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Article

Methylation of 12S rRNA Is Necessary for In Vivo Stability of the Small Subunit of the Mammalian Mitochondrial Ribosome

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SUMMARY

The 3′ end of the rRNA of the small ribosomal subunit contains two extremely highly conserved dimethylated adenines. This modification and the responsible methyltransferases are present in all three domains of life, but its function has remained elusive. We have disrupted the mouse *Tfb1m* gene encoding a mitochondrial protein homologous to bacterial dimethyltransferases and demonstrate here that loss of TFB1M is embryonic lethal. Disruption of *Tfb1m* in heart leads to complete loss of adenine dimethylation of the rRNA of the small mitochondrial ribosomal subunit, impaired assembly of the mitochondrial ribosome, and abolished mitochondrial translation. In addition, we present biochemical evidence that TFB1M does not activate or repress transcription in the presence of TFB2M. Our results thus show that TFB1M is a nonredundant dimethyltransferase in mammalian mitochondria. In addition, we provide a possible explanation for the universal conservation of adenine dimethylation of rRNA by showing a critical role in ribosome maintenance.

INTRODUCTION

The mitochondrial network of mammalian cells harbors the oxidative phosphorylation system, which produces the bulk part of cellular energy in the form of ATP to drive a variety of metabolic reactions (Saraste, 1999). Deficient mitochondrial energy production is heavily implicated in various types of human pathologies, e.g., inherited diseases, age-related chronic diseases, and the aging process (Wallace, 1999; Terzioglu and Larsson, 2007). The biogenesis of the respiratory chain is uniquely dependent on the coordinated expression of both nucleus- and mtDNA-encoded subunits (Scarpulla, 2008). However, only a minority of the respiratory chain subunits are encoded by mtDNA, but these subunits are nevertheless essential, as disruption of mtDNA-expression leads to severely impaired respiratory chain function (Larsson et al., 1998). The regulation of mtDNA expression is under nuclear genetic control (Falkenberg et al., 2007), which further adds to the complexity involved in coordinating cellular energy production.

Mitochondrial transcription factors B1 and B2 (TFB1M and TFB2M, also denoted mtTFB1 and mtTFB2) are important examples of factors involved in regulation of mitochondrial gene expression in mammalian cells (Falkenberg et al., 2002). Both of these factors are nucleus encoded and imported to mitochondria. Their expression is tightly regulated by nuclear transcription factors that control mitochondrial biogenesis, i.e., the PGC-I group of coactivators and the nuclear respiratory factors 1 and 2 (Gleyzer et al., 2005; Spiegelman, 2007; Scarpulla, 2008). In mitochondria, TFB1M and TFB2M form heterodimers with mitochondrial RNA polymerase (POLRMT), and in vitro experiments have demonstrated that both factors can activate mtDNA transcription (Falkenberg et al., 2002). Surprisingly, the TFBM factors may also have a role in translation, as they are related to a class of methyltransferases present in all three domains of life, i.e., in archaebacteria, eubacteria, and eukaryotes (cytosol and mitochondria) (Falkenberg et al., 2002; McCulloch et al., 2002; Shutt and Gray, 2006). These enzymes dimethylate two adjacent adenines in a stem-loop structure close to the 3′ end of the small subunit rRNA (Helser et al., 1971; Poldermans et al., 1979, 1980). This rRNA modification is extremely highly conserved in nature, and only a handful of organisms are known that lack it (Shutt and Gray, 2006; Xu et al., 2008). In bacteria, the modified adenine residues are present in a region of the small subunit of the ribosome that contains the mRNA decoding center and the binding site for the large ribosome subunit (Xu et al., 2008). The critical location of the modified residues within the ribosome and the extreme conservation suggest an important role in regulation of translation. Unexpectedly, *E. coli* lacking this methyltransferase, denoted KsgA, and the corresponding small ribosomal subunit rRNA modification are viable with only a slight growth disadvantage and gain resistance to the antibiotic kasugamycin (Helser et al., 1971; van Buul et al., 1984; Desai and Rife, 2006; Xu et al., 2008). There are thus many questions concerning the role of this highly conserved modification that likely has an important but yet unknown role in ribosome function. Studies of the role for TFB1M and TFB2M in mammalian mitochondria could therefore be a valuable strategy to increase our understanding in this area. Furthermore, there is also a need to definitively resolve the dilemma concerning conflicting data of the distinct roles for TFB1M and TFB2M in mammals. TFB2M has

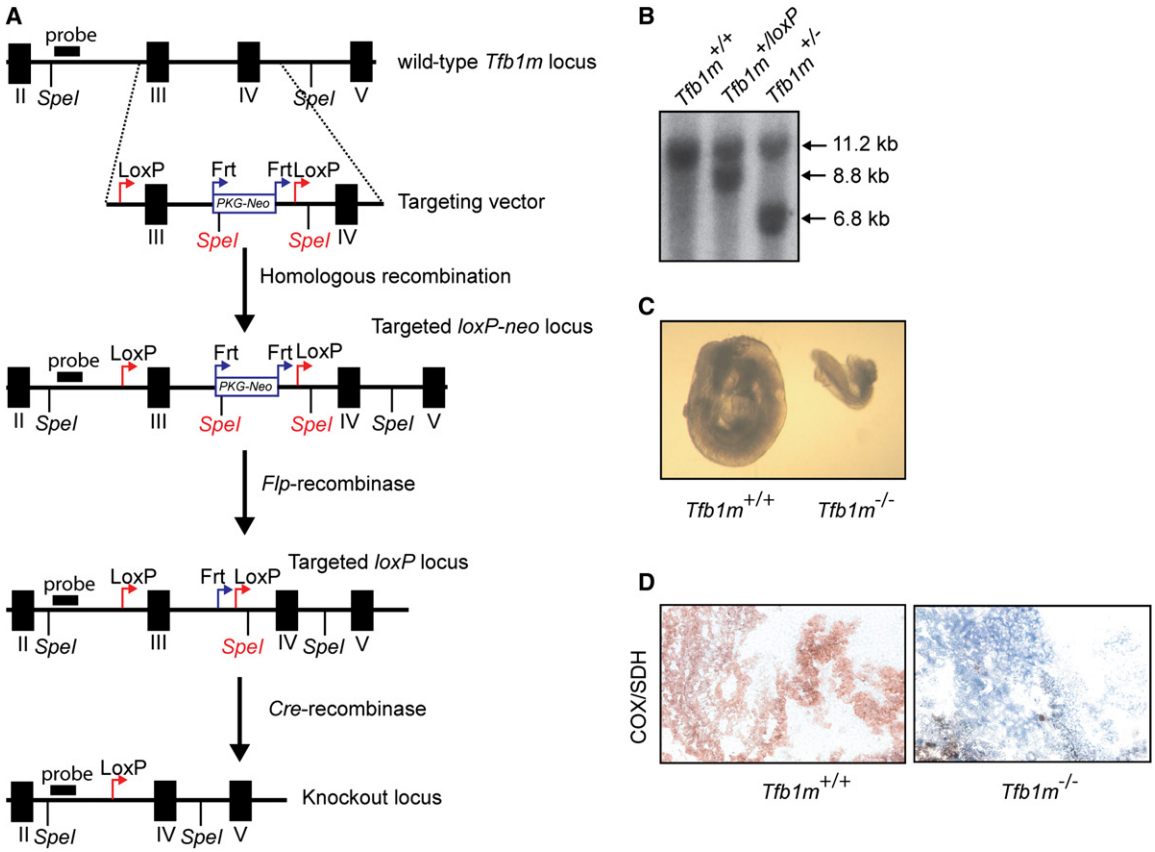

Figure 1. Conditional Knockout of *Tfb1m* and Phenotypes in Homozygous Knockout Embryos

(A) Targeting strategy for disruption of the *Tfb1m* genomic locus. Screening of ESCs was done using the indicated SpeI endonuclease cleavage sites (endogenous: black; introduced: red) and a probe outside the region of homology in the targeting vector.

(B) Southern blot analysis of tissues from wild-type ($Tfb1m^{+/+}$), heterozygous targeted ($Tfb1m^{+/loxP}$), and heterozygous knockout ($Tfb1m^{+/-}$) mice. SpeI digest generates specific fragments of 11.2 kb (wild-type allele), 8.8 kb (targeted allele), and 6.8 kb (knockout allele).

(C) Morphological comparison between wild-type ($Tfb1m^{+/+}$) and knockout ($Tfb1m^{-/-}$) embryos at embryonic day ~E8.5. $Tfb1m^{-/-}$ embryos have a mutant morphology.

(D) Enzyme histochemical double staining for cytochrome *c* oxidase (COX; mitochondria-encoded) and succinate dehydrogenase (SDH; nucleus-encoded) activities in sections from $Tfb1m^{+/+}$ and $Tfb1m^{-/-}$ embryos. Blue staining indicates lack of COX activity in $Tfb1m^{-/-}$ embryos. Magnification, 20×.

been reported to be at least an order of magnitude more active than TFB1M in promoting in vitro transcription of mtDNA in a pure recombinant system (Falkenberg et al., 2002), which suggests that TFB1M is, at best, a very poor transcription factor. Arguing against this interpretation, it has been reported that addition of recombinant TFB1M to a mitochondrial extract dramatically stimulates in vitro transcription of mtDNA (McCulloch et al., 2002; McCulloch and Shadel, 2003). On the other hand, there are reports that both TFB1M and TFB2M act as methyltransferases. First, both proteins have been reported to have the capacity to bind s-adenosyl methionine, which is the methyl donor for the methyltransferase reaction (Seidel-Rogol et al., 2003; Cotney and Shadel, 2006). Second, expression of either TFB1M or TFB2M can restore deficient methylation of the small ribosomal subunit rRNA in bacteria lacking KsgA (Seidel-Rogol et al., 2003; Cotney and Shadel, 2006). To address the role of the highly conserved adenine dimethylation of the small subunit rRNA and to elucidate possible distinct functions of TFB1M and TFB2M, we have conditionally inactivated the *Tfb1m* gene in the mouse.

