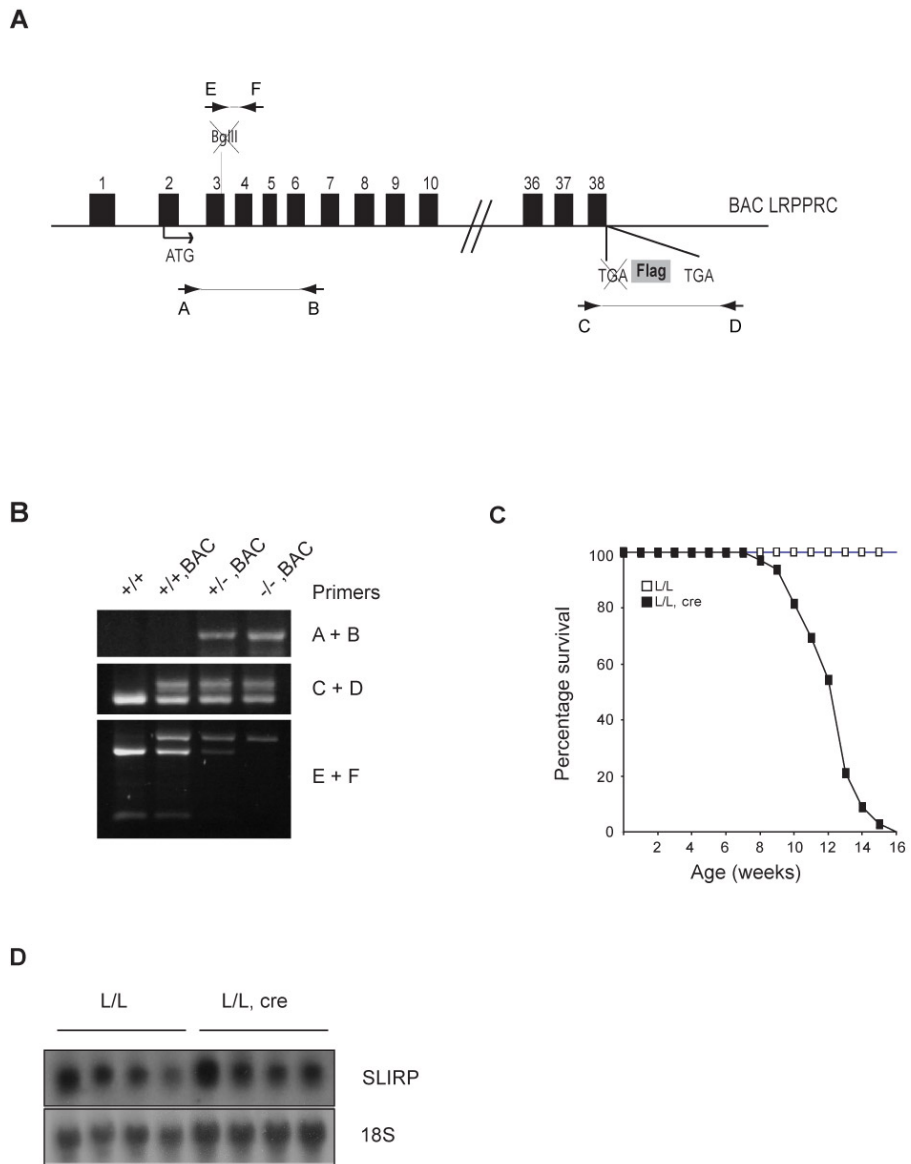


**SUPPLEMENTARY MATERIAL**

**Figure S1**



**Figure S1. Transgenic mice expressing LRPPRC-Flag and characterization of *Lrpprc* heart knockout animals**

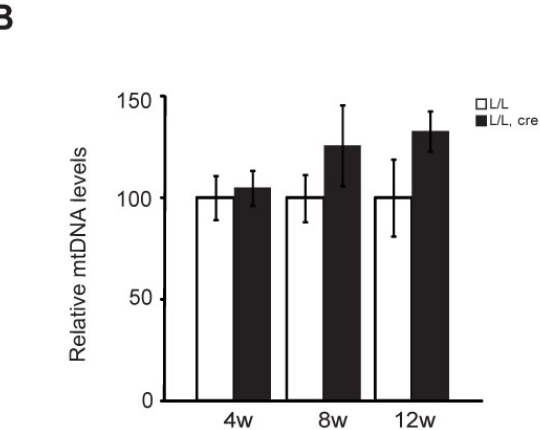
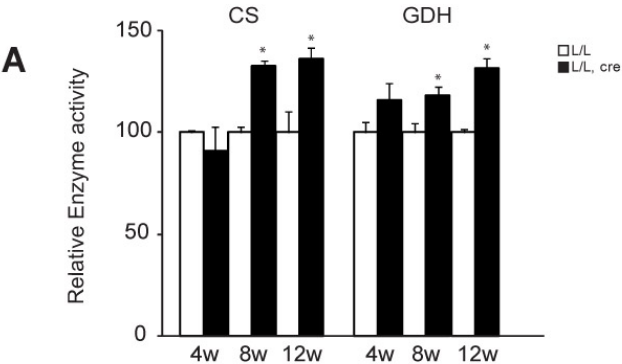
(A) Map of the modified *Lrpprc* BAC with localization of the PCR primers used to genotype the mice. A *Bgl*III site was eliminated by the introduction of a silent point mutation. The stop codon in exon 38 was removed and a nucleotide sequence encoding a Flag-peptide with a downstream stop codon was introduced. PCR primers used for genotyping are indicated by arrows.

(B) PCR genotyping of wild-type (+/+) and homozygous *LRPPRC* germline knockout mice (*Lrpprc*<sup>-/-</sup>), which also transgenically express LRPPRC-Flag (+/BAC-*LRPPRC*-Flag). The rescue mice have the genotype: *Lrpprc*<sup>-/-</sup>, +/BAC-*LRPPRC*-Flag. PCR primers A+B detect the knockout allele in the endogenous *Lrpprc* locus. PCR primers C+D detect the wild-type exon 38 (lower band) and the modified