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NSUN4 Is a Dual Function Mitochondrial Protein Required for Both Methylation of 12S rRNA and Coordination of Mitoribosomal Assembly

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Abstract

Biogenesis of mammalian mitochondrial ribosomes requires a concerted maturation of both the small (SSU) and large subunit (LSU). We demonstrate here that the m5C methyltransferase NSUN4, which forms a complex with MTERF4, is essential in mitochondrial ribosomal biogenesis as mitochondrial translation is abolished in conditional Nsun4 mouse knockouts. Deep sequencing of bisulfite-treated RNA shows that NSUN4 methylates cytosine 911 in 12S rRNA (m5C911) of the SSU. Surprisingly, NSUN4 does not need MTERF4 to generate this modification. Instead, the NSUN4/MTERF4 complex is required to assemble the SSU and LSU to form a monosome. NSUN4 is thus a dual function protein, which on the one hand is needed for 12S rRNA methylation and, on the other hand interacts with MTERF4 to facilitate monosome assembly. The presented data suggest that NSUN4 has a key role in controlling a final step in ribosome biogenesis to ensure that only the mature SSU and LSU are assembled.

Introduction

Expression of mtDNA is essential for production of ATP through oxidative phosphorylation in all eukaryotes. Mammalian mtDNA encodes 13 polypeptides that are translated on mitochondrial ribosomes (mitoribosomes) and are essential for oxidative phosphorylation. The mammalian mitoribosomes are composed of the mtDNA-encoded 12S and 16S rRNAs and more than a hundred different nucleus-encoded ribosomal proteins [1,2]. In bacteria, rRNA modifications are established at precise points of ribosomal assembly and can participate in rRNA processing events as well as in folding and interactions with neighboring proteins [3]. Modifications of rRNA have been also reported in mammalian mitochondria and the best characterized example is the TBP1-mediated dimethylation of two highly conserved adenosines at the 3’-end of mitochondrial 12S rRNA, which is necessary for assembly of the SSU [4]. Other putative methyltransferases, such as RNMTL1, MRMI and MRMM2, were recently proposed to play an important role for mitochondrial rRNA methylation, but the residues which they modify remain unknown [5]. Studies of hamster mitochondria have shown that the SSU rRNA, in addition to the above-mentioned two dimethylated adenosines (m2A), also contains two cytosine methylations (m3C and m2C) and one methylated uracil (m3U), whereas the LSU rRNA carries three ribose methylated nucleotides, one Um and two Gm [6]. The exact role of each of the modified residues is unclear, but it is worth noting that modified residues tend to concentrate in functionally important regions of ribosomes [7].

We have recently identified a novel mitochondrial rRNA methyltransferase, denoted NSUN4, which belongs to the same family of m3C-methyltransferases as the bacterial enzymes RsmB, RsmF and YccW [8–10]. In bacteria, RsmB establishes m3C-methylation on C967 of 16S rRNA, RsmF methylates C1400, C1404 and C1407 of 16S rRNA and YccW is responsible for m3C-methylation on C1962 in 23S rRNA [11–14]. The mitochondrial NSUN4 protein, unlike its bacterial counterparts, lacks PUA- or other types of known RNA-interaction domains. Instead, MTERF4 has been shown to form a stable complex with NSUN4 and target this complex to the LSU of the mitoribosome [8]. Inactivation of the Mterf4 gene leads to inhibition of mitochondrial translation and NSUN4 is no longer targeted to the LSU [8]. Moreover, in the absence of MTERF4, the SSU and LSU are present at increased levels but there is no formation of mature mitoribosomes [8]. The crystal structure of the NSUN4/MTERF4 heterodimeric complex has shown that the very stable interaction between both subunits occurs at the carboxy-terminus

