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Supplemental Data

MTERF3 Is a Negative Regulator of Mammalian mtDNA Transcription

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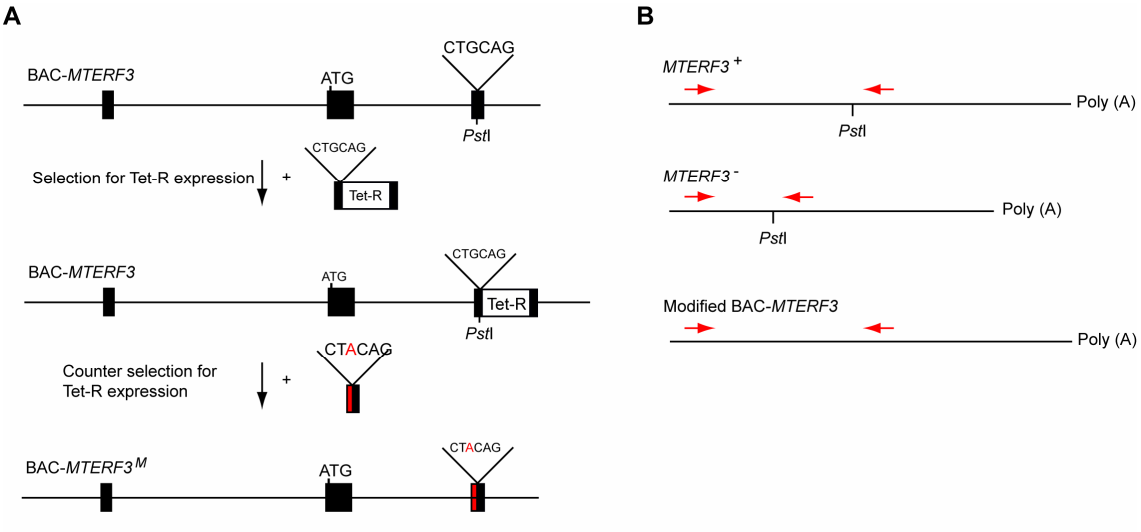
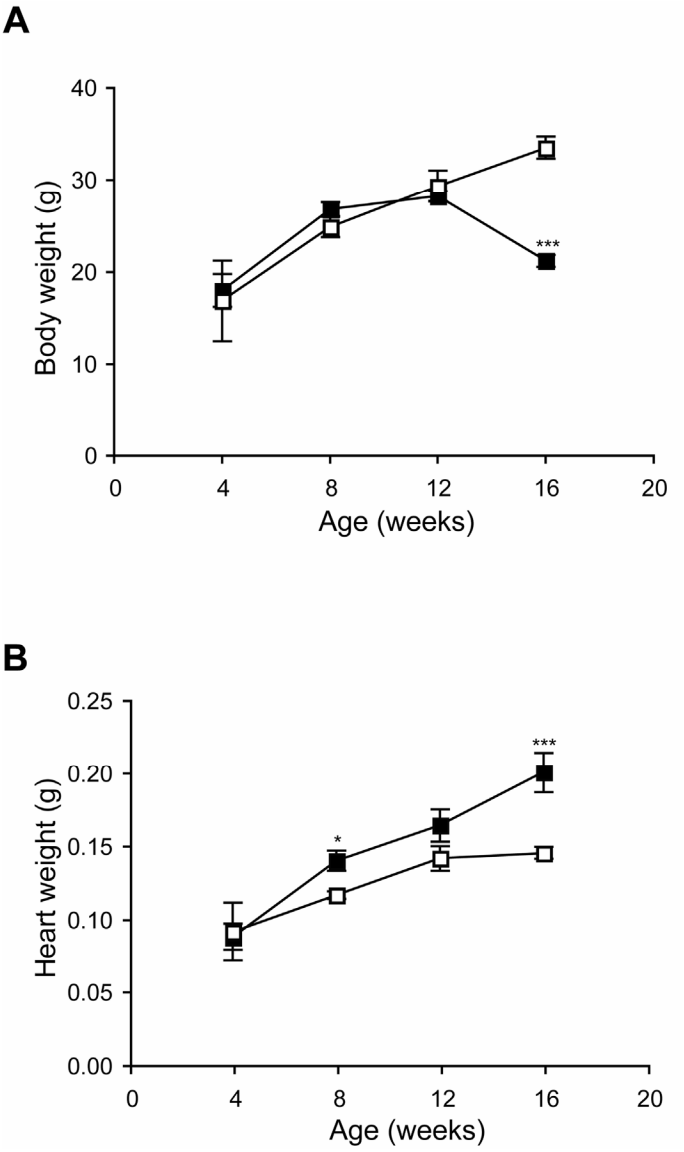
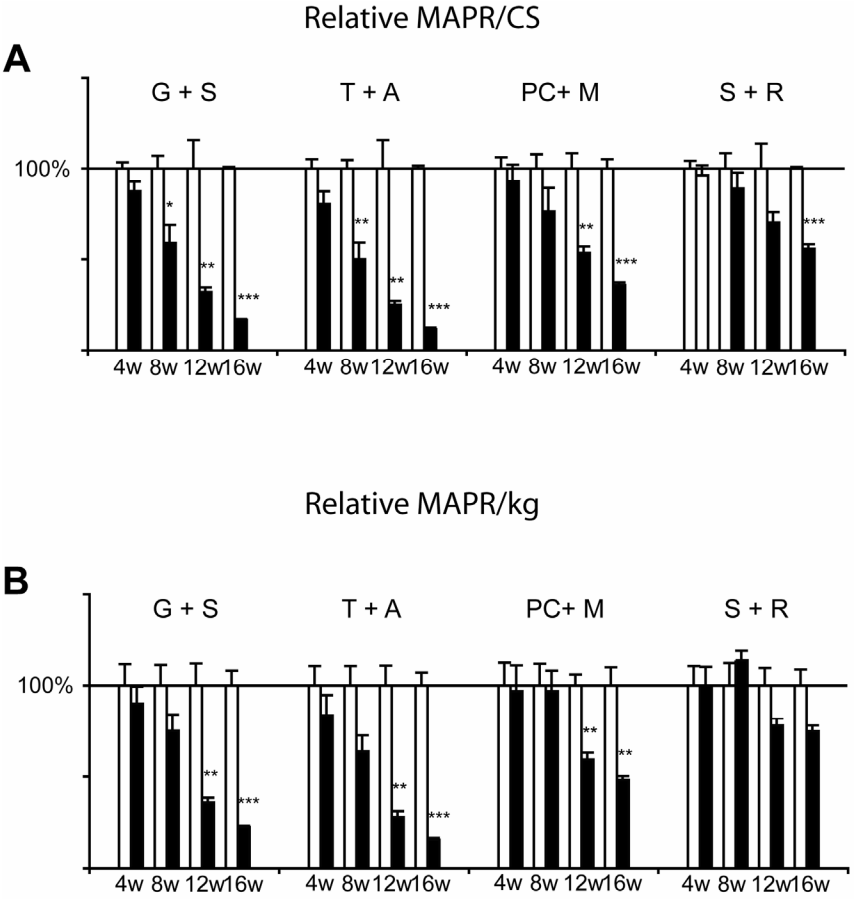
SUPPLEMENTARY FIGURE 1