exon 38 encoding a Flag-tag (upper band). PCR primers E+F and *Bgl*III digestion of the PCR product detects the endogenous *Lrpprc* allele (120bp+400bp) and the *Lrpprc*-Flag BAC allele (520bp).

(C) Life span curves showing percentage of surviving mice at the indicated ages. Open squares represent control mice (*Lrpprc*<sup>loxP/loxP</sup>; L/L; n=50) and filled squares tissue-specific knockout mice (*Lrpprc*<sup>loxP/loxP</sup>, +/Ckmm-cre; L/L,cre; n=33)

(D) Northern blot analyses of the steady-state levels of SLIRP mRNA in heart from control (L/L) and knockout (L/L,cre) mice at 12 weeks of age.

**Figure S2**

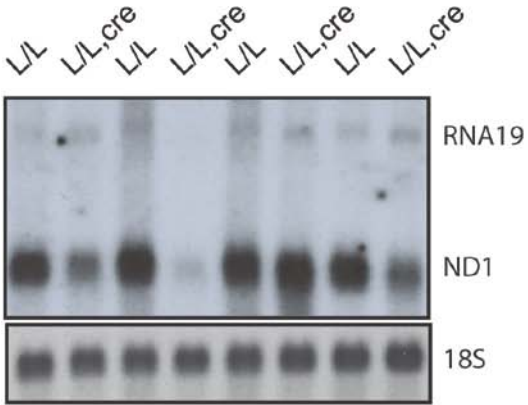


**Figure S2. Mitochondrial mass and levels of mtDNA in *Lrpprc* knockout hearts**

(A) Activity of citrate synthase (CS) and glutamate dehydrogenase (GDH) in heart mitochondria from control (L/L) and *Lrpprc* knockout (L/L,cre) mice of different ages. For each time point the number of analysed animals was n=3 for both genotypes. Error bars indicate SEM; \* = p<0.05, Student's *t* test was used.