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Author Summary

Mitochondria perform a number of essential functions in the cell, including synthesis of ATP via the oxidative phosphorylation (OXPHOS) system. Normal mitochondrial function requires coordinated expression of two genomes: mitochondria’s own genome (mtDNA), which encodes 13 respiratory chain subunits with essential structural and functional roles for the OXPHOS system, and the nuclear genome encoding the remaining ~80 subunits. The mtDNA-encoded polypeptides are synthesized on mitochondrial ribosomes (mitoribosomes) located in the mitochondrial matrix. Biogenesis, maintenance and regulation of the complex mitochondrial translation apparatus are poorly understood despite its fundamental importance for cellular energy homeostasis. Here, we show that inactivation of the Nsun4 gene, encoding a mitochondrial m^5C-methyltransferase, causes embryonic lethality, whereas tissue-specific disruption of Nsun4 in the heart causes cardiomyopathy with mitochondrial dysfunction. By performing sequencing of bisulfite-treated RNA we report that NSUN4 methylates C911 in 12S rRNA of the small ribosomal subunit. Surprisingly, NSUN4 can on its own perform this rRNA modification, whereas interaction with its partner protein MTERF4 is required for assembly of functional ribosomes. NSUN4 thus has dual roles in ribosome maturation and performs an important final quality control step to ensure that only mature mitoribosomal subunits are assembled into functional ribosomes.

Results

Nsun4 is essential for embryonic development in the mouse

We generated mice carrying a conditional knockout allele of the Nsun4 gene to determine the function of NSUN4 in mitochondria (Figure 1A). Nsun4^+/^−/− mice were mated with mice expressing cre-recombinase under the control of the β-actin promoter in order to obtain germine heterozygous knockout mice (Nsun4^+/^−). Inter-crossing of Nsun4^+/− mice did not result in any viable Nsun4^−/− mice, whereas Nsun4^+/^− and Nsun4^+/^− mice were recovered in mendelian proportions, consistent with embryonic lethality. Disruption of essential mitochondrial genes is frequently associated with embryonic lethality at ~E8.5 [4,8,21,22] and we therefore proceeded with analysis of embryos at this stage. Mutant embryos (Nsun4^−/−) exhibited severely retarded growth with no clearly discernible anatomical structures at E8.5, whereas embryos of the other genotypes appeared normal (Figure 1B). These findings show that NSUN4 has an essential in vivo role.

Heart specific knockout of Nsun4 leads to mitochondrial dysfunction

We next proceeded with inactivating the Nsun4 gene in skeletal muscle and cardiomyocytes by crossing Nsun4^+/^−/^−/act^−/− mice with mice expressing cre-recombinase under the control of a creatinine kinase promoter (Ckmm-cre). These conditional knockout mice were viable at birth, but had a much shorter life span than control mice with death before the age of 25 weeks (Figure 2A). We determined the heart to body weight ratio and found a gradual increase with age in the tissue-specific knockout mice (Nsun4^+/^−/act^−/−,+/(+Ckmm-cre), but not in control mice (Nsun4^+/^−/act^−/−) (Figure 2B). Such a progressive cardiomyopathy is commonly seen in mice with heart- and muscle-specific disruption of genes that are essential for oxidative phosphorylation and precedes any detectable phenotypes in skeletal muscle [4,8,21,22]. To assess oxidative phosphorylation capacity in heart, we analyzed the assembly of the five OXPHOS complexes using blue-native PAGE (BN-PAGE) of mitochondrial extracts from control and mutant mice (Figure 2C). This analysis showed that the steady-state levels of assembled OXPHOS complexes were unchanged in 3 weeks-old mice, but were drastically reduced in hearts from 20 weeks-old mutant mice. All complexes containing mtDNA-encoded polypeptides were reduced in abundance, whereas the steady-state levels of mitochondrial transcription factor A (mtTFA) remained unaffected. These findings are consistent with efficient knockout of the Nsun4 gene.

Mitochondrial transcription is increased in Nsun4-knockout hearts

Mitochondrial dysfunction in the heart triggers a compensatory increase in mitochondrial mass, which often is accompanied by an increase in the steady-state levels of mtDNA and an increase in mitochondrial transcription [4,8,15,21]. We determined mtDNA levels with semi-quantitative TaqMan PCR analysis and found a moderate increase in the steady-state levels in 20 weeks old mutant mice compared to age-matched controls (Figure S1A). This observation was corroborated by the finding of an increase in the steady-state levels of mitochondrial transcription factor A.
(TFAM), which typically varies with the levels of mtDNA [4], on western blot analysis (Figure S2B).

