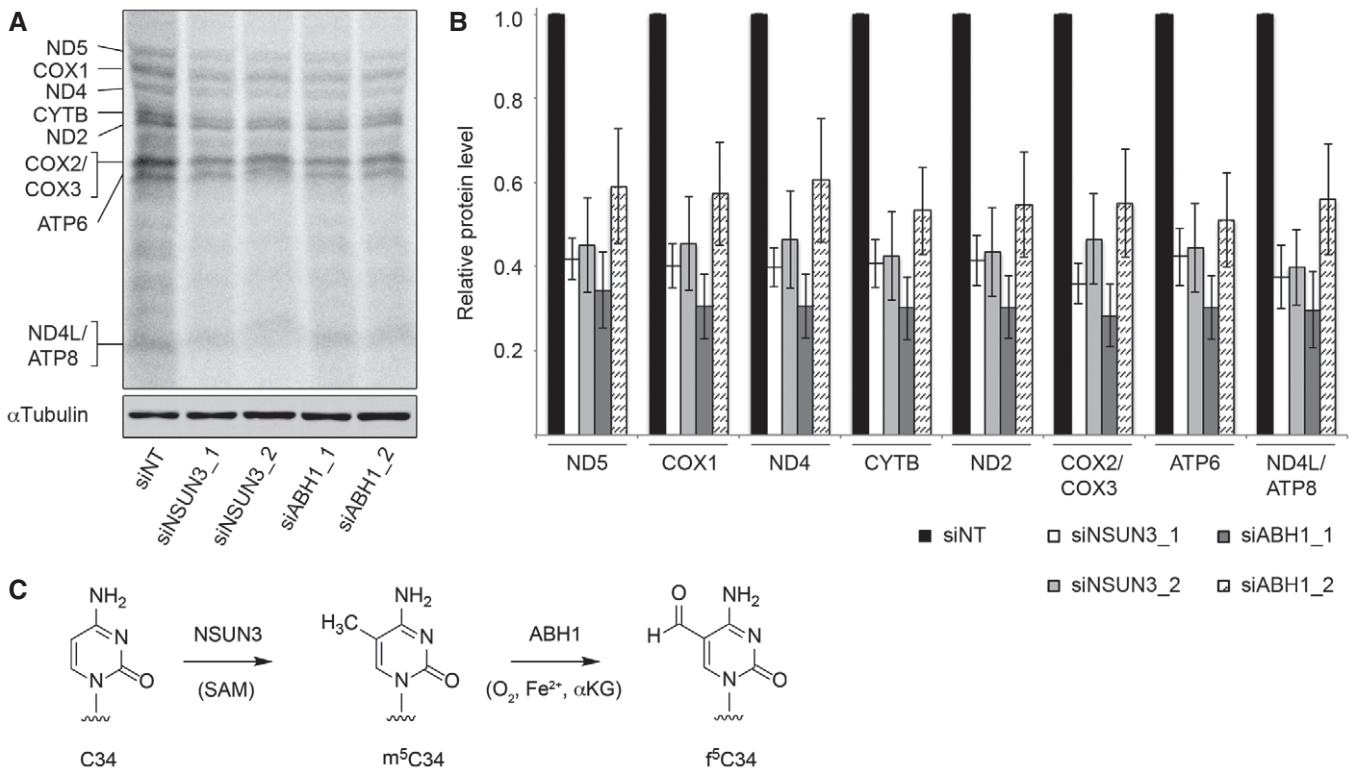

Figure 9. NSUN3 and ABH1 are both required for efficient mitochondrial translation *in vivo*.

A HeLa cells were treated with non-target siRNAs (siNT) or those targeting NSUN3 (siNSUN3_1 or siNSUN3_2) or ABH1 (siABH1_1 or siABH1_2) for 72 h before labelling of mitochondrial translation products with [^{35}S]methionine. Protein samples were separated by SDS–PAGE then transferred to a membrane. Labelled proteins were detected using a phosphorimager, and the levels of tubulin were determined by Western blotting using an antibody against the endogenous protein for normalisation.

B Mitochondrially translated proteins that could be clearly detected were quantified in three independent experiments, and the results are shown graphically as mean ± SD.