RESULTS

TFB1M Is Essential for Embryonic Development in Mouse

We created a conditional *Tfb1m* knockout mouse to address the in vivo function of TFB1M (Figure 1A). Heterozygous mice derived from germline transmission of the targeted $Tfb1m^{+/loxP\text{-}neo}$ allele were mated to *Flp*-deleter mice to remove the neomycin selection marker, thus generating heterozygous $Tfb1^{+/loxP}$ mice, which, in turn, were crossed with mice ubiquitously expressing *Cre* recombinase under the control of the β-actin promoter (Figures 1A and 1B). The resulting heterozygous *Tfb1m* knockout ($Tfb1m^{+/-}$) mice were intercrossed to obtain homozygous knockouts ($Tfb1m^{-/-}$). However, no live $Tfb1m^{-/-}$ mice were born, indicating embryonic lethality. We then proceeded to analyze staged embryos and recovered mutant embryos at a frequency of ~23% at E8.5. The embryos with a mutant phenotype were small, the neural cleft was open, no optic disc was present, and no heart structure was found (Figure 1C). Genotyping showed that the easily recognizable

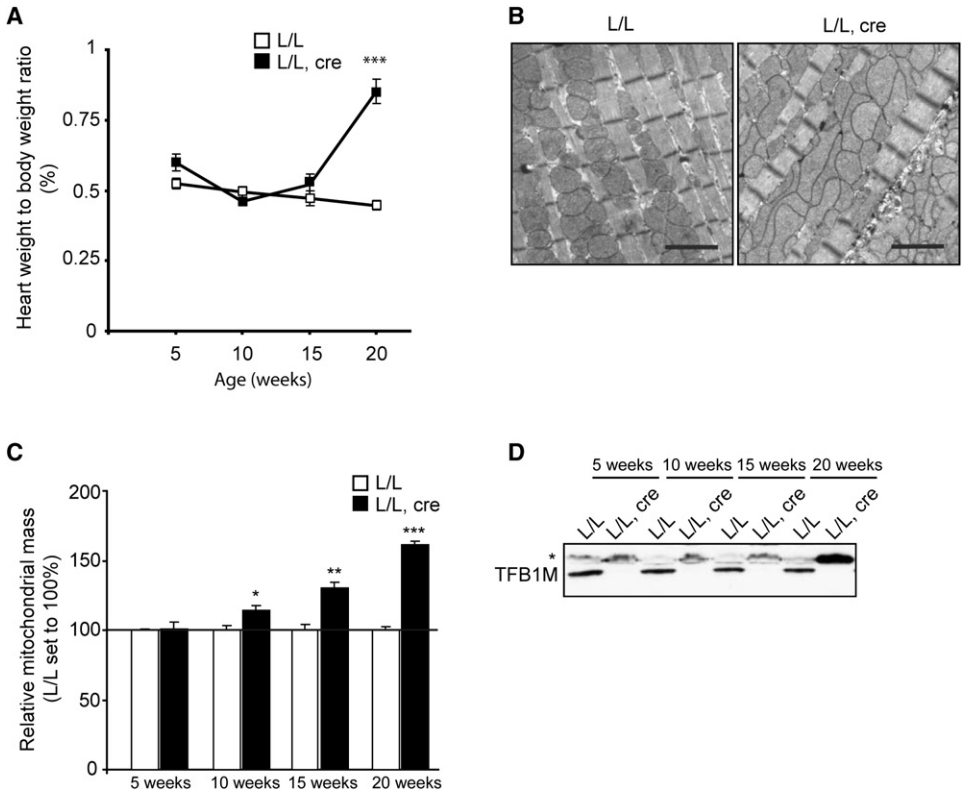

Figure 2. Cardiomyopathy and Mitochondrial Proliferation in TFB1M-Deficient Hearts

(A) Heart weight to body weight ratio in *Tfb1m*$^{loxP/loxP}$ (control, L/L) and tissue-specific *Tfb1m* knockout mice (L/L, cre) at different ages in weeks. At 5 weeks: L/L n = 10, L/L, cre n = 8; 10 weeks: L/L n = 5, L/L, cre n = 6; 15 weeks: L/L = 6, L/L, cre n = 8; 20 weeks: L/L n = 19, L/L, cre n = 16. Error bars indicate SEM; ***p < 0.001; Student's t test.

(B) Electron micrographs of heart tissue of 20-week-old control and knockout mice. Scale bar, 2 μm.

(C) Quantification of mitochondrial mass after electron microscopic analysis of heart tissue from control and tissue-specific *Tfb1m* knockout mice at different ages. Error bars indicate SEM; *p < 0.05; **p < 0.01; ***p < 0.001; Student's t test.

(D) Western blot analysis of TFB1M protein levels in heart mitochondrial extracts from control and *Tfb1m* knockout mice at different ages. *, crossreacting band.

mutant embryos (n = 8) were all homozygous knockouts (*Tfb1m*$^{-/-}$), whereas the remaining normally appearing embryos (n = 27) had the genotype *Tfb1m*$^{+/-}$ (n = 20) or *Tfb1m*$^{+/+}$ (n = 7). We performed staining to simultaneously detect the enzyme activities of cytochrome *c* oxidase (COX; catalytic subunits encoded by mtDNA) and succinate dehydrogenase (SDH; all subunits nucleus encoded) on tissue sections from *Tfb1m*$^{+/+}$ and *Tfb1m*$^{-/-}$ embryos. We found a severe decrease of COX activity, yet preserved SDH activity, in *Tfb1m*$^{-/-}$ embryos (Figure 1D). The *Tfb1m*$^{-/-}$ embryos thus have a severe respiratory chain deficiency and mutant morphology and display embryonic lethality at ~E8.5, which are all typical features of embryos with homozygous disruption of nuclear genes, e.g., *Tfam* (Larsson et al., 1998), *PolgA* (Hance et al., 2005), or *Mterf3* (Park et al., 2007), needed for mtDNA maintenance and expression.

Tissue-Specific Disruption of *Tfb1m* Causes Mitochondrial Cardiomyopathy

We proceeded to study the role of TFB1M in a differentiated mouse tissue by characterizing hearts from mice with disruption of the *Tfb1m* gene in heart and skeletal muscle tissue. These tissue-specific knockout mice (genotype *Tfb1m*$^{loxP/loxP}$ +/*Ckmm-cre*) had a shorter life span, and all of them died before 24 weeks of age. We determined the ratio of heart weight to body weight at different ages and found that it was normal until the age of 15 weeks, and thereafter it increased rapidly in the knockouts (Figure 2A). Electron microscopy analysis of heart tissues from end-stage knockouts showed an increase of mitochondria with abnormal cristae (Figure 2B). We quantified mitochondrial mass in cardiomyocytes and found a progressive increase from age 10 weeks and onward in the knockouts (Figure 2C). Western blot analyses of mitochondrial extracts from knockout hearts showed that TFB1M was drastically depleted already at 5 weeks (Figure 2D). We found no obvious signs of mitochondrial dysfunction in skeletal muscle of *Tfb1m*$^{loxP/loxP}$ +/*Ckmm-cre* mice, consistent with our previous observations that skeletal muscle- and heart-specific disruption of genes controlling mtDNA maintenance and expression mainly results in cardiomyopathy during the short life span when the animals can be observed (Larsson et al., 1998; Wang et al., 1999; Park et al., 2007).

Hearts Lacking TFB1M Are Respiratory Chain Deficient with Increased Mitochondrial Biogenesis

Southern blot analysis of DNA isolated from heart tissues of control and tissue-specific knockout mice revealed a gradual

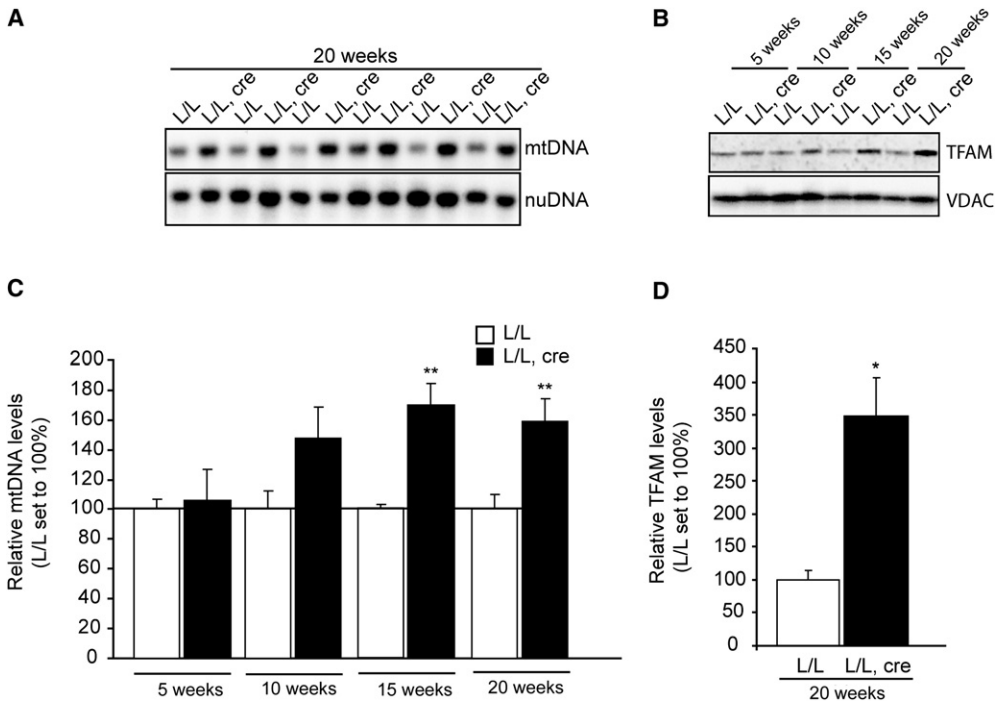

Figure 3. Increased Levels of mtDNA and TFAM in the Absence of TFB1M

(A) Southern blot analysis of total DNA isolated from control (L/L) and tissue-specific *Tfb1* knockout (L/L, cre) hearts at age 20 weeks. mtDNA was detected using *Cox1*-specifc DNA probe, and nuclear DNA (loading control, nuDNA) was detected by an 18S rDNA probe followed by autoradiography.