Next, we examined the steady-state levels of mtDNA-encoded transcripts using northern blotting and autoradiography (Figure S1B, C). All tested mitochondrial transcripts (rRNAs, tRNAs and mRNAs) were significantly increased in mutant mice (Figure S1B, C) consistent with a general upregulation of mitochondrial transcription. Indeed, pulse-labeling of newly synthesized transcripts in isolated mitochondria, followed by autoradiographic analysis of synthesized transcripts, revealed a strong increase in mitochondrial de novo transcription in mutant mice compared to controls (Figure S2A). Consistent with these data, western blot analyses showed an increase in the steady-state levels of the mitochondrial transcription factor TFB2M and TFAM in mitochondrial extracts (Figure S2B). Additionally, mutant mitochondria had increased steady-state levels of LRPPRC (Figure S2C), a stability factor for mitochondrial mRNAs, whose steady-state levels correlate with the levels of mitochondrial mRNAs [22]. Thus, knockout of Nsun4 in the heart leads to increased de novo synthesis and increased steady-state levels of mitochondrial transcripts.

Mitochondrial assembly and mitochondrial translation are inhibited in the absence of Nsun4

We have previously shown that NSUN4 is targeted to the LSU through its physical interaction with MTERF4 and that absence of MTERF4 leads to defective ribosomal assembly and inhibition of mitochondrial translation [8]. We therefore proceeded to determine if mitochondrial translation also was inhibited in Nsun4-knockout hearts. De novo labeling of mitochondrial translation products, followed by gel electrophoresis and autoradiography, revealed a strong inhibition of translation in mutant mitochondria (Figure 3A). Consistent with this, the steady-state levels of the mitochondrially encoded ATP6 and COX II proteins were decreased in knockout hearts (Figure 3B).

Next, we analyzed the assembly of the mitoribosome by ultracentrifugation of control and mutant mitochondrial extracts through linear-density sucrose gradients (Figure 3C). Sedimentation of the SSU and LSU was determined by immunological detection of the protein markers MRPS15 and MRPL13, respectively. In control extracts, we observed a typical sedimentation pattern of the separate SSU (28S) and LSU (39S) as well as of co-sedimentation of both subunits in fully assembled (55S) ribosomes [4,8,22]. In contrast, knockout mice exhibited an accumulation of assembled SSU and LSU without a corresponding increase in assembled ribosomes (Figure 3C). Furthermore, the steady-state levels of proteins from the LSU and SSU were increased in knockout mice (Figure 3D). We have previously reported that when ribosomal assembly and/or function are not inhibited, an accumulation of both the SSU and LSU coincides with accumulation of assembled ribosomes [22]. The data we present here therefore indicate that lack of NSUN4 inhibits the
association between the SSU and LSU to form functional ribosomes.

NSUN4 methylates C911 in 12S rRNA

Previous reports have shown that NSUN4 is an m^5C-methyltransferase proposed to methylate an unknown residue in 16S rRNA [8–10]. However, studies on hamster mitochondria have shown that the 17S rRNA of the LSU does not contain any C5-methylated residues, unlike 13S rRNA of the SSU, which contains one m^4C and one m^5C at relative position 911 and 913, respectively [6]. We revisited these data by attempting to map all m^5C residues in mouse 12S and 16S rRNA by using sequencing of cDNA generated from bisulfite treated RNA. The bisulfite method is based on the chemical conversion of

Figure 2. Conditional inactivation of the Nsun4 gene in heart and skeletal muscle. A. Decreased lifespan of mice with a muscle-specific knockout of Nsun4. Survival curve for control (L/L; n = 20; open squares) and mutant mice (L/L, cre; n = 16; filled squares). B. Heart- to body-weight ratio in control (L/L; open squares) and mutant mice (L/L, cre; filled squares). Number of analyzed animals at 5 weeks L/L n = 6, L/L, cre n = 6; at 10 weeks L/L n = 7, L/L, cre n = 8; at 15 weeks L/L n = 5, L/L, cre n = 7; at 20 weeks L/L n = 12, L/L, cre n = 12. Data are represented as mean +/- SEM. *, p<0.05; **, p<0.01; ***,p<0.001. Student's t test. C. BN-PAGE analysis of levels of assembled respiratory chain complexes in control (L/L) and knockout (L/L, cre) mice at different ages. D. Western immunoblotting of steady-state levels of NSUN4 in heart mitochondrial extracts from control (L/L) and knockout (L/L, cre) mice at different ages. VDAC was used as a loading control. doi:10.1371/journal.pgen.1004110.g002
Figure 3. Mitochondrial translation and ribosome assembly in Nsun4 knockout hearts. A. Pulse-labeling of mitochondrial translation products in isolated heart mitochondria from 20 weeks-old control (L/L) and knockout mice (L/L, cre). The Coomassie-stained gel is a loading control. Known mitochondrial polypeptides are indicated. B. Western blot analysis of steady-state levels of mitochondrially and nucleus-encoded OXPHOS proteins in mitochondria from control and knockout hearts at different ages. VDAC was used as a loading control. *, cross reaction. C. Analysis of mitoribosomal assembly by sucrose gradient ultracentrifugation of heart mitochondrial extracts from control (L/L) and mutant (L/L, cre) mice. Sedimentation of 28S (SSU, fraction 6), 39S (LSU, fraction 8) and 55S (assembled ribosomes, fraction 10) was determined by western blot analysis using MRPS15- and MRPL13-specific antibodies. D. Western blot analysis of steady-state levels of MRPL13 and MRPS15 in heart mitochondrial extracts from control (L/L) and knockout (L/L, cre) mice at different ages. VDAC was used as a loading control.