(B) Southern blot quantification of mtDNA levels in hearts from control and tissue-specific knockout mice at different ages. A similar number of control (n=5) and knockout animals (n=5) were analysed at age 4 weeks, 8 weeks and 12 weeks. Error bars indicate SEM.

**Figure S3**



**Figure S3. Steady-state levels of a precursor transcript containing the 16S rRNA and the ND1 mRNA (RNA19)**

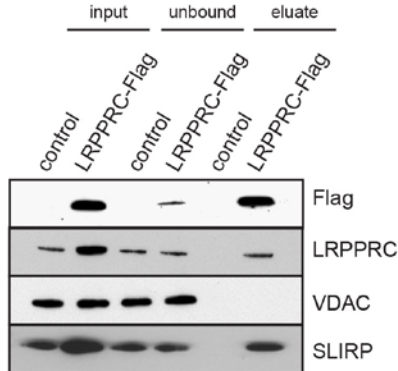
Northern blot analysis of heart RNA from control (L/L) and *LRPPRC* knockout (L/L,cre) mice at 12 weeks of age. RNA19 was detected by a probe specific for ND1.

**Figure S4**

**A**

MAALLRSARWLLRAGAAPRLPLSLRLLPGGPGRLLHAASYLPAAR  
AGPVAGLLSPARLYAIAAKEKDIQEESTFSSRKISNQFDWALMR  
LDLSVRRTGRIPKLLQKVFNDTCRSGGLGG

**B**



**Figure S4. Amino-terminal sequencing of the mature form of hLRPPRC and immunoprecipitation experiments.**

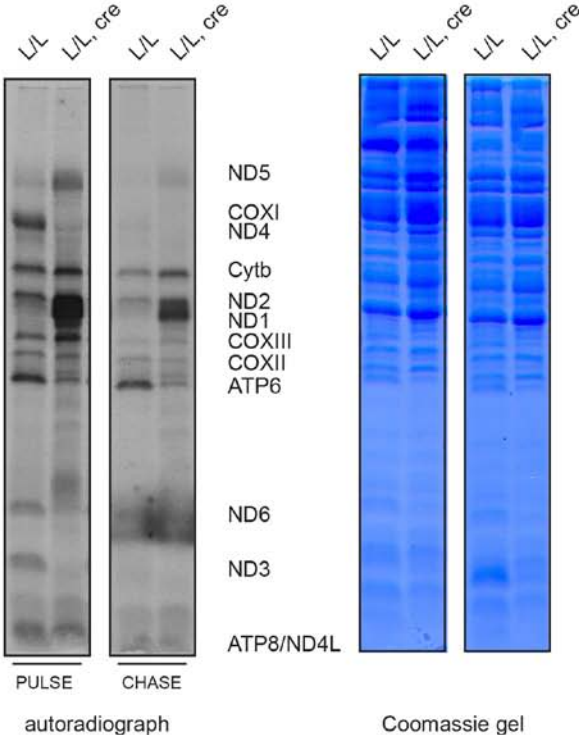
(A) Edman degradation was performed on hLRPPRC-Flag protein that had been affinity purified from transfected HeLa cells. The amino terminal sequence of intramitochondrial LRPPRC is indicated by black letters. The mitochondrial leader peptide is indicated by grey letters. The cleavage site to remove the leader peptide is indicated by an arrow.

(B) Mitochondrial extracts from HeLa cells expressing hLRPPRC-Flag protein were used for immunoprecipitation. Co-immunoprecipitation between hLRPPRC-Flag and SLIRP was observed. LRPPRC and SLIRP were detected by immunoblotting using specific antibodies. Mitochondrial extracts from untransfected HeLa cells were used as negative control (control). Input, unbound and eluate fractions are shown.