(B) Western blot analysis of heart mitochondrial extracts from control and knockout mice at different ages. TFAM and VDAC (loading control) were detected using specific polyclonal and monoclonal antisera, respectively.

(C) Quantification of mtDNA levels in heart of control and knockout mice at different ages. At 5 weeks, n = 4; 10 weeks, n = 3; 15 weeks, n = 4; 20 weeks, n = 6.

(D) TFAM steady-state levels were quantified by western immunoblotting in 20-week-old mice (control and knockout) and normalized to the levels of VDAC in the same samples. TFAM levels in knockout hearts are shown as percentage of the levels in control mice. n = 3. Error bars in (C) and (D) indicate SEM; *p < 0.05; **p < 0.01; ***p < 0.001; Student's t test.

increase in mtDNA steady-state levels in the absence of TFB1M, reaching maximum in 15- to 20-week-old knockout mice (Figures 3A and 3C). We performed western blot analyses and found a gradual increase of TFAM protein levels (Figures 3B and 3D), consistent with previous experimental work showing that levels of TFAM protein directly regulate steady-state levels of mtDNA (Larsson et al., 1998; Ekstrand et al., 2004). Measurement of respiratory chain function and mitochondrial ATP production rates in hearts of 5- and 20-week-old knockouts showed a progressive deficiency (Figures S1A and S1B). We found increased enzyme activities of the mitochondrial matrix enzyme citrate synthase (Figure S1C), consistent with the observed increase of mitochondrial mass by electron microscopy (Figures 2B and 2C). Most likely, the respiratory chain deficiency secondarily elicits mitochondrial biogenesis as a futile compensatory response. This assumption is well in line with numerous reports showing that increased mitochondrial biogenesis is a common response in humans and mice with mitochondrial disease (Smeitink et al., 2001; Wredenberg et al., 2002; Wenz et al., 2008).

Deficiency of 12S rRNA despite Increased Mitochondrial Transcription

We performed an extensive survey of steady-state levels of mitochondrial transcripts in TFB1M-deficient hearts by northern blotting (Figure 4A) and found a dramatic decline of 12S rRNA levels already in 5-week-old knockouts with constantly low levels over the whole period of the sampling (Figures 4B and 4C). This finding suggests that TFB1M may directly modify the 12S rRNA and thereby influences its stability. The levels of 16S rRNA and ND1 mRNA were substantially increased, whereas the other studied mRNAs remained similar to control steady-state levels (Figures 4B and 4C). The increase in steady-state levels of ND1 and 16S rRNA can be due to either the increased stability or increased de novo transcription in the absence of TFB1M. We therefore proceeded to determine steady-state levels of mitochondrial tRNAs (Figure 4A), as levels of tRNAs are much less subject to posttranscriptional control than mRNAs (Park et al., 2007). The steady-state levels of all studied tRNAs were elevated in knockout mice, regardless of whether they were transcribed from the HSP or LSP promoter (Figures 4D and 4E). These findings are consistent with a global increase of transcription initiation from the promoters of both strands of mtDNA. We tested this possibility further by γ-^{32}P-UTP labeling of newly synthesized transcripts and found a several-fold increase of de novo transcription in isolated mitochondria lacking TFB1M (Figures 5A and 5B). We investigated the expression of MTERF3, a negative regulator of mammalian mtDNA transcription (Park et al., 2007), and found normal *Mterf3* mRNA levels (Figure S2). However, the MTERF3 protein levels were

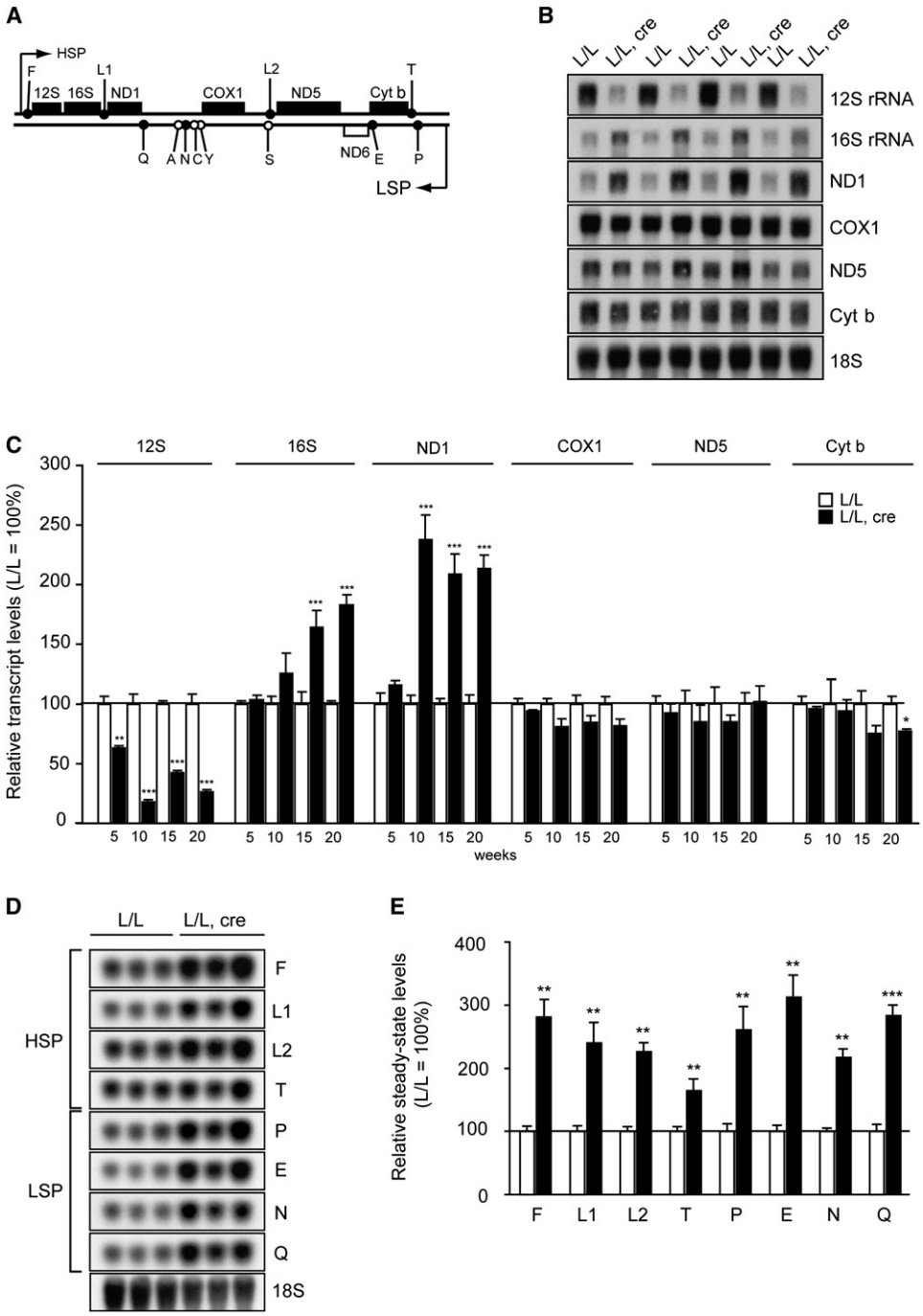

Figure 4. Variable Steady-State Levels of Mitochondrial Transcripts in TFB1M-Deficient Hearts

(A) Linear map of mouse mtDNA indicating the localization of the genes encoding rRNAs (12S, 16S), some mRNAs (ND1, COX1, ND5, Cyt b, ND6), and some tRNAs (F, L1, L2, etc.).

(B) Northern blot analysis of RNA, isolated from heart tissues of control (L/L) and knockout (L/L, cre) mice at 20 weeks of age (n = 4 for each genotype). Nuclear 18S rRNA was used as a loading control.

(C) Quantification by northern blot analyses of mitochondrial transcripts from control and tissue-specific knockout mice at different ages. Steady-state levels of individual transcripts were normalized to the levels of 18S and are shown as percentage from the control. Error bars represent SEM. Number of independent samples for each genotype was: 5 weeks, n = 4; 10 weeks, n = 5; 15 weeks, n = 4; and 20 weeks, n = 4. Error bars represent SEM; *p < 0.05; **p < 0.01; ***p < 0.001; Student's t test.

(D) Northern blot analysis of mitochondrial tRNA steady-state levels in hearts from 20-week-old control (L/L) and knockout mice (L/L, cre). Number of animals from each genotype, n = 3. 18S rRNA was used as a loading control.

(E) Quantification of steady-state levels of tRNAs shown as a percentage of the levels in controls after normalization to 18S rRNA levels. Error bars represent SEM; *p < 0.05; **p < 0.01; ***p < 0.001; Student's t test.