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non-methylated cytosine to uracil, whereas m^5C residues are resistant to this treatment and remain unchanged in the treated RNA [23]. Other modifications, like m^4C can also be detected, but with a lower frequency. Using this approach we probed the methylation status of heart mitochondrial rRNA from control mice (Figure 4A) and detected a single C5-methylated cytosine residue at position 911 in 12S rRNA (methylation rate 87.5 ± 0.9%). Next, we assessed the methylation status of heart mitochondrial rRNA from Nsun4 knockout mice and found that methylation on C911 was now essentially absent (methylation rate 2.5 ± 2.4%; Figure 4B). Alignment of the mouse and hamster sequences revealed that m^5C911 in mouse 12S rRNA corresponds to the previously reported m^5C913 in hamster 13S rRNA (Figure S3A). Thus, NSUN4 methylates C911 in 12S rRNA, and this modification is likely the only m^5C-methylation present in mammalian mitochondrial rRNAs.

In addition to m^5C911, there was also a cytosine at position 909 (C909) in mouse 12S rRNA that showed partial resistance to bisulfite treatment (methylation rate 23.6 ± 1.7%; Figure 4A). The corresponding nucleotide in hamster 13S rRNA has previously been reported to harbor an m^4-methylation [6]. The m^4C909 modification in mouse 12S rRNA was not affected (methylation

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Figure 4. Analysis of rRNA methylation in control, Nsun4 and Mterf4 heart tissue specific knockout. A. Relative methylation levels of 12S and 16S rRNA determined after sequencing of cDNA obtained from bisulfite treated RNA from heart mitochondria of control (L/L; n = 3) mice at age 20 weeks. Nucleotide numbers are relative to the 5'-end of the mouse mtDNA gene for tRNA^Phe. B. Relative methylation levels of 12S and 16S rRNA in NSUN4 knockout (L/L, cre; n = 3) at age 20 weeks. Analysis performed as in panel a. C. Relative methylation levels of 12S rRNA in control (N = 1) and MTERF4 knockout (N = 2) at age 15 weeks and in control (N = 1) and MTERF4 knockout (N = 2) at age 20 weeks.

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Figure 5. rRNA binding by NSUN4 and MTERF4 and model of the role of the NSUN4/MTERF4 complex in regulation of ribosome assembly. A. Location, along mtDNA, of the RNA fragments identified after CLIP analysis on HeLa cells expressing “trap” mutant of NSUN4, NSUN4C258A-FLAG. B. Location, along mtDNA, of the RNA fragments identified after PAR-CLIP analysis on HeLa cells expressing MTERF4-FLAG. C. Model for the role of NSUN4 in mitoribosomal assembly. (1) NSUN4 methylates 12S rRNA. Red star denotes the methyl group donated by SAM. (2) The NSUN4/MTERF4 complex is incorporated into the LSU. (3) Release of the NSUN4/MTERF4 complex from LSU enables the interaction between both subunits.