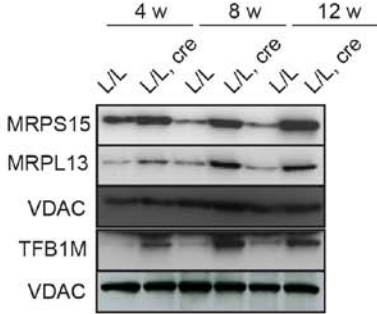


Figure S5

A



B

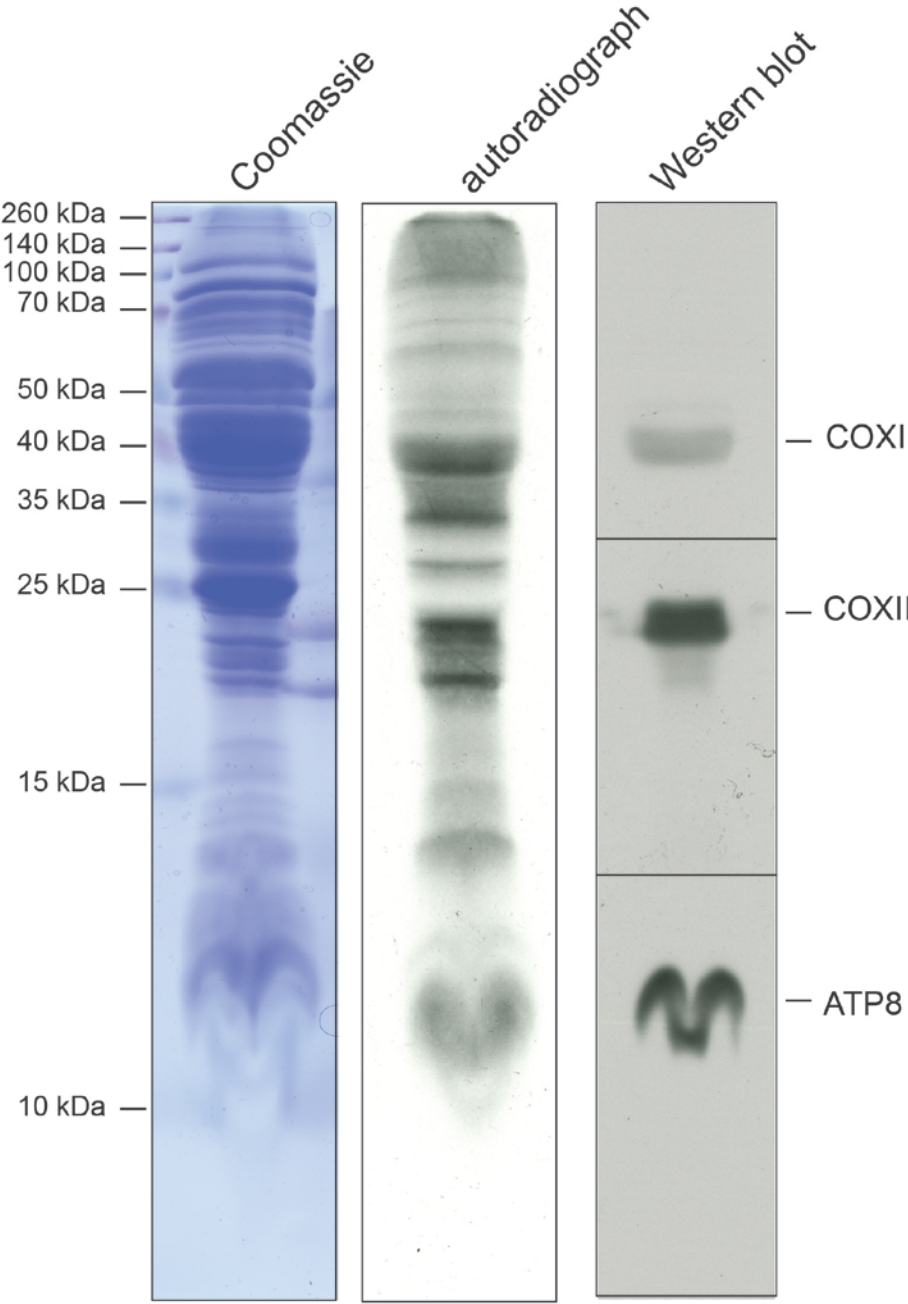


**Figure S5. Translation in *Lrpprc* knockout heart mitochondria**

(A) Mitochondria from control (L/L) and *Lrpprc* knockout (L/L,cre) mice were pulse-labeled with [<sup>35</sup>S]-methionine and [<sup>35</sup>S]-cysteine for 1 hour, followed by a cold chase for 3 hours. Coomassie blue staining of SDS PAGE separated proteins is shown as loading control. The two panels of the autoradiograph and the Coomassie gel are derived from a single contiguous gel.