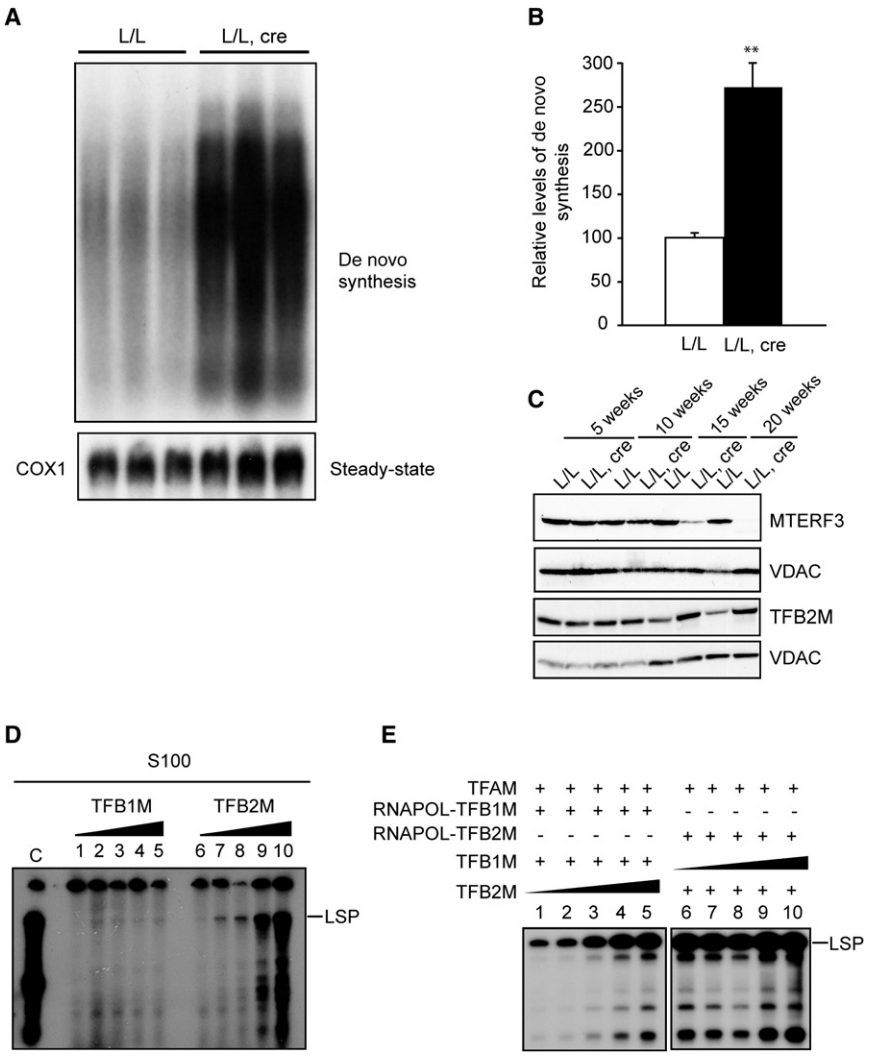

Figure 5. Increased Transcription Initiation in the Absence of TFB1M and Lack of In Vitro Transcription Inhibition by TFB1M

(A) In organello transcription in heart mitochondria isolated from 20-week-old control (L/L) and tissue-specific *Tfb1m* knockout (L/L, cre) mice. De novo synthesized transcripts were labeled using α-^{32}P-UTP and separated by agarose gel electrophoresis. COX1 mRNA, present in the mitochondrial preparations, was detected using α-^{32}P-dCTP-labeled *Cox1*-specific DNA probe to assess levels of steady-state transcripts.

(B) Quantification of the newly synthesized transcription products in control and tissue-specific knockout heart mitochondria. Number of independent samples, n = 3. Error bars represent SEM; **p < 0.01; Student's t test.

(C) Western blot analysis of MTERF3 and TFB2M protein levels in heart mitochondrial extracts isolated from control and *Tfb1m* knockout mice at different ages. MTERF3 and TFB2M were detected using peptide-specific polyclonal antibodies. VDAC was used as a loading control.

(D) Transcription assay using S100 mitochondrial extract and varying concentrations of either TFB1M or TFB2M. Increasing amounts of purified, recombinant TFB1M or TFB2M were added to S100 extracts from HeLa cell mitochondria in the presence of α-^{32}P-UTP. De novo synthesized LSP transcripts were detected by autoradiography. The in vitro transcription reactions contain: lanes 1 and 6, 0 fmol; lanes 2 and 7, 250 fmol; lanes 3 and 8, 500 fmol; lanes 4 and 9, 1000 fmol; and lanes 5 and 10, 2000 fmol of TFB1M or TFB2M, respectively. Lane C, control reaction (see Experimental Procedures). Transcription products were detected by autoradiography.

(E) In vitro transcription assay using varying amounts of recombinant TFB1M or TFB2M proteins. Reactions containing LSP promoter DNA template (85 fmol), TFAM (2.5 pmol), RNAPOL (400 fmol) copurified with TFB1M or TFB2M (RNAPOL-TFB1M or RNAPOL-TFB2M, respectively), and either TFB1M (400 fmol) or TFB2M (400 fmol) were supplemented with varying amounts of recombinant TFB2M or TFB1M: lanes 1 and 6, 100 fmol; lanes 2 and 7, 200 fmol; lanes 3 and 8, 400 fmol; lanes 4 and 9, 800 fmol; and lanes 5 and 10, 1600 fmol. Labeling was carried out in the presence of α-^{32}P-UTP, and transcription products were detected by autoradiography.

decreased (Figure 5C), consistent with posttranslational regulation of the activity of this repressor. In addition, the expression of the TFB2M protein was increased (Figure 5C). The observed increase of mitochondrial transcription is thus probably regulated by MTERF3 and TFB2M and is likely part of a mitochondrial biogenesis response induced by the severe respiratory chain deficiency accompanying TFB1M deficiency, as discussed above.

TFB1M Does Not Repress mtDNA Transcription In Vitro

It has been reported that addition of recombinant TFB1M protein to mitochondrial extracts dramatically stimulates mtDNA transcription (McCulloch et al., 2002; McCulloch and Shadel, 2003). These findings seem inconsistent with our results, as we found dramatically increased transcription of mtDNA after genetic ablation of TFB1M in the mouse heart (Figure 5A). We therefore repeated the experiments previously published by others (McCulloch et al., 2002; McCulloch and Shadel, 2003), and we were surprised to find no increase of in vitro transcription after addition of recombinant TFB1M to mitochondrial extracts (Figure 5D). It should be mentioned that the TFB1M protein preparation we used for this experiment has transcription initiation activity in the pure recombinant in vitro transcription system (Falkenberg et al., 2002). We also ensured that the mitochondrial extract we used was active by demonstrating that addition of recombinant TFB2M resulted in a dramatic increase of in vitro transcription (Figure 5D).

It is a formal possibility that TFB1M may compete with TFB2M by acting as a transcriptional repressor, which would explain the observed increase of mtDNA transcription in *Tfb1m* knockout mice. We have previously addressed this issue in a series of unpublished experiments and found no evidence that TFB1M represses transcription. However, given the importance of this issue, we repeated these in vitro transcription experiments with mtDNA promoter templates and pure recombinant TFB1M, TFB2M, POLRMT, and TFAM proteins

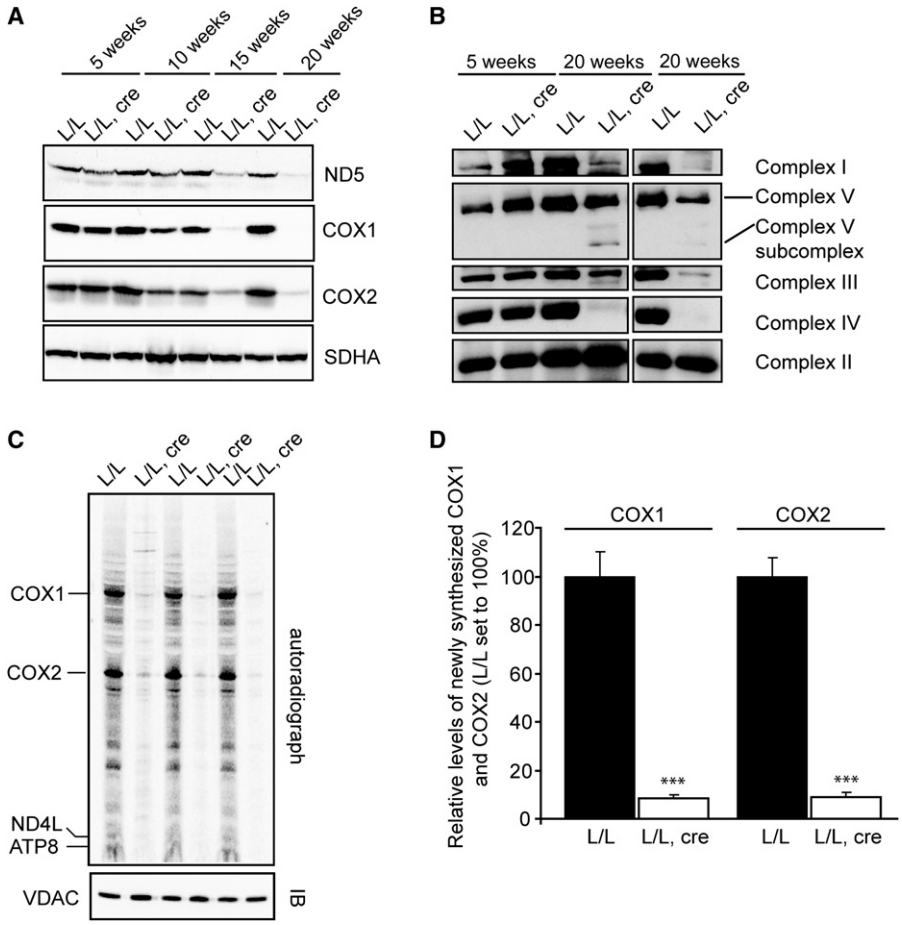

Figure 6. Reduced Formation of Respiratory Chain Enzymes Due to Inhibited Mitochondrial Translation in the Absence of TFB1M

(A) Steady-state levels of mitochondrial- and nuclear-encoded subunits of the respiratory chain complexes detected by immunoblotting of mitochondrial extracts from control (L/L) and tissue-specific *Tfb1m* knockout (L/L, cre) hearts at different ages. ND5 (complex I), COX1, and COX2 (complex IV) are encoded and translated in mitochondria, whereas the SDHA (complex II) is nuclear encoded.