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showing that the m5C911 modification is specifically generated by SSU, resulting from loss of m5C, could possibly affect these maturation of the SSU [4]. Assembly defects of the mitoribosomal RNA may interact with two different LSU regions simultaneously. Unfortunately, there is no atomic resolution structure the mitochondrial ribosome and we can therefore not exactly pinpoint the location of these regions. The RNA sequences identified by the CLIP experiments are predicted to form mixed double- and single-stranded structures [26] and we therefore tested whether the MTERF4/NSUN4 complex has any structural preferences when binding RNA. We performed gel shift experiments with short RNA fragments incubated with the recombinant NSUN4/ MTERF4 complex or the recombinant NSUN4 protein. The NSUN4/MTERF4 complex binds RNA fragments with a pronounced double-stranded conformation (Figure S3A; right panel), whereas it poorly binds single-stranded RNA fragments derived from the same double-stranded RNA fragment (Figure S3A; left panels). These findings indicate that NSUN4/MTERF4 preferentially binds double-stranded RNA. In view of our finding that NSUN4 is able to methylate C911 independent of its interaction with MTERF4, we also tested if NSUN4 directly can bind an RNA fragment (ds12S: 878–949) containing the methylation site and indeed found such a specific interaction (Figure S5B), showing that NSUN4 can bind RNA on its own in the absence of MTERF4.

**Discussion**

We demonstrate here that NSUN4 unexpectedly is a dual function protein. On the one hand, NSUN4 can alone methylate C911 in 12S rRNA and on the other hand NSUN4 in complex with MTERF4 is targeted to the LSU to regulate mitoribosomal assembly. The role for NSUN4 in rRNA modification can be uncoupled from its role in mitoribosomal assembly as Mterf4 knockout mice cannot assemble functional ribosomes despite the presence of the methylated C911 residue. Also other methyltransferases have been suggested to have dual functions. The yeast methyltransferase, Dim1p, which generates the highly conserved m5Am6A on cytoplasmic SSU rRNA, also has a role in rRNA processing [27]. With the use of different dim1 alleles both functions can be uncoupled, revealing that dimethylation is dispensable for growth whereas the role in rRNA processing is essential [28]. The bacterial ortholog of Dim1p, KsgA, has also been suggested to have dual roles in SSU biogenesis by establishing the m6A2Am6A modification of the SSU rRNA and by sterically blocking access for binding of the LSU and the initiation factor 3 to regulate the formation of assembled bacterial ribosomes [29,30]. Moreover, conformational changes upon binding of the methyltransferase RsmC to its substrate, G1207, were proposed to protect rRNA against misfolding during LSU assembly [31]. Similar to the examples above, we hypothesize that the NSUN4/MTERF4 methylation complex plays a critical structural role during LSU assembly through its affinity for double-stranded rRNA. The NSUN4/MTERF4 complex, when bound to the LSU, may inhibit the formation of assembled ribosomes by two possible, but not necessarily mutually exclusive, mechanisms: i) by physically preventing interaction between both ribosomal subunits and ii) by occluding a putative 16S rRNA fragment required for subunit interaction or LSU activation. Ribosomal assembly in bacteria is characterized by continuous structural rearrangements of rRNA, which sometimes transit through misfolded states stabilized through interactions with ribosomal proteins or ribosomal assembly factors [32]. Later, these states are resolved through refolding of rRNA, release of the ribosomal assembly factors or both. It is possible that release of the NSUN4/MTERF4 complex results in conformational changes in 16S rRNA, which in turn can activate
the LSU to enable and stabilize its interaction with the SSU. Thus the NSUN4/MTERF4 complex may also play an important structural role by preventing premature entry into translation.

The exact mechanistic function of m$^3$-methylation on cytosine in rRNA is generally unclear. An m$^3$-methylation does not impair base pairing nor does it appear to induce conformational changes in the modified nucleotide. However, studies on rRNA$^{32}$P and rRNA$^{3H}/$AMC have suggested that m$^3$C, likely in cooperation with other modified residues, is involved in the stabilization of rRNA folding under physiological conditions [33–35]. Similarly, m$^3$C911 may cooperate with the nearby m$^4$C909 and other rRNA modifications in stabilization of 12S rRNA folding, thereby facilitating mitoribosomal assembly. Detailed crystallographic analyses on mitoribosomal structure are lacking, which hinders a detailed understanding of the importance of C911 for mitoribosomal assembly.

Based on the analyses of our mutant mouse strains, we have attempted to build a preliminary model for the late stages of mitoribosomal subunit assembly and the formation of translation-competent assembled ribosomes (Figure 5C). According to this model, NSUN4 methylates C911 in 12S rRNA of SSU (Figure 5C: step 1). This step does not require interaction with MTERF4, suggesting that NSUN4 may be targeted by either interacting directly with the rRNA substrate or by interacting with an unknown SSU protein. Other modifications, like m$^6$A1006 and m$^2$A2A1007 by TEB1M, are established independently of m$^3$C911 and stabilize the newly formed SSUs. NSUN4/MTERF4 complexes are incorporated into the LSU during its assembly (Figure 5C: step 2). LSU-bound NSUN4/MTERF4 complexes prevent partially assembled LSU from forming abortive 55S complexes with available SSUs. Finally, LSU-bound NSUN4/MTERF4 complexes are dissociated from the LSU to enable its interaction with the SSU to form translation-competent ribosomes (Figure 5C; step 3).