C Overview of the modification pathway of C34 in mt-tRNA^{Met}. NSUN3 introduces an m^{5}C methylation on C34 using S-adenosylmethionine (SAM) as methyl group donor, and this can be further oxidised by ABH1 in the presence of O_2, Fe(II) (Fe^{2+}) and alpha-ketoglutarate (αKG) to produce f^{5}C34.

Together, our data indicate that the different modification states of cytosine 34 in mt-tRNA^{Met} can expand the ability of the single tRNA^{Met} to read the different codons encoding methionine in mitochondrial translation initiation and elongation.

The modification state of C34 in mt-tRNA^{Met} 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^{5}C34 and f^{5}C34 in human mt-tRNA^{Met} (Nakano *et al*, 2016; Van Haute *et al*, 2016). Our findings imply that *in vivo* a large fraction of the m^{5}C34-containing mt-tRNA^{Met} is oxidised by ABH1, which is in line with previous reports that found the f^{5}C34 modification in mt-tRNA^{Met} (Moriya *et al*, 1994; Takemoto *et al*, 2009; Suzuki & Suzuki, 2014). However, we also observed that mt-tRNA^{Met} carrying m^{5}C34 is present *in vivo*, which is supported by findings of Van Haute *et al* (2016), and that this modification state of mt-tRNA^{Met} is efficiently recruited to the P site of the ribosome *in vitro*. Importantly, mutations in mt-tRNA^{Met} 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-tRNA^{Met} methylation by NSUN3. These results indicate that NSUN3 malfunction and a lack in mt-tRNA^{Met} modification might represent the molecular cause of such diseases. An important role of the modifications installed in mt-tRNA^{Met} 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-tRNA^{Met} 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-tRNA^{Met} 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-tRNA^{Met} 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% CO_2 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 pcDNA5 vector with C-terminal GFP or His-PreScission protease cleavage site-2×FLAG (HisPrcFlag) 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 proteinase 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 ^{32}P-5′ end-labelled probes (anti-mt-tRNA^{Met}, anti-mt-tRNA^{Glu}, anti-mt-tRNA^{Pro}, anti-tRNA$_i^{Met}$, anti-tRNA$_e^{Met}$; 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).

$NaBH_4$ treatment and bisulphite reaction

To analyse the cytosine modification status of mt-tRNA^{Met}, DNase I treated total RNA from wild-type, NSUN3 or ABH1 knock-down cells was either directly subjected to bisulphite sequencing (Schaefer *et al*, 2009) or treated with 0.25 M $NaBH_4$ in 200 mM Tris–HCl pH 7.5, 20 mM $MgCl_2$, 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-tRNA^{Met}_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-tRNA^{Met} 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 His_{14}-MBP-tag (Weis *et al*, 2014) and the CDS of MTIF2 or TUFM into a pQE80 derivative encoding a C-terminal His_{10}-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-tRNA^{Met}, mt-tRNA^{Glu}, mt-tRNA^{Pro}, tRNA$_i^{Met}$ and tRNA$_e^{Met}$ 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 *Bsa*I 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 *Bsa*I-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 tetrabutylammonium 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 f^5C34 or hm^5C-containing mt-tRNA^{Met}, ligation was performed with m^5C34-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 $NaBH_4$ (see $NaBH_4$ treatment). The modified ASLs were PAGE purified and their homogeneity and identity were confirmed by anion exchange HPLC and ESI-MS. Labelling of f^5C-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 tRNA or 10 μg of total RNA in 1× methylation buffer (50 mM Tris–HCl pH 7.0, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT) and 1.7 μM [^{3}H]-SAM (Hartmann), 1 unit/ml RiboLock (Thermo) were incubated at 22°C for 2 h. After addition of proteinase 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 amplify 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^{Met} or mt-tRNA^{Met} ASL were labelled with a [^{3}H]-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_{14}-MBP-ABH1, MBP or His_{14}-MBP-FTO in the presence of 50 mM HEPES pH 6.9, 5 mM $MgCl_2$, 4 mM ascorbic acid and 100 μM $Fe(NH_4)_2(SO_4)_2$ 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 [^{3}H] 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^5C34 ASL for preparation of mt-tRNA^{Met} 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 ^{35}S methionine for 30 min. Cells were harvested, and proteins were separated on a 10–18% Tricin–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.

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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.

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