(B) Steady-state levels of small (MRPS15) and large (MRPL13) ribosomal subunit proteins in heart mitochondrial extracts from control (L/L) and knockout (L/L,cre) mice at different ages. Also the protein levels of the ribosomal adenine methyltransferase TFB1M are shown. VDAC was used as loading control.

**FIGURE S6**



**Figure S6. Western blot analyses to identify specific *in organello* translation products in mouse heart mitochondria.**

The *in organello* translation reactions were performed by using wild-type mouse heart mitochondria. The total mitochondrial protein mixture after radioactive labeling was separated in a SDS-page gel. One lane was stained with Coomassie (left panel) and then used for autoradiography to identify radioactively labeled polypeptides (middle panel). A second adjacent lane from the same gel was transferred to nitrocellulose by western blotting. After transfer, the membrane was cut into three different portions, each containing separated proteins of different molecular weights. The three different membranes were probed with a monoclonal antibody against COXI (right panel, top), a polyclonal antibody against COXII (right panel, middle) and a polyclonal antibody against ATP8 (right panel, bottom), respectively. The molecular weight markers for all three panels are shown on the left side.

**Table T1****Table T1. Description of the structure of mitochondrial rRNAs and mRNAs as determined by sequencing of circularized RNA molecules.**

	<b>5' UTR</b>	<b>Start position (codon)</b>	<b>Stop position (codon)</b>	<b>Number of A required to form STOP</b>	<b>3' UTR</b>
<b><u>rRNAs</u></b>					
<b>12S</b>	-	69 (none)	1024 (none)	-	-
<b>16S</b>		1094 (none)	2674 (none)	-	-
<b><u>mRNAs</u></b>					
<b>ND1</b>	-	2715 (GTG)	3705 (T)	2	0
<b>ND2</b>	-	3914 (ATA)	4949 (T)	2	0
<b>CoxI</b>	t	5328 (ATG)	6871 (TA)	1	71nt
<b>CoxII</b>	t	7013 (ATG)	7696 (TAA)	0	3nt
<b>ATP8<sup>1</sup></b>	t	7766 (ATG)	7969 (TAA)	0	ATP6
<b>ATP6<sup>1</sup></b>	ATP8	7927 (ATG)	8606 (TA)	1	0
<b>CoxIII</b>	-	8607 (ATG)	9390 (T)	2	0
<b>ND3</b>	-	9459 (ATC)	9806 (TAA)	0	1nt
<b>ND4L<sup>2</sup></b>	t	9877 (ATG)	10174 (TAA)	0	ND4
<b>ND4<sup>2</sup></b>	ND4L	10167 (ATG)	11544 (T)	2	0
<b>ND5</b>	-	11742 (ATC)	13565 (TAA)	0	> 155nt
<b>ND6</b>	-	14070 (ATG)	13522 (TAA)	0	626nt
<b>Cytb</b>	accta	14145 (ATG)	15288 (T)	2	0

<sup>1</sup>Encoded by a bicistronic mRNA containing the ATP6 and ATP8 open reading frames.

<sup>2</sup>Encoded by a bicistronic mRNA containing the ND4L and ND4 open reading frames.