(B) Levels of assembled respiratory chain enzyme complexes analyzed by BN-PAGE of mitochondrial extracts from control and tissue-specific *Tfb1m* knockout mice at different ages.

(C) Mitochondrial translation in isolated heart mitochondria from control (L/L) and knockout (L/L, cre) mice at 20 weeks of age. Mitochondrial translation products were labeled using Redivue *L*-[^{35}S]-Methionine and detected by autoradiography after SDS-PAGE and transfer on nitrocellulose membranes. VDAC was detected by western immunoblotting (IB) and used as a loading control for steady-state levels of proteins in the mitochondrial extract.

(D) Levels of newly synthesized COX1 and COX2 in knockout mitochondria shown as percentage from the corresponding levels in control mitochondria. The signals for COX1 and COX2 in both genotypes were normalized to the signal for VDAC as detected by immunoblotting. Number of samples for each genotype, n = 3. Error bar indicates SEM; ***p < 0.001; Student's t test.

(Figure 5E). We confirmed our previous finding that TFB2M is several orders of magnitude more active than TFB1M in promoting transcription initiation in this system (Falkenberg et al., 2002). We tested the possibility that TFB1M may act as a transcriptional inhibitor by adding excess recombinant TFB1M to transcription reactions containing TFB2M. We found no evidence that a molar excess of TFB1M can inhibit a transcription reaction dependent on TFB2M (Figure 5E, right panel). Consistent with these findings, we found strong activation of transcription when we added excess recombinant TFB2M to transcription reactions containing TFB1M (Figure 5E, left panel). Based on these results, we conclude that the observed increase of mitochondrial transcription in *Tfb1m* knockout mice is not explained by a transcription repression role for TFB1M.

Loss of TFB1M Severely Impairs Mitochondrial Translation

Western blot analyses of mitochondrial extracts from control and tissue-specific knockout mice revealed a gradual decrease in the steady-state levels of the mitochondrially encoded respiratory chain subunits ND5 (complex I), COX1, and COX2 (complex IV), whereas levels of the nuclear SDHA (complex II) subunit remained unchanged (Figure 6A). We assessed levels of assembled respiratory chain complexes with BN-PAGE analyses and found a defective assembly of complex I, III, and IV and, to a lesser extent, complex V in 20-week-old tissue-specific *Tfb1m* knockout mice (Figure 6B), consistent with the observed decrease in respiratory chain function and mitochondrial ATP rates (Figures S1A and S1B). We investigated the cause for this reduction in levels of mtDNA-encoded respiratory chain

subunits by performing translation assays with mitochondria isolated from control and tissue-specific knockout hearts. Autoradiographic analysis of newly synthesized mitochondrial translation products showed severely impaired translation (>90% decrease) in mitochondria from 20-week-old tissue-specific *Tfb1m* knockout mice (Figures 6C and 6D).

Decreased Methylation of 12S rRNA Impairs Stability of the Small Ribosomal Subunit

We performed primer extension analyses to check for the presence of adenine dimethylation in the hairpin loop at the 3′ end of 12S rRNA (Figure 7A), as previously described (Lafontaine et al., 1995, 1998). Due to the decrease in the steady-state levels of 12S rRNA, our primer extension assay estimates the degree of methylation on the remaining rRNA molecules. We found a severe decrease of the primer extension stop in end-stage knockouts, demonstrating that loss of TFB1M completely abolishes 12S rRNA adenine dimethylation (Figures 7B and 7C). This finding also demonstrates that TFB2M or other enzymes cannot compensate for the severe loss of methyltransferase activity that occurs in *Tfb1m* knockouts. Methylation of 12S rRNA was only mildly reduced at 5 weeks of age (Figure 7C) despite the profound depletion of TFB1M protein at the same age (Figure 2D). These findings suggest that turnover of methylated 12S rRNA may take several weeks, and the *Tfb1m* knockout animals will likely not manifest a major phenotype until most of the methylated 12S rRNAs have been turned over.

Next, we examined the steady-state levels of proteins from the 28S and the 39S ribosomal subunits of the mammalian mitochondrial ribosome by using western blot analyses (Figure 7D). MRPS15, homologous to *E. coli* Rps15 (Cavdar Koc et al., 2001), and MRPS16 are both protein components of the small ribosomal subunit and were present at decreased steady-state levels in 10-, 15-, and 20-week-old *Tfb1m* knockout mice (Figure 7D). In contrast, MRPL13 and MRPL48, which are both protein components of the large ribosomal subunit, were present at increased levels in *Tfb1m* knockouts (Figure 7D), in good agreement with the observed increase of steady-state levels of 16S rRNA (Figures 4B and 4C). In 5-week-old knockouts, MRPS15 was increased in abundance, which may represent a compensatory increase in its expression in response to an emerging translational deficiency.

We carried out gradient sedimentation analyses of mitochondrial extracts from knockout and control mice to investigate the integrity of the mitochondrial ribosome (Figure 7E). With this method, we detected the 28S and 39S subunits and the 55S assembled mitochondrial ribosome as determined by western blot analyses of gradient fractions (Figure 7E). We used MRPS15 as a marker for the 28S ribosomal subunit and comigration of MRPL13 and MRPL48 as markers for the 39S subunit. Comigration of MRPS15, MRPL13, and MRPL48 were used as markers for the assembled 55S mitochondrial ribosome. Analyses of ribosome assembly patterns in *Tfb1m* knockout mice showed a deficiency of assembled small ribosomal subunits (Figure 7E). Based on these results, we conclude that absence of adenine dimethylation in 12S rRNA causes a specific loss of 28S subribosomal particles, whereas 39S subunits are assembled and present at increased steady-state levels. Although we cannot exclude the possibility that individual proteins from the 39S subunits are absent, our results suggest that the assembly and stability of 39S particles is mostly independent of the presence of assembled small ribosomal subunits. We analyzed the distribution of TFB1M protein by gradient sedimentation analyses of mitochondrial extracts from control mice and found that a fraction of TFB1M was associated with the small subunit of the ribosome (Figure 7F), consistent with the above described role in 12S rRNA methylation.

DISCUSSION

The functional role of adenine dimethylation at the 3′ end of the rRNA situated in the small ribosomal subunit appears to be relatively limited in unicellular organisms, despite the extremely high conservation in all domains of life (Shutt and Gray, 2006). In *E. coli*, lack of the *KsgA* methyltransferase leads to resistance to the aminoglycoside kasugamycin and only marginal growth retardation (Helser et al., 1971; Poldermans et al., 1979; van Buul et al., 1984). In *S. cerevisiae*, adenine dimethylation is dispensable in vivo, as Dim1p mutant yeast cells grow normally despite the absence of cytoplasmic rRNA adenosine dimethylation (Lafontaine et al., 1998). In vitro experiments with extracts from KsgA-deficient *E. coli* cells have shown decreased affinity between the ribosomal subunits (Poldermans et al., 1980) and aberrant regulation of in vitro translation (Poldermans et al., 1979), consistent with the observation that the dimethylated adenines are present at the interface of the two ribosomal subunits (Xu et al., 2008).

In contrast to these earlier studies in unicellular organisms, we show here that the dimethylation of a stem-loop structure of 12S rRNA is of critical importance for normal function and integrity of the mammalian mitochondrial ribosome. The gene encoding the TFB1M methyltransferase is essential for embryonic development in mouse. Loss of function of TFB1M in a differentiated tissue, such as heart, leads to loss of dimethylation on A1006 and A1007 of 12S rRNA, decreased steady-state levels of 12S rRNA, instability of the small subunit of the ribosome, and abolished mitochondrial translation.

Lack of dimethylation on 12S rRNA leads to a drastic decrease in levels of assembled 28S ribosomal subunits, whereas the levels of the 39S subunits are increased. It is therefore possible that 12S rRNA methylation is a mandatory step in the biogenesis of the 28S subunit. However, this hypothesis is not supported by results from studies in bacteria, because functional bacterial ribosomes can be assembled and are stable in the absence of rRNA dimethylation (Poldermans et al., 1979, 1980). Another possibility is that the 28S subunit is formed normally but becomes unstable in the absence of rRNA dimethylation. It has been shown that KsgA is able to bind assembled bacterial small (30S) ribosomal subunits, which could provide a checkpoint mechanism prior to assembly of fully active ribosomes (Desai and Rife, 2006; Xu et al., 2008). It is possible that KsgA binding could ensure that only 30S subunits that are translationally competent are present in fully assembled ribosomes (Desai and Rife, 2006; Xu et al., 2008). We found cosedimentation of TFB1M and the small ribosomal subunit, and it is therefore possible that TFB1M functions in a manner similar to KsgA and binds the interface between the mitochondrial ribosomal subunits to provide a checkpoint prior to initiation of