In conclusion, we show here that NSUN4 is a dual function protein involved in coordinating ribosomal biogenesis in mammalian mitochondria by methylation C911 on the 12S rRNA and by interacting with MTERF4 to regulate the assembly of the two ribosomal subunits. We propose that NSUN4 functions as a quality control step late in ribosomal biogenesis to ensure that only mature SSUs and LSU are assembled into functional mitoribosomes.

**Methods**

**Ethics statement**

This study was performed in strict accordance with the recommendations and guidelines of the Federation of European Laboratory Animal Science Associations (FELASA). The protocol was approved by the “Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen”.

**Generation of Nsun4 knockout mice**

The $Nsun4^{loxP/loxP}$ mice, that have exon II flanked by loxP-sites, were generated at TacomicArtemis GmbH (Cologne, Germany). The $Nsun4^{loxP/loxP}$ mice were mated with mice ubiquitously expressing cre-recombinase to generate heterozygous knockout mice ($Nsun4^{+/loxP}$). Heart- and skeletal muscle-specific knockout mice were generated as described previously [4,8,21,22]. $Nsun4^{loxP/loxP}$ mice were crossed with transgenic mice expressing cre-recombinase under the control of the muscle creatinine kinase promoter ($Cmmv$-cre). The resulting double heterozygous mice ($Nsun4^{loxP/loxP}, +/Cmmv$-cre) and control ($Nsun4^{loxP/loxP}, +/Cmmv$-cre) mice to generate tissue-specific knockout ($Nsun4^{loxP/loxP}$, +/Cmmv-cre) and control ($Nsun4^{loxP/loxP}, +/Cmmv-cre$) mice.

**Bisulfite mapping of m$^5$C residues in mitochondrial rRNA**

RNA for bisulfite treatment was isolated using the miRNeasy Mini Kit (QIAGEN) and treated with TURBO DNase (Ambion) to remove mitochondrial DNA. Bisulfite treatment was performed as described previously [34]. Treated RNA was converted to cDNA and sequenced using the Illumina platform. Reads were submitted to fastqc for quality analysis. Fastq files were clipped with fastx_trimmer (fastx toolkit, http://hannonlab.cshl.edu/fastx_toolkit/) to remove the first 4 and last 35 bases, which were composed of lower quality bases at the start and end of the sequences. Clipped paired reads were aligned to the mouse reference genome with the Bismark software [36]. Bismark performs bisulfite mapping and allows calculation of methylation rates via perl scripts. Bisulfite reads are transformed into a C-to-T and G-to-A version (reverse strand) which are aligned to equivalently pre-converted forms of the reference genome using four parallel instances of the Bowtie aligner [37]. Duplicate sequences were removed and data were reevaluated. Methylation rates were calculated using Perl scripts from the Bismark website, (http://www.bioinformatics.babraham.ac.uk/projects/bismark/).

**BN-PAGE and in organello experiments**

BN-PAGE experiments were performed using NativePAGE Novex Bis-Tris Gel System (Invitrogen) according to the manufacturer’s recommendations. Heart mitochondria (40 μg) were solubilized with NativePAGE Sample Buffer containing 1% dodecylmaltoside (DDM). After 20 minutes on ice, samples were centrifuged (30 min, 16000 g, 4°C). Supernatants were supplemented with NativePAGE G250 Sample Additive and fractionated through 4–16% NativePAGE Novex Bis-Tris Gel. Respiratory chain complexes were transferred onto PVDF membrane and detected using specific antibodies. Pulse labeling of mitochondrial transcription products was performed in isolated mitochondria according to [38,39]. In organello translation was performed as previously described [22,40].

**Linear-density sucrose gradients**

Assembly of 28S and 39S ribosomal subunits as well as 55S monosomes was assayed using ultracentrifugation through a 10–30% linear-density sucrose gradient as described previously [22].