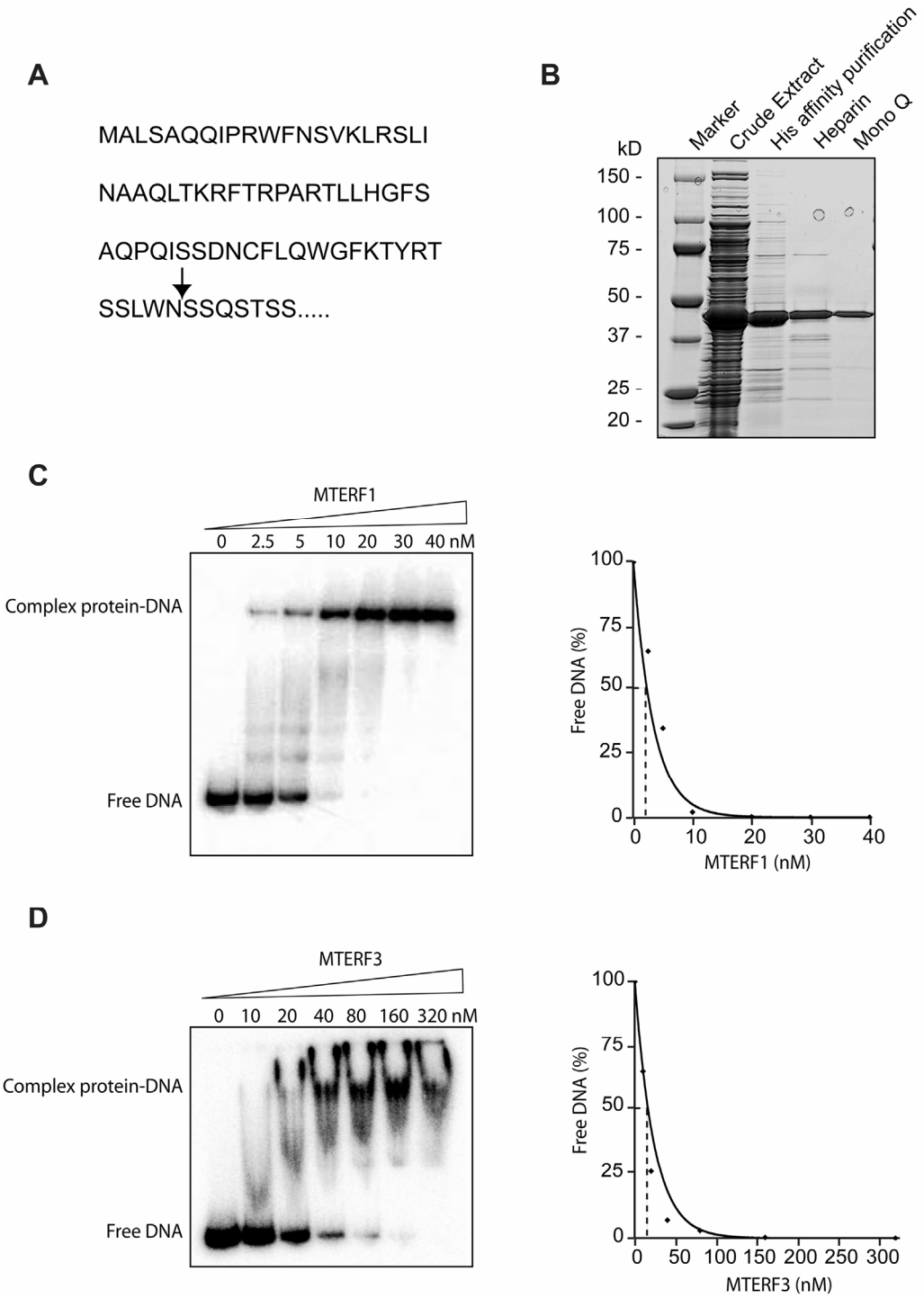
SUPPLEMENTARY FIGURE 2



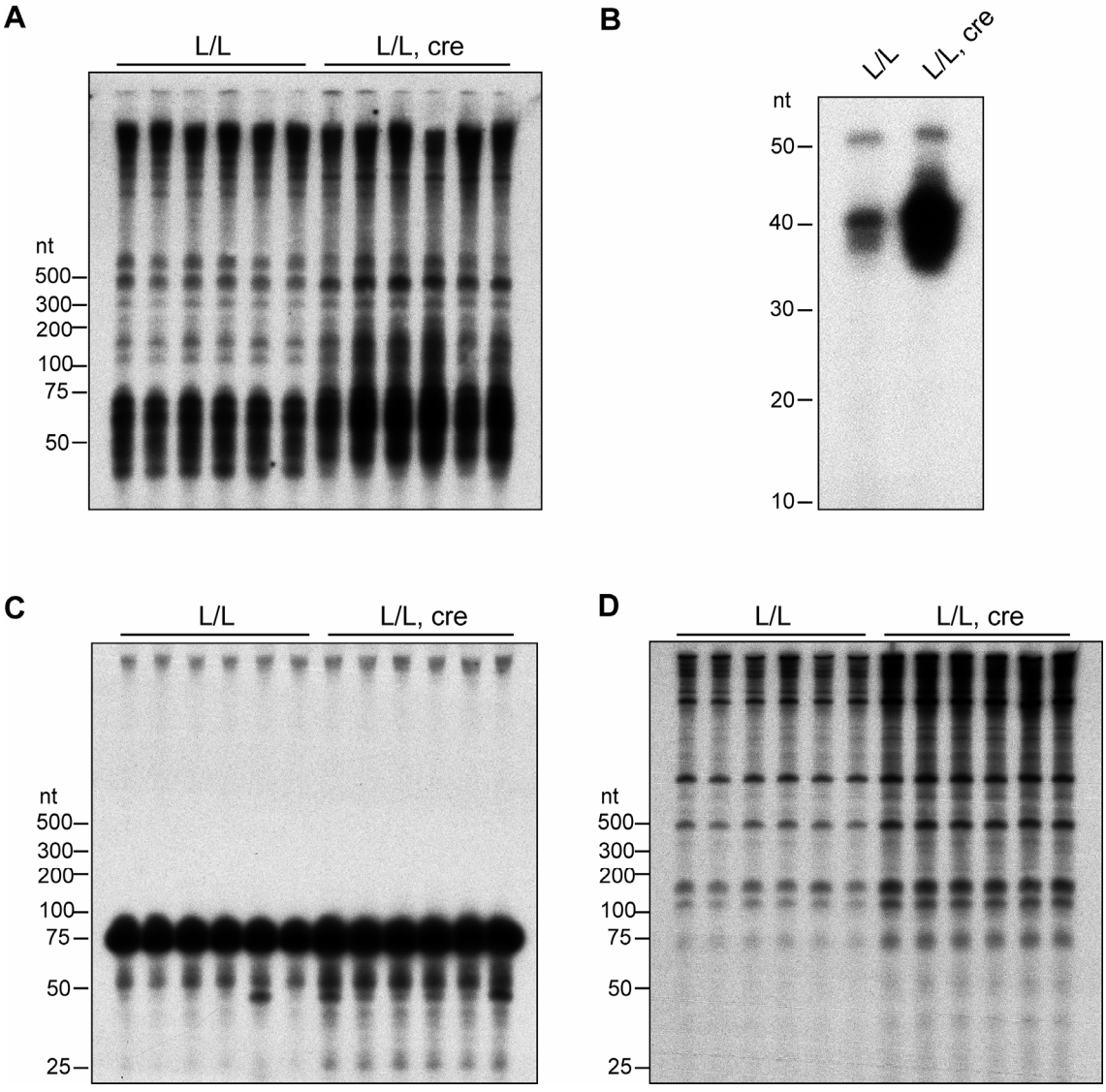
SUPPLEMENTARY FIGURE 3



SUPPLEMENTARY FIGURE 4



SUPPLEMENTARY FIGURE 5



LEGENDS TO SUPPLEMENTARY FIGURES

Supplementary Figure 1. Construction of modified *MTERF3* BACs. **A**. A PCR product consisting of the 5' portion of exon 3, the tetracycline resistance gene (*Tet-R*) and the 3' portion of exon 3 were used to replace the endogenous exon 3 in the BAC thus creating *BAC-MTERF3*$^{\text{Tet-R}}$. Next, *Tet-R* was replaced by using an oligonucleotide containing homology to the part of exon 3 that is adjacent to the 5' end of *Tet-R* and homology to the part of exon 3 that is adjacent to the 3' end of *Tet-R.* This oligonucleotide creates a synonymous codon change in exon 3 that abolish a *Pst*I restriction site, thus creating *BAC-MTERF3*$^{\text{M}}$. **B.** Analysis of the expression of *BAC-MTERF3*$^{\text{M}}$. PCR amplification of an *MTERF3* cDNA fragment followed by *Pst*I digestion produces a 588 bp fragment corresponding the wild-type allele (*MTERF3*$^{+}$), a 248 bp fragment of the knockout (*MTERF3*$^{-}$) allele and a 744 bp fragment of the *BAC-MTERF3*^{M} allele. The locations of PCR primers are indicated by arrows.