## EXPERIMENTAL PROCEDURES

### Southern, Northern and Western Blot Analysis

For mtDNA quantification, total cellular DNA was isolated with the DNeasy Blood & Tissue Kit (QIAGEN), and 1-5 µg of DNA was digested with *SacI* endonuclease, electrophoresed in 0.8% agarose gels and transferred onto nylon membranes by Southern blotting. The membranes were probed with [ $\alpha$ -<sup>32</sup>P]-dCTP-labeled COXI and 18S DNA. Around 1-2 µg of total RNA was denatured in NorthernMax®-Gly Sample Loading Dye (Ambion), electrophoresed in 1.2% agarose gels containing formaldehyde and transferred to nylon membranes. Individual transcripts were detected using [ $\alpha$ -<sup>32</sup>P]-dCTP-labeled DNA probes. tRNAs were detected using specific oligonucleotides labeled with  $\gamma$ -<sup>32</sup>P-ATP. Radioactive signals were detected by autoradiography and quantification was performed using Molecular Imager FX (Bio-Rad) and Quantity One software (Bio-Rad). Protein samples were separated using SDS-PAGE and transferred to Hybond-C nitrocellulose membranes (GE Healthcare). Immunodetection of COXI and COXIV (Complex IV), SDHA (Complex II), UQCRC2 (Complex III), NDUFA9 (Complex I), ATP5A1 (Complex V) was performed with specific monoclonal antibodies (Molecular Probes). VDAC (porin) antibodies were purchased from Calbiochem and polyclonal antisera were used to detect TFAM, COXII and ATP8 (Larsson et al, 1998), MTERF3 (Park et al, 2007), TFB1M, TFB2M, MRPS15 and MRPL13 (Metodiev et al, 2009). Polyclonal antibodies against both N- and C-terminal regions of LRPPRC were generated in rabbits using synthetic peptides: VYLQNEYKFSPTDFLAK and TAKNLKLLDDLFLKRYA (Agrisera) (Tsuchiya et al, 2002). Antibodies against the human SLIRP protein were purchased from Abcam while monoclonal antibodies against mouse SLIRP protein were generated by AbD Serotec.

### **Analyses of *de novo* transcription and translation in isolated mitochondria**

*In organello* transcription assays were performed on mitochondria isolated from heart tissue as previously described (Enriquez et al, 1996) with modifications. Mouse heart mitochondria were isolated by differential centrifugation in isolation buffer A. The protein concentration was measured by using Bradford Reagent (Sigma) and ~800  $\mu\text{g}$  of mouse heart mitochondria were washed in 1 ml of transcription incubation buffer (25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM  $\text{K}_2\text{HPO}_4$ , 50  $\mu\text{M}$  EDTA, 5 mM  $\text{MgCl}_2$ , 1 mM ADP, 10 mM glutamate, 2.5 mM malate, 10 mM Tris-HCl pH 7.4 and 1 mg/ml BSA). Mitochondria were pelleted by centrifugation at 10.000xg for 3 min at 4°C, resuspended in 500  $\mu\text{l}$  of transcription incubation buffer supplemented with 50  $\mu\text{Ci}$  of  $\alpha$   $^{32}\text{P}$ -UTP (Perkin-Elmer) and incubated for 1 h at 37°C with gentle rotation. After incubation, mitochondria were washed twice with 1 ml of washing buffer (10% glycerol, 10 mM Tris-HCl pH 6.8 and 0.15 mM  $\text{MgCl}_2$ ) and resuspended in 1 ml of Trizol Reagent (Invitrogen) for RNA isolation. Isolated RNA was analysed by northern blotting and radiolabeled transcripts were visualized by autoradiography. For pulse-chase experiments, labeled mitochondria were washed twice with 1 ml of transcription incubation buffer, resuspended in 500  $\mu\text{l}$  of the same buffer and incubated for 75 and 150 min at 37°C. After incubation, mitochondria were washed with washing buffer and RNA was isolated as previously described.

*In organello* translation was performed as previously described (Cote et al, 1989) with some modifications. Mitochondria were isolated by differential centrifugation in isolation buffer A and incubated at 37°C with gentle rotation for 5 min in translation buffer containing 6  $\mu\text{g}/\text{ml}$  of all amino acids except methionine. Easy Tag Express $^{35}\text{S}$  Protein labeling mix (a mix of [ $^{35}\text{S}$ ]-Methionine and [ $^{35}\text{S}$ ]-Cysteine; Perkin Elmer) was added to a final concentration of 0.35 mCi/ml and after 1h mitochondria were washed with isolation buffer A and resuspended in a conventional SDS-PAGE loading buffer. For pulse-chase experiments, labeled mitochondria