**Quantification of mitochondrial DNA using TaqMan RT-PCR**

For quantification of mtDNA, total DNA was isolated from heart tissue using the DNeasy Blood & Tissue Kit (QIAGEN). Semi-quantitative RT-PCR was carried out on 4 ng of total DNA in a 7900HT Real Time PCR system (Applied Biosystems), using TaqMan probes specific for the CoxI and 18S genes (Applied Biosystems).

**Gel shift assays**

Purification of human recombinant NSUN4/MTERF4 complex for gel shift experiments was done according to [9]. RNA fragments were purchased from Thermo Scientific or Eurofins MWG Operon. Gel shift experiments were carried out using varying quantities of NSUN4/MTERF4, and in the presence of 40 ng RNA essentially as described in [15]. Fragment ds12S:878–949, which carries C911 in human 12S rRNA, contains three G and three C residues at the 5’- and 3’-end, respectively, to prevent

**NSUN4 Regulates Mitoribosomal Biogenesis**

![Image](http://www.plosgenetics.org/hs1000110/fg_1.png)
formation of single-stranded termini. See Table S3 for sequences of the RNA fragments used for gel shift experiments.

**Primer extension.** Primer extensions were performed on total RNA from heart tissue as described previously [4]. Samples were analyzed by size fractionation through a 6% polyacrylamide-7M urea gel and subjected to autoradiography.

**Nucleic acid sequence alignments**

Sequence alignments were performed using Clustal Omega [41] at default settings and were visualized using GeneDoc at shade level 1 [42]. Sequence accession numbers are given in figure legends.

**Northern blot analysis**

RNA for northern blot analysis was isolated using Trizol Reagent (Invitrogen) and resuspended in formamide (Ambion). For detection of mitochondrial transcripts, 1–2 μg of total RNA was denatured in NorthernMax-Gly Sample Loading Dye (Ambion), separated in 1.2% agarose gels containing formaldehyde (SIGMA-Aldrich) and transferred to Hybond-N membranes (GE Healthcare). DNA probes, for the detection of mitochondrial mRNAs and rRNAs, were radiolabeled with α-32P-dCTP using the Prime-It II random primer labeling kit (Stratagene). For detection of tRNAs, oligonucleotides were labeled with γ-32P-ATP using T4-poly nucleotide kinase (NEB).

**Western blot analysis**

Rabbit polyclonal antisera were used for the detection of TFAM, ATP6 and COXII [43]. A rabbit polyclonal antisera was used for detection of LRPPRC [22]. Affinity purified rabbit polyclonal antibodies were used for the detection of TF2BM [4], MRPS15 [4] and MRPL13 [8]. Monoclonal antibodies against VDAC were purchased from Calbiochem. Immunodetection of NDUFa9, SDHA, UQCRC2, COX IV and ATP5A1 was performed with monoclonal antibodies from MitoSciences. Monoclonal antibodies against mouse NSUN4 were generated by AbD Serotec.

**DNA constructs and cell lines**

The HeLa cells stably transfected with pTreTight-hMterf4-FLAG DNA construct are described previously [8]. HeLa cell clones stably transfected with pTreTight-hNSUN4-FLAG and pTreTight-hNSUN4C258A-FLAG were generated as described [8].

**Crosslinking Immunoprecipitation (CLIP)**

Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP) was performed by combining and adapting previously described methods [44,45]. HeLa cells transfected with doxycycline-inducible MTERF4-FLAG, NSUN4-FLAG and NSUN4C258A-FLAG containing DNA constructs were induced with 1μg/ml doxycycline and grown in the presence of 4-thiouridine (4-SU; final concentration of 100 μM) for 14 hours. The cells from 20 culture dishes (500 cm² each) were washed with PBS, crosslinked at 365 nm, collected and frozen in liquid nitrogen. Next, cells were lysed, treated with RNase T1 and used for immunoprecipitation experiments with ANTI-FLAG M2 magnetic beads according to the manufacturer’s recommendations (Sigma). The samples were further processed according to [44].

