APPENDIX

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

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Table S1: Oligonucleotides used in this study

Name	Sequence (5'-3')
NSUN3_qPCR_fwd	TGGGTCTGTTTGGAATCCTATT
NSUN3_qPCR_rev	TGCACCACCTTAAATCATTGTTAC
ABH1_qPCR_fwd	CACCATTCTGCTGTGCCCTA
ABH1_qPCR_rev	CAAGCTGCCTACCCTCAGAC
GAPDH_qPCR_fwd	CTGGCGTCTTCACCACCATGG
GAPDH_qPCR_rev	CATCACGCCACAGTTTCCCGG
mt-tRNA^{Met}_RT	TAATAATACAAAAAAAATATAACCAAC
anti-mt-tRNA^{Met}	GGGAAGGGTATAACCAACATTTTCGGGGTATGGGCCCGATAGCTTATTTAGCTGACC
anti-mt-tRNA^{Glu}	CACGGACTACAACCACGACCAATGATATGAAAAACCATCGTTGTATTTCAACTACAAG
anti-mt-tRNA^{Pro}	GAGAAAAAGTCTTTAACTCCACCATTAGCACCCAAAGCTAAGATTCTAATTTAAACTATTCTC
anti-tRNA$_i^{Met}$	GGTTTCGATCCATCGaCCTCTGGGTTATGGGCCCAGCACGCTTCCGCTGCG
anti-tRNA$_e^{Met}$	GAGGCTTGAACTCAGGaCCTTCAGATTATGAGACTGACGCGCTGCCAGCTG

Table S2: Antibodies used in this study

Name	Source/Reference
ABH1	Abcam (ab128895)
TIM44	Proteintech (13859-1-AP)
TIM23	Dennerlein et al, 2015
TOM70	Dennerlein et al, 2015
FLAG	Sigma Aldrich (F3165)
Tubulin	Sigma Aldrich (T6199)

Table S3: siRNAs used in this study

Name	Sequence (5'-3')
siABH1_1	UGACCAGAAUAGCGAAGUA
siABH1_3	GUGGUGACAUCAUGAUAAU
siNSUN3_1	GGACUUGCUCCCACGACUU
siNSUN3_2	GCAAAGUUGUGUUGGAUCA
siNT	UCGUAAGUAAGCGCAACCC

Supplementary Methods

Crosslinking and analysis of cDNA (CRAC)

Crosslinking and analysis of cDNA experiments were performed as previously described (Bohnsack et al, 2012). In brief, HEK293 cells expressing NSUN3-HisPrcFlag, NSUN3-C265A-HisPrcFLAG, ABH1-HisPrcFLAG or the HisPrcFlag tag alone were induced using 1 µg/mL tetracycline for 24 h. UV crosslinking was carried out using a Stratalinker (Stratagene) as previously described (Sloan et al, 2015). For chemical crosslinking cells were incubated in growth media complemented with 4 µM 5-azacytidine (Sigma) as previously described (Haag et al, 2015). Cells were harvested in buffer containing 50 mM Tris/HCl pH 7.6, 150 mM NaCl, 0.1% NP-40, 5 mM β-mercaptoethanol and protease inhibitors, and lysed by sonication. Protein-RNA complexes were affinity purified using anti-FLAG magnetic beads (Sigma) before elution using PreScission Protease. A partial RNase digest (1 U RNAceIT (Agilent) for 30 sec at 37°C) was performed and complexes were purified under denaturing conditions (6 M guanidium-HCl) on Ni-NTA (Qiagen). Co-purified RNAs were radiolabelled using T4 PNK (Thermo Scientific) and ^{32}P-γ-ATP (Perkin Elmer) and RNA-protein complexes were separated by NuPAGE gel electrophoresis then transferred to a nitrocellulose membrane. Radioactive signals were detected by exposure to an X-ray film. For Illumina sequencing, 3´ and 5´ adapters were ligated to the co-precipitated RNA and a cDNA library was prepared by reverse transcription of the RNA using Superscript III Reverse Transcriptase (Thermo) and amplification using primers containing randomised five-nucleotide sequence to enable PCR templates to be distinguished. Before mapping Flexbar was used to remove 3´-adapter sequences and bases with a phred quality score <13 (95% base call accuracy) and identical sequence reads were collapsed using python scripts. Bowtie2 was used to map the remaining reads on the human ensembl genome version GRCh 37.75 with an 18-nucleotide cut-off.

Expression and purification of recombinant proteins

Expression of His_{14}-MBP-NSUN3 or His_{14}-MBP-ABH1 (and mutants thereof) in *Escherichia coli* (DE3) Rosetta pLysS cells was induced by addition of 0.5 mM IPTG for 16 h at 18°C. Cells were harvested and lysed by sonication in a buffer containing 30 mM KPi pH 7.0, 300 mM KCl, 10% (v/v) glycerol, 10 mM imidazole, 0.1 mM dithiotreitol (DTT) and protease inhibitors (complete mini, Roche). The lysate was cleared by centrifugation and His-tagged proteins were retrieved on cOmplete His-Tag purification resin (Roche). After washing steps with lysis buffer, proteins were eluted in a buffer composed of 30 mM KPi pH 7.0, 300 mM KCl, 10% glycerol, 200 mM imidazole and 0.1 mM DTT. In the case of NUSN3, the eluate was diluted five-fold in a buffer containing 20 mM KPi pH 7.0, 200 mM KCl, 10% (v/v) glycerol, 2 mM beta-mercaptoethanol and then incubated with amylose resin. Elution of bound proteins was achieved using the dilution buffer supplement with 10 mM maltose. All proteins were dialysed against a buffer containing 30 mM KPi pH 7.0, 100 mM KCl, 50% glycerol, 1 mM DTT, and 0.1 mM EDTA for storage.