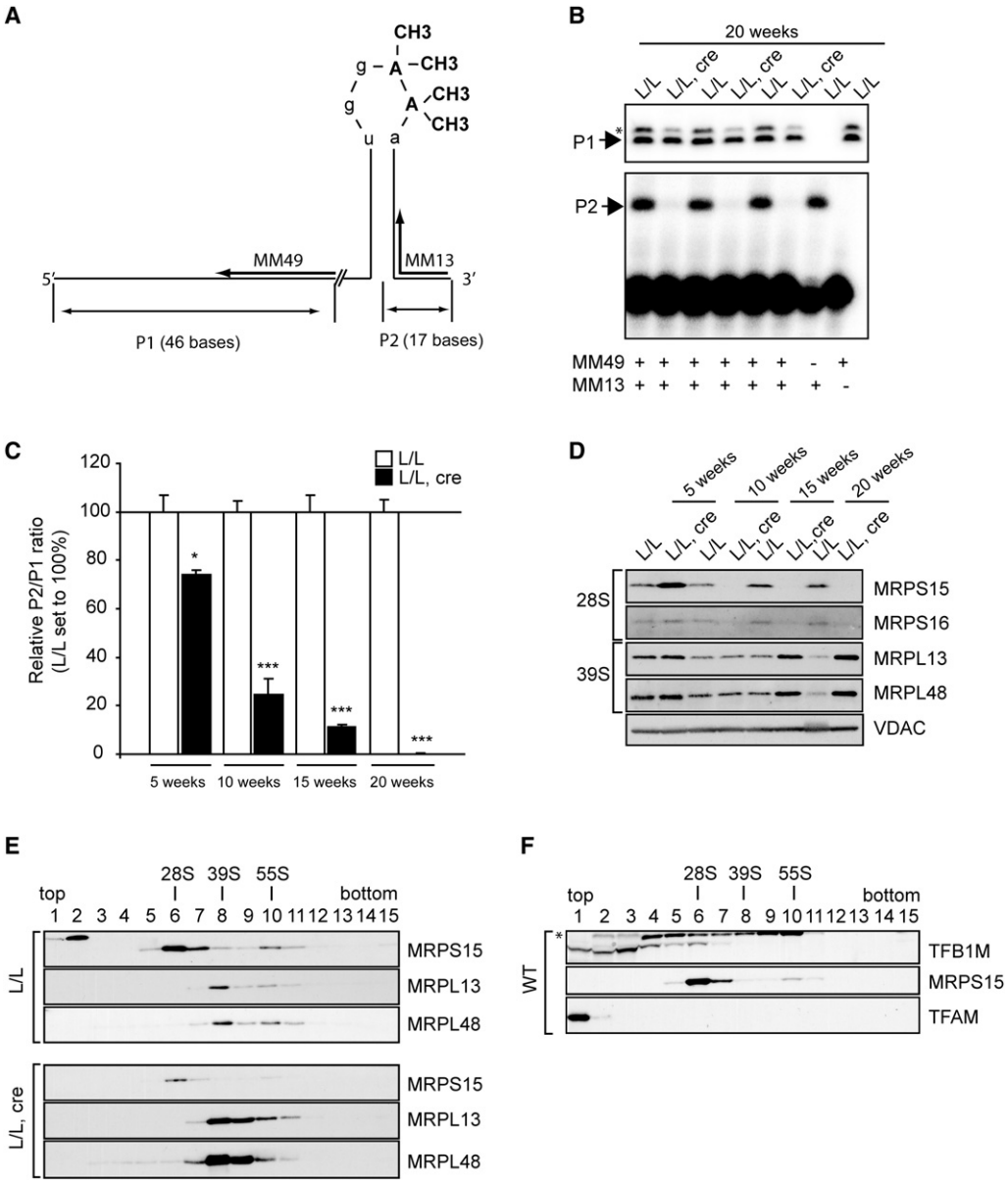

Figure 7. Loss of 12S rRNA Dimethylation Results in Reduced Steady-State Levels of the Small Ribosomal Subunit in Tissue-Specific Knockout of *Tfb1m*

(A) Experimental set up for measuring the presence of dimethylation on A1006 and A1007 residues in 12S rRNA (counted from the 5′ end of tRNA-Phe). A set of two primers (MM49 and MM13) was used for primer extension. MM49 (12 bases) is complementary to a region close to the 5′ end of 12S rRNA, and its extension product (P1; 46 bases) was used as a 12S rRNA loading control. MM13 (12 bases) is complementary to a region positioned downstream of the dimethylated A1006 and A1007 (shown in bold capital letters). Extension of this primer is inhibited by the presence of methylation and leads to accumulation of an extension product P2 (17 bases).

(B) Primer extension analysis of 12S rRNA in RNA samples isolated from heart tissues of control (L/L) and knockout (L/L,cre) mice at 20 weeks of age. RNA from knockout mice was used in 2-fold excess of RNA from control mice. Control reactions using MM13 or MM49 alone are shown. *, a putative precursor form of 12S rRNA.

(C) Relative ratio between primer extension products P2 and P1 in RNA samples from control and tissue-specific *Tfb1m* knockout mice at different ages. Error bars represent SEM. Number of independent samples from each genotype: 5 weeks, n = 3; 10 weeks, n = 3; 15 weeks, n = 4; 20 weeks, n = 3. *$p < 0.05$; ***$p < 0.001$; Student's t test.

(D) Steady-state levels of proteins from the small (28S) and large (39S) ribosomal subunits, detected by western immunoblotting of mitochondrial extracts from control and tissue-specific *Tfb1m* knockout mice at different ages. MRPS15 and MRPS16 are subunits of the 28S ribosomal particle. MRPL13 and MRPL48 are subunits from the 39S ribosomal particle. VDAC was used as a loading control.

(E) Sedimentation analysis of 28S and 39S subribosomal particles by centrifugation through linear 10%–30% sucrose density gradient. The migration of both ribosomal particles was determined by immunoblotting for MRPS15, MRPL13, and MRPL48 after SDS-PAGE. Representative from three independent experiments is shown. Fraction numbers are indicated.

mitochondrial translation. This model predicts that 28S subunits with unmethylated 12S rRNA will fail to interact with the 39S subunit and instead will be degraded.

The results presented here are inconsistent with previous reports suggesting that TFB1M is a strong transcriptional activator (McCulloch et al., 2002; McCulloch and Shadel, 2003). If TFB1M indeed would be a strong transcription factor, knockout of the *Tfb1m* gene should lead to decreased mtDNA transcription. However, at variance with this prediction, we instead report that loss of TFB1M leads to increased transcription initiation. Transcription from both mtDNA promoters, LSP and HSP, generates polycistronic transcripts that are processed to release the individual mRNAs and tRNAs. The steady-state levels of mRNAs are much more subjected to posttranscriptional control than the steady-state levels of tRNAs (Park et al., 2007). In line with this prediction, we found normal or near normal steady-state levels of most mRNAs, except for the ND1 mRNA, whereas the steady-state levels of all tRNAs were significantly increased. Importantly, the levels of tRNAs were increased regardless of whether the corresponding gene was transcribed from the LSP or HSP promoter. In vitro transcription experiments indeed confirmed a strong activation of de novo transcription in mouse heart mitochondria lacking TFB1M. The observed reduction of MTERF3 protein, a known repressor of mtDNA transcription (Park et al., 2007), and increase of TFB2M protein provide a mechanism to explain the increased mtDNA transcription in TFB1M knockouts. We also provide evidence that TFB1M does not act as a transcriptional repressor that competes with TFB2M in vitro. Instead, we find it likely that the increased mitochondrial transcription is part of a secondary response to increase mitochondrial biogenesis as a cellular attempt to compensate for the severe respiratory chain deficiency caused by the abolished mitochondrial translation in the absence of TFB1M. Consistent with this interpretation, we report a concerted increase of mitochondrial mass, mtDNA, TFAM, and mtDNA transcription in the TFB1M-deficient hearts. Such an increase of mitochondrial biogenesis is a well-documented response in animals and humans with respiratory chain deficiency (Larsson et al., 1998; Wredenberg et al., 2002; Park et al., 2007; Scarpulla, 2008). Based on our data, we propose that TFB1M functions as an important regulator of ribosomal biogenesis in mammalian mitochondria. We further propose that activation of the expression of the nuclear genes encoding TFB1M and TFB2M is a central component of the mitochondrial biogenesis response to ensure that there is a concerted increase of both mitochondrial transcription and translation to increase the expression of the essential mtDNA-encoded respiratory chain subunits. This hypothesis could be tested by performing correlative studies in mammalian tissues induced to increase mitochondrial biogenesis. More importantly, experimental studies aimed at simultaneously regulating the expression of both TFB1M and TFB2M in mice should be performed. Increased understanding of molecular mechanisms regulating mtDNA expression may well lead to the opening of novel avenues leading toward treatment of age-associated diseases and amelioration of aging.

In summary, we show here that TFB1M is an essential dimethyltransferase in mammalian mitochondria that dimethylates adenines at the 3′ end of 12S rRNA. We present evidence that this modification is essential for the stability of the small subunit of the mitochondrial ribosome. In addition, our study provides a possible explanation for the universal conservation of adenine dimethylation of the small subunit rRNA in a variety of organisms and presents evidence for a critical role in regulating ribosome maintenance and translation.