Supplementary Figure 2. Body weight and heart weight of control (white squares) and tissue-specific MTERF3 knockout (black squares) mice. **A**. Body weights of control (L/L, genotype *MTERF3*$^{\text{loxP}}$/*MTERF3*$^{\text{loxP}}$) mice (*n*=60) and tissue-specific knockout (L/L, cre; *genotype MTERF3*$^{\text{loxP}}$/*MTERF3*$^{\text{loxP}}$;+/*Ckmm-cre*) mice (n=20). **B**. Heart weights of control (L/L) mice (*n*=60) and tissue-specific knockout (L/L, cre) mice (n=20) mice. *, $P < 0.05$; ***, $P < 0.001$, Student's *t*-test. All error bars indicate s.e.m.

Supplementary Figure 3. Mitochondrial ATP-production rate (MAPR) in the heart of control (white bars) and tissue-specific MTERF3 knockout (blsck bars) mice. **A**. Measurements of MAPR per unit of citrate synthase (CS) activity, by using substrates that enter the respiratory chain at different points. The relative MAPR/CS presented as 100% in the figure corresponds to the following absolute ratios of MAPR/unit of CS activity at 4, 8, 12 and 16 weeks (w) of age, respectively: glutamate plus succinate (G + S), 0.22, 0.20, 0.20 and 0.23; TMPD plus ascorbate (T + A), 0.22, 0.18, 0.19 and 0.23; palmitoyl-L-carnitine plus malate (PC + M), 0.12, 0.10, 0.11 and 0.12; succinate plus rotenone (S + R), 0.04, 0.04, 0.04 and 0.05. **B**. Measurements of MAPR per kg of heart weight. The relative MAPR/kg presented as 100% in the figure corresponds to the following absolute ratios of MAPR/kg heart (mmol/ATP/min/kg heart) at 4, 8, 12 and 16 weeks (w) of age, respectively: G + S, 63, 55, 73, 69; T + A, 64, 51, 69 and 69; PC + M, 36, 27, 39 and 35; S + R, 12, 11, 16, and 15.

Supplementary Figure 4. Analyses of recombinant proteins. **A.** Amino terminal sequencing to determine the processing site (arrow) of the mitochondrial form of MTERF3. **B.** Purification of recombinant MTERF3. **C.** Electrophoresis mobility shift assay to determine the apparent K_d for non-sequence-specific binding of MTERF1 to dsDNA, The probe is a 20-bp fragment of the human D-loop region. **D.** Electrophoresis mobility shift assay to determine the apparent K_d for binding of MTERF3 to unspecific dsDNA,

Supplementary Figure 5. Analysis of promoter proximal mtDNA transcripts. **A, C, D**. Northern-blot analyses of mitochondrial transcripts in control (L/L) and MTERF3 knockout

(L/L, cre) hearts at 16 weeks of age (pairs of animals analysed, n=6). Oligonucleotides specific to the proximal region of the light-strand promoter (**A**), the heavy-strand promoter 1 (**C**), and the heavy-strand promoter 2 (**D**) were used. **B.** S1-mapping analysis of transcripts proximal to the light-strand promoter.