were washed twice with 1 ml of translation buffer, resuspended in 500  $\mu$ l of the same buffer and incubated for 3 hours at 37°C. Translation products were separated by SDS-PAGE, the gel was stained with Coomassie blue (GE Health Care), treated with Amplify™ (Amersham) and dried. Newly synthesized, radiolabeled polypeptides were detected by autoradiography. The identities of the translation products corresponding to COXI, COXII and ATP8 were verified by western blots with specific antibodies (Figure S6). The identification of the other translation products was done according to previous publications taking into account the predicted size and migration of the translation product (Sasarmann et al., 2010, Fernandez-Silva et al., 2007).

### **Protein immunoprecipitation and RNA Immunoprecipitation Assay (RIP)**

Protein immunoprecipitation and RNA immunoprecipitation assay were performed as previously described (Camara et al, 2011), but without crosslinking with formaldehyde.

### **RNA circularization and RT-PCR**

An RNA circularization protocol was performed as described previously (Stewart & Beckenbach, 2009). Approximately 6 ng total mitochondrial RNA was circularized with 5 U T4 RNA ligase in a volume 200  $\mu$ l at 16°C for at least 16 h in the manufacturer-supplied buffer (NEB). The circularized RNA was precipitated with an equal volume of isopropanol, incubated at -20°C for at least 4 h, and centrifuged for 20 min at top speed in a bench top centrifuge. The entire precipitate was used for complementary DNA synthesis with gene specific primers using GeneAmp® RNA PCR kit (Applied Biosystems). PCR products were purified using ExoSAP-IT (Affymetrix) and sequenced. Some PCR products were cloned and sequenced in order to confirm the results from direct sequencing. Primer sequences for RT-PCR and subsequent PCR are available on request.



### **Size exclusion chromatography**

Liver mitochondria were isolated by differential centrifugation in isolation buffer A (320 mM Sucrose, 1 mM EDTA, 10 mM Tris-HCl pH 7.4) containing 1x complete protease inhibitor cocktail (Roche). Mitochondria were lysed at a concentration of 5 mg/ml in lysis buffer B (50mM TrisHCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 5% glycerol, 0.5% TritonX and 1x complete protease inhibitor cocktail for 20 min on ice followed by centrifugation at 13000xg for 45 min at 4°C. Next, 1 mg of the precleared lysate was subjected to size exclusion chromatography through Superose 6 column (GE Healthcare), which had been pre-equilibrated with lysis buffer B. Fractions of 1 ml were collected, TCA precipitated and analyzed by SDS-PAGE and immunoblotting. Mitochondrial lysis with buffers containing varying concentrations of NaCl (500 mM), EDTA (100 mM) or supplemented with RNase was also performed.

### **Ribosomal and RNA analysis using sucrose density ultracentrifugation**

Sucrose density ultracentrifugation of ribosomal particles was performed as described previously (Metodieiev et al, 2009) with some modifications. Mitochondria (0.9-1.2 mg) were lysed in lysis buffer containing 260 mM sucrose, 100 mM KCl, 20 mM MgCl<sub>2</sub>, 10 mM Tris-HCl pH 7.5, 0.08 U/ml RNasin<sup>®</sup> (Promega), 0.5 % Triton X100 and supplemented with EDTA-free complete protease inhibitor cocktail (Roche). Mitochondrial lysates (typically 0.9 mg) were overlaid on top of 10%-30% linear sucrose gradients prepared in 100 KCl, 20 mM MgCl<sub>2</sub>, 10 mM Tris-HCl, pH 7.5 and EDTA-free complete protease inhibitor cocktail (Roche). For analysis of rRNA and mRNA sedimentation profiles, RNA was isolated from one third of each fraction using Trizol<sup>®</sup>SL (Invitrogen<sup>™</sup>) according to manufacturer's recommendations. The samples were subjected to DNase treatment (Ambion) and reverse transcribed to cDNA using High-Capacity cDNA Archive kit (Applied Biosystems). For the detection of each mitochondrial transcripts TaqMan gene expression assays were used (Applied Biosystems).