EXPERIMENTAL PROCEDURES

Generation of Conditional *Tfb1m* Knockout Mice

We designed a targeting vector with exon III of the *Tfb1m* locus, flanked by two *loxP* sites, to generate conditional knockout *Tfb1m* mice (Figure 1A). Genomic clones containing mouse *Tfb1m* were isolated from a 129Sv RPCI-22M BAC Library (Invitrogen). DNA fragments containing exon III and exon IV of the *Tfb1m* locus were isolated from BAC clones using XhoI-BamHI and BamHI endonuclease digestion, respectively. pBluescript II SK+ (pBS; Stratagene) vector was modified by ligation with oligonucleotides containing XhoI and BamHI restriction sites to create the pNH7 plasmid. In pNH7, sites not compatible with downstream cloning steps were thus eliminated. A DNA fragment containing exon III of *Tfb1m* was cloned into the BamHI-XhoI sites of the pNH7 vector to create the pNH10 plasmid. The BamHI genomic fragment containing exon IV of *Tfb1m* was cloned into the BamHI site of pBluescript II SK+, giving rise to the pNH9 plasmid. Next, pNH10 was linearized with XbaI, and a *loxP* sequence was introduced upstream of exon III by ligation with oligonucleotides containing this sequence, thus generating vector pNH11. A DNA fragment containing the neomycin resistance gene and a *loxP* site was cloned into pNH10 using AatII and ClaI endonuclease restriction sites, resulting in vector pNH12. Oligonucleotides containing NotI and SalI restriction sites were ligated into a ClaI-PacI-digested pNH12 vector. The resulting pNH13 vector was digested using NotI and SalI endonucleases. Finally, the DNA fragment containing exon IV was isolated from pNH9 using NotI-SalI endonuclease digestion and cloned into linearized pNH13. This construct, pNH14, was linearized using SalI endonuclease and electroporated in 129R1 ESCs. Targeted clones were identified by SpeI restriction digestion of genomic DNA and hybridization with an α-^{32}P-dCTP-labeled probe located outside of the targeted region. Positive ESC clones were used for blastocyst injection, and germline transmission was obtained by mating the chimeric mice to B6C57 mice. The *PKG-neo* gene was removed by mating $Tfb1m^{+/loxP\text{-}neo}$ mice to mice ubiquitously expressing *Flp* recombinase (Rodriguez et al., 2000), thus generating $Tfb1m^{+/loxP}$ mice. These mice, in turn, were mated to mice ubiquitously expressing *Cre* recombinase (β-actin-*cre*) (Larsson et al., 1998) to obtain heterozygous knockout ($Tfb1m^{+/-}$) mice.

Tissue-Specific Knockout of *Tfb1m*

Heart- and skeletal muscle-specific knockout mice were generated as described previously (Larsson et al., 1998; Hance et al., 2005; Park et al., 2007). Briefly, heterozygous $Tfb1m^{+/loxP}$ mice were mated to heterozygous transgenic mice expressing *Cre* recombinase under the control of a heart- and muscle-specific promoter (+/*Ckmm-cre*). Mice with the genotype $Tfb1m^{+/loxP}$, +/*Ckmm-cre* were recovered from this cross and mated to $Tfb1m^{loxP/loxP}$ mice to generate $Tfb1m^{loxP/loxP}$, +/*Ckmm-cre* (tissue-specific knockouts) and $Tfb1m^{loxP/loxP}$ (control) mice.

(F) Sedimentation analysis of heart mitochondrial extracts from wild-type (WT) mice by centrifugation through linear 10%–30% sucrose density gradient. The partial comigration of TFB1M and the small ribosomal subunit was determined by immunoblotting with specific antibodies after SDS-PAGE. MRPS15 was used as a marker for the sedimentation of the small ribosomal subunit. TFAM was used as a negative control. TFB1M is the lower band in the upper panel; a cross reacting band is indicated by *.

Southern, Northern, and Western Blot Analysis

For the determination of mtDNA levels, total DNA was isolated using the DNeasy Blood & Tissue Kit (QIAGEN), and 1 μg DNA was digested with SacI endonuclease. The digested DNA was electrophoresed in agarose, blotted, and hybridized to α-^{32}P-dCTP-labeled probes to detect mtDNA (CoxI probe) and nuclear DNA as loading control (18S rDNA probe).

RNA for northern blot analyses was isolated using the ToTALLY RNA Total RNA isolation kit (catalogue number AM1910; Ambion) and resuspended in formamide. Samples of 1–2 μg RNA were electrophoresed in formaldehyde agarose gels and transferred to Hybond-N+ membranes (GE Healthcare). Individual RNA species were detected using α-^{32}P-dCTP-labeled DNA probes. Different tRNAs were detected using specific oligonucleotides labeled with γ-^{32}P-dATP (Park et al., 2007). Quantifications of Southern and northern blots were performed after autoradiographic detection using Molecular Imager FX (Bio-Rad).

Rabbit polyclonal antisera were used for detection of the TFAM, COX1, COX2, and ND5 proteins. Antisera specific for NDUFA9 (complex III), SDHA (70 kDa subunit of complex II), F1α (complex V), and subunit IV (complex IV) were purchased from Molecular Probes (Eugene, OR). VDAC (Porin) antibodies were purchased from Calbiochem (San Diego; CA). Mouse polyclonal MRPL48-specific antiserum was purchased from the Abnova Corporation. Polyclonal antisera against mouse TFB1M, TFB2M, and MRPS15 were generated in rabbits by using a single peptide or a mixture of two peptides corresponding to each protein (TFB1M: N-MAASGKLGTFRLC-C and N-CFTYNFRE ELKOKKS-C; TFB2M: N-CIEPLPDSDLEESSPWTSRNRS-C; MRPS15: N-KIL RQTNYDVFEKTC-C and N-CPENPSNAVPEKTQVN-C). Antisera against TFB1M and TFB2M were additionally purified by immunoadsorption using the antigenic peptides. The MTERF3 polyclonal antibody was generated as described previously (Park et al., 2007). The MRPL13 antiserum was provided by Doctor Linda Spremulli and the MRPS16 rabbit polyclonal antibody by Doctor Ann Saada. Quantifications of chemiluminescent signals were performed using a CCD camera (Bio-Rad).

Mitochondrial Isolation and In Organello Translation

Mitochondria were isolated by differential centrifugation in isolation buffer A (320 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA [pH 7.4]). After three washes with isolation buffer, mitochondria were either used immediately or stored at −80°C for later use. Mitochondria for in organello translation were isolated in isolation buffer B (220 mM mannitol, 70 mM sucrose, 20 mM HEPES, 2 mM EGTA, 0.1% BSA [pH 7.4]) (Mattiazzi et al., 2002). Translation in isolated mitochondria was performed as described previously, with some modifications (Cote et al., 1989). Mitochondria (100 μg) were resuspended in translation buffer EGS+ (Cote et al., 1989) containing 60 μg/ml of each amino acid, minus methionine, and incubated for 5 min at 30°C. Redivue *L*-[^{35}S]-Methionine (GE Healthcare) was added to 0.5 mCi/ml to the mitochondrial suspension, and translation was allowed to proceed for 40 min at 30°C. Mitochondria were reisolated by centrifugation (5 min/7000× g/4°C) and washed twice with isolation buffer A. Mitochondrial pellets were resuspended in SDS-PAGE loading buffer and subjected to SDS-PAGE. Labeled mitochondrial translation products were transferred on Hybond-C Extra nitrocellulose membranes (GE Healthcare) and detected by autoradiography.

Sucrose Density Gradient Analysis of 28S and 39S Ribosomal Subunits

Sucrose density gradient analysis of ribosomal subunits was performed as described in Matthews et al., 1982, with modifications. Heart mitochondria (1.2 mg), isolated in isolation buffer A without EDTA and supplemented with Complete EDTA-free Protease Inhibitor Cocktail (Roche), were reisolated by centrifugation (10 min/9300× *g*/4°C) and resuspended at concentration 10 mg/ml in lysis buffer (260 mM sucrose, 100 mM KCl, 20 mM $MgCl_2$, 10 mM Tris-HCl [pH 7.5], 5 mM β-mercaptoethanol, 1% Triton X-100) supplemented with Complete EDTA-Free Protease Inhibitor Cocktail (Roche). After incubation for 20 min on ice, lysates were cleared by centrifugation (45 min/9300× *g*/4°C). The subribosomal particles in 100 μl of cleared mitochondrial lysate were separated by sedimentation through 10%–30% linear sucrose gradients by centrifugation for 15 hr at 71,000× *g* (4°C). Gradients were prepared in buffer A without Triton X-100. Gradients were formed using Gradient Master (Biocomp Instruments, Inc.) in 14 × 89 mm Ultra-Clear centrifuge tubes (Beckman Instruments, Inc.) according to instructions in the operator's manual provided for Gradient Master. Prior to loading of the samples, a single 400 μl fraction was removed from the top of the gradients to prevent sample losses incurred during the acceleration and deceleration phase of the centrifugation. Fractions (750 μl) were collected from the top of the gradients, and proteins in each fraction were precipitated with trichloracetic acid and subjected to SDS-PAGE followed by immunoblotting. Subribosomal particles were detected using antisera specific for individual proteins from the 28S and 39S ribosomal subunits.

Primer Extension Analysis

Primer extension analyses were performed using a pair of primers in each primer extension reaction: MM49 (12 bases, 5′-GTGTAATTTTAC-3′) complementary to a sequence close to the 5′-end of 12S rRNA, whose extension product (46 bases) was used as a loading control, and MM13 (12 bases, 5′-ATT ATTCCAAGC-3′) complementary to a region downstream of the 3′ end of 12S rRNA, close to the dimethylated A1006 and A1007. Primers were labeled with γ-^{32}P-ATP by using T4 polynucleotide kinase. Labeled primers were purified using MicroSpin G-25 chromatography columns (GE Healthcare) and annealed to RNA from heart tissue of control and tissue-specific *Tfb1m* knockout mice. Reactions were incubated at 65°C for 5 min and placed on ice. M-MuLV Reverse Transcriptase (Stratagene) was added to 5 U/μl together with dNTPs (0.25 mM/μl each). The reactions were incubated for 1 hr at 37°C. Extension products were precipitated and resuspended in loading buffer (formamide, 1 mg/ml bromphenol blue, 1 mg/ml xylene cyanol, 10 mM EDTA [pH 8.0]). Primer extension products were resolved using denaturing Urea-PAGE and detected by autoradiography. To compensate for the decrease in the 12S rRNA levels, RNA isolated from tissue-specific *Tfb1m* knockout mice was used in a 2- to 3-fold excess in comparison with control samples.