For MTIF2-His_6 or TUFM-His_6, expression in BL21 Codon Plus cells was induced by addition of 1 mM IPTG for 4 h at 37°C before cells were harvested. Cells were resuspended in a buffer containing 50 mM Tris-HCl pH 8.0, 60 mM NH_4Cl, 7 mM $MgCl_2$, 300 mM KCl, 10 mM imidazol, 10% (v/v) glycerol, 5 mM beta-mercaptoethanol, 50 µM GDP and lysed using a “French press”. After pelleting cell debris by centrifugation, the cleared lysate was incubated with Ni-NTA (Qiagen). Washing steps were performed with lysis buffer before elution in buffer composed of 50 mM Tris-HCl pH 8.0, 60 mM NH_4Cl, 7 mM $MgCl_2$, 300 mM KCl, 250 mM imidazol, 10% (v/v) glycerol, 5 mM beta-mercaptoethanol, 50 µM GDP. Proteins were further purified on a Sephadex 75 column equilibrated in 50 mM Tris-HCl pH 7.5, 70 mM NH_4Cl, 7 mM $MgCl_2$, 30 mM KCl and fractions containing the MTIF2 or TUFM were pooled and concentrated.

Preparation of [^{14}C]Met-tRNA^{Met} and ribosome binding assays

Aminoacylation of mitochondrial tRNAs was carried out in 160 µL reaction volume containing 50 mM HEPES pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 11 mM $MgCl_2$, 3 mM ATP, 2 mM β-mercaptoethanol, 5 A_{260} units tRNAs (unmodified, m^5C, f^5C, hm^5C), 20 µM [^{14}C]Methionine, and 15% (v/v) of *Escherichia coli* methionine tRNA synthetase (MetRS). After incubation for 30 min at 37°C, aminoacylated tRNA was extracted with phenol and precipitated with 2% (w/v) KAc pH 5.0 and cold ethanol.

Ribosome binding experiments were carried out in buffer $TAKM_7$ (50 mM Tris-HCl pH 7.5, 50 mM NH_4Cl, 50 mM KCl, 7 mM $MgCl_2$, 1 mM DTT) if not stated otherwise.

Ribosomes, EF-Tu, and fMet-tRNA^{fMet} from *E. coli* were prepared as described (Rodnina and Wintermeyer, 1995; Gromadski et al, 2006; Milon et al, 2007). Preparation of POST complex was carried out as previously described (Milon et al, 2007; Belardinelli et al, 2016). Ribosomes (4 μM) were incubated with a two-fold excess of mRNA (GGCAAGGAGGUAAAUA AUG UUU AUA GUU AC, or GGCAAGGAGGUAAAUA AUG UUU AUG GUU AC (IBA Göttingen); codon occupying the A-site is underlined) in the presence of 6 μM initiation factors IF1, IF2, IF3, 6 μM f[^{3}H]Met-tRNA^{fMet}, and 1 mM GTP in TAKM$_7$ for 30 min at 37°C. Ternary complexes of EF-Tu–GTP–Phe-tRNA^{Phe} were prepared by incubating EF-Tu with 1 mM GTP, 3 mM phosphophenolpyruvate and 0.1 mg/ml pyruvate kinase for 15 min at 37 °C prior to the addition of Phe-tRNA^{Phe}. The POST complex was formed by mixing initiation complex with ternary complex for 1 min at RT and with EF-G (0.1 μM) for an additional 1 min at room temperature. The resulting POST complexes were purified by centrifugation through a 1.1 M sucrose cushion in buffer A. For mitochondrial [^{14}C]Met-tRNA^{Met} binding to the A site, ternary complexes of TUFM–GTP–[^{14}C]Met-tRNA^{Met} were prepared by incubating TUFM with 1 mM GTP, 3 mM phosphophenolpyruvate, 0.1 mg/ml pyruvate kinase and [^{14}C]Met-tRNA^{Met} prepared from unmodified, m^{5}C, hm^{5}C or f^{5}C tRNAs for 20 min at 30°C. The ternary complex was added to the POST complex and incubated for 10 min at room temperature. The amount of f[^{3}H]Met-Phe-[^{14}C]Met bound to ribosomes was determined by nitrocellulose filtration.

For mitochondrial [^{14}C]Met-tRNA^{Met} binding to the P-site, 1 μM ribosomes were incubated with a two-fold excess of mRNA (GGCAAGGAGGUAAAUA AUG AAA UUU GUU AC, or GGCAAGGAGGUAAAUA AUA AAA UUU GUU AC, or GGCAAGGAGGUAAAUA AUU UUU UAA AGU UAC (IBA Göttingen); codon occupying the P-site is underlined) in the presence of 2 μM human mitochondrial initiation factor 2 (MTIF2), 0.15 μM [^{14}C]Met-tRNA^{Met} prepared from unmodified, m^{5}C, hm^{5}C or f^{5}C tRNAs and 1 mM GTP in TAKM$_7$ for 10 min at 30°C. The amount of [^{14}C]Met bound to ribosomes was determined by nitrocellulose filtration and scintillation counting.

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