## **Biochemistry and BN-PAGE**

Enzyme activities of the respiratory chain complexes, mitochondrial ATP production rate (MAPR) were measured as previously described (Wibom et al, 2002). BN-PAGE and subsequent immunodetection of assembled complexes was performed as previously described (Park et al, 2007).

## **Transmission electron microscopy and volume density measurements**

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<sub>2</sub> (pH 7.4) at room temperature for 30 min, followed by 24h at 4°C. Specimens were rinsed in a buffer containing 0.15M sodiumcacodylate and 3mM CaCl<sub>2</sub> (pH 7.4), postfixed in 2% osmiumtetroxide, 0.07M sodiumcacodylate, 1.5mM CaCl<sub>2</sub> (pH 7.4) at 4°C for 2h, dehydrated in ethanol followed by acetone and embedded in LX-112 (Ladd, Burlington, VT). Ultra-thin sections (40-50 nm) from longitudinal parts were cut and examined in a Tecnai 10 transmission electron microscope (Fei, Eindhoven, The Netherlands) at 80 kV. Digital images at a final magnification of 8200X were randomly taken on myofibrils from sections of the myocardium. Printed digital images were used and the volume density (V<sub>v</sub>) of mitochondria was calculated by point counting using a 2 cm square lattice according to Weibel (1979). A pilot study was performed to determine the number of blocks and images needed for an appropriate sample using cumulative mean plot for evaluation (Weibel 1979). Thus, two different blocks from one animal were sectioned and 10 random images were collected from each block.

## **Electrophoresis of mitochondrial protein complexes**

1-D BNE (blue native electrophoresis), and 2-D SDS-PAGE were performed as described (Schagger, 2006; Wittig et al, 2006). Briefly, 240  $\mu$ g mitochondria were resuspended in 70  $\mu$ l solubilization buffer (50 mM NaCl, 50 mM imidazole/HCl, 2 mM 6-aminohexanoic acid, 1 mM EDTA, pH 7.0) and solubilized with a DDM/protein ratio of 2.5 g/g. After centrifugation 15 min at 100,000g, the supernatant was supplemented with 4  $\mu$ l of a 5% Coomassie blue G-250 suspension in 500 mM 6-aminohexanoic acid. 25  $\mu$ l of each sample, corresponding to 80  $\mu$ g mitochondrial protein, were loaded to a 4 to 13 % acrylamide gradient gel. Following BNE, native gel strips were placed on top of a 16 % Tricine–SDS gel for second dimension electrophoresis. 2-D SDS gels were silver stained (Rais et al, 2004).

## **Generation of stable HeLa TetON cell lines expressing hLRPPRC-Flag**

The stop codon of a full-length cDNA clone of human *LRPPRC* (hLRPPRC, Origene) was changed to a *XbaI* site and the cDNA was fused to an oligonucleotide encoding for the Flag-tag, inserted as a *XbaI*-fragment. This cDNA, encoding hLRPPRC-Flag, was cloned into the pTRE-Tight vector (Clontech) to generate the pTRE-hLRPPRC-Flag plasmid. HeLa TetON cells (Clontech) were cotransfected with the pTRE-hLRPPRC-Flag vector and a linear hygromycin resistance marker (Clontech). Stably transfected clones (hLRPPRC-Flag-HeLa) were obtained by culturing the cells in GlutaMax DMEM medium (Gibco) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 200  $\mu$ g/ml hygromycin. Induction of hLRPPRC-Flag expression was achieved by addition of 2  $\mu$ g/ml doxycycline to the incubation medium for 48-72 h.

## **N-terminal sequencing**

Expression of the hLRPPRC-Flag protein was induced in stably transfected HeLa cells for 48h. Mitochondria from  $\sim 4 \times 10^8$  cells were isolated by differential centrifugation, hLRPPRC-Flag protein was purified using ANTI-FLAG M2 affinity gel (Sigma) and subjected to automated chemical (Edman) protein sequencing using a Procise 494 instrument from Applied Biosystems as described (Tempst et al, 1994).

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