In Vitro Transcription, Transcription in S100 Mitochondrial Fraction, and Transcription in Isolated Mitochondria

Mitochondrial S100 fractions were isolated as described previously (Micol et al., 1996). Recombinant POLRMT, TFAM, TFB1M, and TFB2M were purified as described previously (Falkenberg et al., 2002). Transcription reactions with S100 extracts were carried out using varying amounts of recombinant TFB1M or TFB2M. Control reactions were carried out in the presence of 85 fmol LSP-containing DNA template and recombinant TFB2M (500 fmol), TFAM (2.5 pmol), and POLRMT (500 fmol) instead of S100 mitochondrial extracts. In vitro transcription reactions were carried out essentially as previously described (Falkenberg et al., 2002). Transcription in isolated mitochondria (in organello) was performed as described previously (Enriquez et al., 1996; Park et al., 2007).

Biochemistry, BN-PAGE, and Electron Microscopy

BN-PAGE was performed as described in Park et al., 2007. Respiratory chain enzyme activities and MAPR were measured in mitochondria isolated from the left ventricle tissue as described (Wibom et al., 2002). Electron microscopy and quantification of mitochondrial volume density was performed as described (Park et al., 2007).

SUPPLEMENTAL DATA

Supplemental Data include two figures and can be found online at http://www.cell.com/cellmetabolism/supplemental/S1550-4131(09)00061-8.

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REFERENCES

Cavdar Koc, E., Burkhart, W., Blackburn, K., Moseley, A., and Spremulli, L.L. (2001). The small subunit of the mammalian mitochondrial ribosome. Identification of the full complement of ribosomal proteins present. J. Biol. Chem. *276*, 19363–19374.

Cote, C., Poirier, J., and Boulet, D. (1989). Expression of the mammalian mitochondrial genome. Stability of mitochondrial translation products as a function of membrane potential. J. Biol. Chem. *264*, 8487–8490.

Cotney, J., and Shadel, G.S. (2006). Evidence for an early gene duplication event in the evolution of the mitochondrial transcription factor B family and maintenance of rRNA methyltransferase activity in human mtTFB1 and mtTFB2. J. Mol. Evol. *63*, 707–717.

Desai, P.M., and Rife, J.P. (2006). The adenosine dimethyltransferase KsgA recognizes a specific conformational state of the 30S ribosomal subunit. Arch. Biochem. Biophys. *449*, 57–63.

Ekstrand, M.I., Falkenberg, M., Rantanen, A., Park, C.B., Gaspari, M., Hultenby, K., Rustin, P., Gustafsson, C.M., and Larsson, N.G. (2004). Mitochondrial transcription factor A regulates mtDNA copy number in mammals. Hum. Mol. Genet. *13*, 935–944.

Enriquez, J.A., Perez-Martos, A., Lopez-Perez, M.J., and Montoya, J. (1996). In organello RNA synthesis system from mammalian liver and brain. Methods Enzymol. *264*, 50–57.

Falkenberg, M., Gaspari, M., Rantanen, A., Trifunovic, A., Larsson, N.G., and Gustafsson, C.M. (2002). Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA. Nat. Genet. *31*, 289–294.

Falkenberg, M., Larsson, N.G., and Gustafsson, C.M. (2007). DNA replication and transcription in mammalian mitochondria. Annu. Rev. Biochem. *76*, 679–699.

Gleyzer, N., Vercauteren, K., and Scarpulla, R.C. (2005). Control of mitochondrial transcription specificity factors (TFB1M and TFB2M) by nuclear respiratory factors (NRF-1 and NRF-2) and PGC-1 family coactivators. Mol. Cell. Biol. *25*, 1354–1366.

Hance, N., Ekstrand, M.I., and Trifunovic, A. (2005). Mitochondrial DNA polymerase gamma is essential for mammalian embryogenesis. Hum. Mol. Genet. *14*, 1775–1783.

Helser, T.L., Davies, J.E., and Dahlberg, J.E. (1971). Change in methylation of 16S ribosomal RNA associated with mutation to kasugamycin resistance in Escherichia coli. Nat. New Biol. *233*, 12–14.

Lafontaine, D., Vandenhaute, J., and Tollervey, D. (1995). The 18S rRNA dimethylase Dim1p is required for pre-ribosomal RNA processing in yeast. Genes Dev. *9*, 2470–2481.

Lafontaine, D.L., Preiss, T., and Tollervey, D. (1998). Yeast 18S rRNA dimethylase Dim1p: a quality control mechanism in ribosome synthesis? Mol. Cell. Biol. *18*, 2360–2370.

Larsson, N.G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., Barsh, G.S., and Clayton, D.A. (1998). Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. Nat. Genet. *18*, 231–236.

Matthews, D.E., Hessler, R.A., Denslow, N.D., Edwards, J.S., and O'Brien, T.W. (1982). Protein composition of the bovine mitochondrial ribosome. J. Biol. Chem. *257*, 8788–8794.

Mattiazzi, M., D'Aurelio, M., Gajewski, C.D., Martushova, K., Kiaei, M., Beal, M.F., and Manfredi, G. (2002). Mutated human SOD1 causes dysfunction of oxidative phosphorylation in mitochondria of transgenic mice. J. Biol. Chem. *277*, 29626–29633.

McCulloch, V., and Shadel, G.S. (2003). Human mitochondrial transcription factor B1 interacts with the C-terminal activation region of h-mtTFA and stimulates transcription independently of its RNA methyltransferase activity. Mol. Cell. Biol. *23*, 5816–5824.

McCulloch, V., Seidel-Rogol, B.L., and Shadel, G.S. (2002). A human mitochondrial transcription factor is related to RNA adenine methyltransferases and binds S-adenosylmethionine. Mol. Cell. Biol. *22*, 1116–1125.

Micol, V., Fernandez-Silva, P., and Attardi, G. (1996). Isolation and assay of mitochondrial transcription termination factor from human cells. Methods Enzymol. *264*, 158–173.

Park, C.B., Asin-Cayuela, J., Camara, Y., Shi, Y., Pellegrini, M., Gaspari, M., Wibom, R., Hultenby, K., Erdjument-Bromage, H., Tempst, P., et al. (2007). MTERF3 is a negative regulator of mammalian mtDNA transcription. Cell *130*, 273–285.

Poldermans, B., Van Buul, C.P., and Van Knippenberg, P.H. (1979). Studies on the function of two adjacent N6,N6-dimethyladenosines near the 3′ end of 16 S ribosomal RNA of Escherichia coli. II. The effect of the absence of the methyl groups on initiation of protein biosynthesis. J. Biol. Chem. *254*, 9090–9093.

Poldermans, B., Bakker, H., and Van Knippenberg, P.H. (1980). Studies on the function of two adjacent N6,N6-dimethyladenosines near the 3′ end of 16S ribosomal RNA of Escherichia coli. IV. The effect of the methylgroups on ribosomal subunit interaction. Nucleic Acids Res. *8*, 143–151.

Rodriguez, C.I., Buchholz, F., Galloway, J., Sequerra, R., Kasper, J., Ayala, R., Stewart, A.F., and Dymecki, S.M. (2000). High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. Nat. Genet. *25*, 139–140.

Saraste, M. (1999). Oxidative phosphorylation at the fin de siecle. Science *283*, 1488–1493.

Scarpulla, R.C. (2008). Transcriptional paradigms in mammalian mitochondrial biogenesis and function. Physiol. Rev. *88*, 611–638.

Seidel-Rogol, B.L., McCulloch, V., and Shadel, G.S. (2003). Human mitochondrial transcription factor B1 methylates ribosomal RNA at a conserved stem-loop. Nat. Genet. *33*, 23–24.

Shutt, T.E., and Gray, M.W. (2006). Homologs of mitochondrial transcription factor B, sparsely distributed within the eukaryotic radiation, are likely derived from the dimethyladenosine methyltransferase of the mitochondrial endosymbiont. Mol. Biol. Evol. *23*, 1169–1179.

Smeitink, J., van den Heuvel, L., and DiMauro, S. (2001). The genetics and pathology of oxidative phosphorylation. Nat. Rev. Genet. *2*, 342–352.

Spiegelman, B.M. (2007). Transcriptional control of mitochondrial energy metabolism through the PGC1 coactivators. Novartis Found. Symp. *287*, 60–63.

Terzioglu, M., and Larsson, N.G. (2007). Mitochondrial dysfunction in mammalian ageing. Novartis Found. Symp. *287*, 197–208.

van Buul, C.P., Visser, W., and van Knippenberg, P.H. (1984). Increased translational fidelity caused by the antibiotic kasugamycin and ribosomal ambiguity in mutants harbouring the ksgA gene. FEBS Lett. *177*, 119–124.

Wallace, D.C. (1999). Mitochondrial diseases in man and mouse. Science *283*, 1482–1488.

Wang, J., Wilhelmsson, H., Graff, C., Li, H., Oldfors, A., Rustin, P., Bruning, J.C., Kahn, C.R., Clayton, D.A., Barsh, G.S., et al. (1999). Dilated cardiomyopathy and atrioventricular conduction blocks induced by heart-specific inactivation of mitochondrial DNA gene expression. Nat. Genet. *21*, 133–137.

Wenz, T., Diaz, F., Spiegelman, B.M., and Moraes, C.T. (2008). Activation of the PPAR/PGC-1alpha pathway prevents a bioenergetic deficit and effectively improves a mitochondrial myopathy phenotype. Cell Metab. *8*, 249–256.

Wibom, R., Hagenfeldt, L., and von Dobeln, U. (2002). Measurement of ATP production and respiratory chain enzyme activities in mitochondria isolated from small muscle biopsy samples. Anal. Biochem. *311*, 139–151.

Wredenberg, A., Wibom, R., Wilhelmsson, H., Graff, C., Wiener, H.H., Burden, S.J., Oldfors, A., Westerblad, H., and Larsson, N.G. (2002). Increased mitochondrial mass in mitochondrial myopathy mice. Proc. Natl. Acad. Sci. USA *99*, 15066–15071.

Xu, Z., O'Farrell, H.C., Rife, J.P., and Culver, G.M. (2008). A conserved rRNA methyltransferase regulates ribosome biogenesis. Nat. Struct. Mol. Biol. *15*, 534–536.