**Supporting Information**

**Figure S1** Steady-state levels of mtDNA and mitochondrial transcripts. A. Steady-state levels of mtDNA in hearts from 20 weeks-old control (L/L; n = 3) and knockout (L/L, cre; n = 3) mice. mtDNA was detected using a C911-specific TaqMan probe. 18S rRNA was used as a loading control. Data are represented as mean ±/− SEM. **p<0.001; Student’s t test. B. Northern blot analysis of the steady-state levels of mitochondrially encoded rRNAs, mRNAs and tRNAs from 20-week-old control (L/L) and mutant (L/L, cre) mice. Nucleus-encoded 18S rRNA is used as a loading control. C. Quantification of the steady-state levels of rRNA, mRNA and tRNA detected by autoradiography. For rRNA and mRNA: number of analyzed animals at 5 weeks, L/L n = 3; L/L, cre n = 3; at 10 weeks, L/L n = 4; L/L, cre n = 4; at 15 weeks, L/L n = 4; L/L, cre n = 4; 20 weeks, L/L n = 4; L/L, cre n = 4. For tRNA: number of analyzed animals at 20 weeks, L/L n = 4; L/L, cre n = 4. Data are represented as mean ±/− SEM. *p<0.05; **p<0.01; ***p<0.001. Student’s t test. (TIF)

**Figure S2** De novo transcription in NSUN4 knockout hearts. A. Analysis of mitochondrial transcription after pulse labeling in isolated heart mitochondria from 20-week-old control (L/L) and knockout mice (L/L, cre). B. Western blot analysis to determine steady-state levels of TF2BM and TFAM in mitochondrial extracts from control (L/L) and knockout (L/L, cre) mice at 5 and 20 weeks of age. C. Western blot analysis of LRPPRC levels in mitochondrial extracts from control (L/L) and knockout (L/L, cre) mice at 5, 10, 15 and 20 weeks of age. (TIF)

**Figure S3** Sequence alignments and analysis of adenine dimethylation in 12S rRNA. A. Nucleotide sequence alignment of the rRNA of the SSU containing the m4C and m5C modification in hamster (M. auroa, NC_013276.1), mouse (M. musculus, NC_005089.1) and human (H. sapiens, NC_012920.1). The location of m4C and m5C is indicated. Nucleotide numbering is from the 5′-end of tRNA^Pro^. B. Primer extension analysis of 12S rRNA from control (L/L) and tissue-specific NSUN4 knockout mouse (L/L, cre). Autoradiography of samples separated in a polyacrylamide-urea gel is shown. Primer extension with an oligonucleotide annealing close to the 5′-end of 12S rRNA gives rise to the extension product P1, which is used as a loading control. Dimethylation of 12S rRNA at A1006 and A1007 cause a partial stop of the primer extension reaction and thereby generates the extension product P2. Unextended primers are indicated in the bottom of the figure. C. Quantification of the ratio of primer extension products P2 to P1 (see panel A) in control (L/L, n = 4) and tissue-specific NSUN4 knockout (L/L, cre, n = 4) mice. Data represent mean ±/− SEM. (TIF)

**Figure S4** C911 methylation rate in Mterf3-knockout mice. Relative methylation rate of 12S rRNA in control (N = 1) and Mterf3 knockout (N = 2) hearts at 14 weeks of age. (TIF)

**Figure S5** rRNA binding by NSUN4 and NSUN4/MTERF4 complex tested by EMSA. A. Gel shift assays to determine binding of the recombinant NSUN4/MTERF4 complex to ssRNA and dsRNA of 16S rRNA. Filled triangles denote increasing concentrations of recombinant proteins: 0, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56 μM. Nucleotide numbering is relative to the 5′-end of the human mitochondrial gene for tRNAPhe. ss, single-stranded; ds, double-stranded (first row). B. Gel shift assays to determine binding of the recombinant NSUN4 to a double-stranded fragment from 12 rRNA containing the methylation substrate C911. Analysis was performed as in A. (TIF)
Table S1: Sequences of the RNA fragments identified after CLIP experiments performed on HeLa cells expressing NSUN4C258A-FLAG. Positions of the RNA fragments along mtDNA relative to the beginning of rNA16 are indicated. C911 is indicated in red.

(DOC)

Table S2: Sequences of the RNA fragments identified after PAR-CLIP experiments performed on HeLa cells expressing MTERF4-FLAG. Positions of the RNA fragments along mtDNA relative to the beginning of rNA16 are indicated. Results from two independent experiments were pooled.

(DOC)

Table S3: Sequences of the RNA fragments used for gel shift experiments. Positions and sequences of the RNA fragments used for the gel shift experiments are listed in the table.

(DOC)

Author Contributions
Conceived and designed the experiments: MDM BR NGL. Performed the experiments: MDM BR HS PLP CB JA. Analyzed the data: MDM BR CM BH. Wrote the paper: MDM BR NGL.

References