SUPPPLEMENTARY TABLE 1

Primers used for ChIP analysis

PCR product	Forward primer		Reverse primer	
	Location (nt)	Sequence (5'-3')	Location (nt)	Sequence (5'-3')
A	13785-13805	cctctacctaaaactcacagc	13932-13912	gatgctagggtagaatccgag
B	14394-14413	ctaaaacactcaccaagacc	14490-14471	ggaatgatggttgtctttgg
C	15604-15623	caaactaggaggcgtccttg	15773-15754	ctggttgtcctccgattcag
D	16205-16225	caagtacagcaatcaaccctc	16363-16343	gacgagaagggatttgactgt
E	16400-16419	ccaccatcctccgtgaaatc	16520-16501	gaccctgaagtaggaaccag
F	1-19	gatcacaggtctatcaccc	130-112	cagatactgcgacataggg
G	325-345	cacagcacttaaacacatctc	420-400	gtgcataccgccaaaagataa
H	583-602	gtagcttacctcctcaaagc	728-709	gagggtgaactcactggaac
I	1141-1160	cactacgagccacagcttaa	1283-1264	tcagggtttgctgaagatgg
J	1782-1800	gtaccgcaagggaaagatg	1899-1880	cttagctttggctctccttg
K	2435-2454	ggcatgctcataaggaaagg	2570-2551	ggccgttaaacatgtgtcac
L	2983-3001	gacctcgatgttggatcag	3099-3080	gaaaccgacctggattactc
M	3603-3622	cctaggcctcctatttattc	3754-3736	gaatgatggctagggtgac
N	4182-4201	cttcctaccactcaccctag	4360-4341	ctcagggatgggttcgattc
O	4803-4821	cacttctgagtcccagagg	4942-4924	gagagagtgaggagaaggc
P	5349-5368	ctacgcctaatctactccac	5549-5530	ctttgaaggctcttggtctg
Q	5985-6004	gtcctaggcacagctctaag	6153-6133	gaactagtcagttgccaaagc
R	6610-6630	gatttttcggtcaccctgaag	6729-6709	ctcagaccatacctatgtatc

S	7185-7204	caacactttctcggcctatc	7340-7321	cttcgaagcgaaggcttctc
T	7782-7801	ctatcctgcccgccatcatc	7936-7917	gattagtccgccgtagtcgg
U	8409-8429	ccatactccttacactattcc	8529-8509	cattttggttctcagggtttg
V	9001-9020	cgcctaaccgctaacattac	9145-9125	cgacagcgatttctaggatag
W	9605-9625	cacatccgtattactcgcatc	9752-9732	gaagtactctgaggcttgtag
X	10165-10184	cttacgagtgcggcttcgac	10368-10349	aggccagacttagggctagg
Y	10793-10813	ctaccactgacatgactttcc	10980-10961	ggtaggagtcaggtagttag
Z	11390-11409	ggactccacttatgactccc	11513-11493	ggttgagaatgagtgtgaggc
AA	11967-11986	cagccctatactccctctac	12110-12091	ggttgagggataggaggaga
BB	12603-12622	catccctgtagcattgttcg	12770-12751	tctcagccgatgaacagttg
CC	13181-13201	ctatcaccactctgttcgcag	13303-13283	gtggttggttgatgccgattg

SUPPLEMENTARY MATERIALS AND METHODS

Bioinformatics prediction of subcellular protein localization.

We used the following software tools to predict subcellular localization of human MTERF3: MitoProt II 1.0a4 (http://ihg.gsf.de/ihg/mitoprot.html), PSORT (http://psort.ims.u-tokyo.ac.jp/form2.html) and TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP-1.1).

Confocal microscopy

We analyzed subcellular localization of MTERF3 with confocal microscopy. A *Xho*I -*Sac*II fragment encoding the complete human MTERF3 protein sequence was cloned into the *Xho*I and *Sac*II restriction sites of the EGFP-N3 plasmid (Clontech). The resulting plasmid (MTERF3-GFP) encodes a fusion protein consisting of MTERF3 with an in-frame addition of green fluorescent protein (GFP) to its carboxy-terminus. We transfected HeLa cells with pEGFP-N3, MTERF3-GFP and a control plasmid containing the mitochondrial targeting peptide of ornithine transcarbamylase added in frame to the aminoterminus of GFP (OTC-GFP). We transiently transfected HeLa cells and used a laser scanning confocal microscope to monitor expression of GFP. We observed excitation and emission of GFP at 488 nm and 400-440 nm, respectively. We labelled mitochondria by adding 25 nM MitoTracker Red CMXRos (Molecular Probes) to living cells for 20 min and observed excitation at 568 nm and emission at 580-640 nm.

Mitochondrial protein import assays

The full-length human MTERF3 protein was expressed *in vitro* using the TnT® Coupled Reticulocyte Lysate System (Promega) following the manufacturer's instructions in the presence of 30 μCi of [^{35}S]methionine. Rat liver mitochondria were obtained by differential centrifugation and the protein concentration was determined by the Bradford assay. Mitochondria were washed twice in import buffer (10 mM tris-HCl, 25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM KH_2PO_4, 0.05 mM EDTA, 5 mM $MgCl_2$, pH 7.4) and resuspended in the same buffer at 2 mg protein/ml. For the import reaction, 5 μl of TnT reaction were added to 40 μl of mitochondrial suspension supplemented with 1mg/ml bovine serum albumin, 2 mM Na succinate, 1 mM methionine and 1 mM ATP, to a final volume of 50 μl. In some experiments, carbonyl cyanide m-chlorophenylhydrazone (CCCP) was included in the reaction mixture at 1.7 μg/ml. The import reaction was carried out for 30 min at 37^{o}C in a rotary shaker. In some experiments, the import reaction was allowed to proceed for additional 10 minutes in the presence of trypsin (10 μg/ml final concentration). After completed import, mitochondria were washed twice in import buffer and disrupted in 2xSDS sample buffer (100 mM Tris-HCl, 4% sodium dodecyl sulfate, 0.2% bromophenol blue, 20% glycerol, 200 mM dithiothreitol) for 3 min at 95^{o}C. The mitochondrial lysates were separated in a 12.5% SDS-PAGE gel. The gel was fixed, treated with Amplify™ (Amersham), washed in 7% acetic acid/ 7% methanol/ 1% glycerol, dried and used for autoradiographic detection of labelled proteins.

Transmission electron microscopy

Electron micrographs of mitochondria were obtained as previously described (Hansson et al., 2004). Briefly, small pieces from the left myocardium were fixed in 2% glutaraldehyde, 0.5% paraformaldehyde, 0.1M sodiumcacodylate, 0.1M sucrose, and 3mM $CaCl_2$ (pH 7.4) at room temperature for 30 min, followed by 24h at 4 ^oC. Specimens were rinsed in a buffer containing 0.15M sodiumcacodylate and 3mM $CaCl_2$ (pH 7.4), postfixed in 2% osmiumtetroxide, 0.07M sodiumcacodylate, 1.5mM $CaCl_2$ (pH 7.4) at 4 ^oC for 2h, dehydrated in ethanol followed by acetone and embedded in LX-112 (Ladd, Burlington, VT). Ultra-thin sections (40-50 nm) from longitude parts were cut and examined in a Tecnai 10 transmission electron microscope (Fei, Eindhoven, The Netherlands) at 80 kV.

In vitro transcription.

As template for in vitro transcription, a linear DNA fragment encompassing nucleotides 15910 to 728 of human mtDNA was used. Individual reaction mixtures (25 µl) contained 3.5 µl S-100 extract supplemented with 2.5 pmol TFAM to boost transcription activity. In addition, the reactions contained 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, 100 µg/ml bovine serum albumin, 4U RNAGuard® (Roche), 0.4 mM ATP, 0.15 mM CTP, 0.15 mM GTP, 0.01 mM UTP, 1.25 µCi [α^{32}P]-UTP, and 85 fmol DNA template. After incubation at 32^oC for 30 min, the reactions were stopped by adding 200 µl stop buffer (10 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 1 mM EDTA and 0.1 mg/ml glycogen). Samples were then incubated with 100 µg/ml proteinase K in the presence of 0.5 % SDS at 42^oC for 45 min and the labeled transcripts were precipitated with 0.6 ml ice-cold EtOH. Pellets were resuspended in 10 µl loading buffer (98% formamide, 10 mM EDTA, pH 8.0, 0.025 %

xylene cyanol, 0.025 % bromophenol blue), heated at 95°C for 4 min, kept on ice for 3 min and loaded on a 4% polyacrylamide/7M urea gel in 1 x TBE. After electrophoresis, the gels were dried and exposed to a Hyperfilm® (Amersham Biosciences) or, for quantification purposes, to a Phosphorimager® screen and scanned with a Personal Molecular Imager FX (Bio-Rad). Quantification was carried out with Quantity One 4.6 software (Bio-Rad).

References

Hansson, A., Hance, N., Dufour, E., Rantanen, A., Hultenby, K., Clayton, D. A., Wibom, R., and Larsson, N. G. (2004). A switch in metabolism precedes increased mitochondrial biogenesis in respiratory chain-deficient mouse hearts. Proc Natl Acad Sci USA *101*, 3136-3141.