

Supplemental Figures

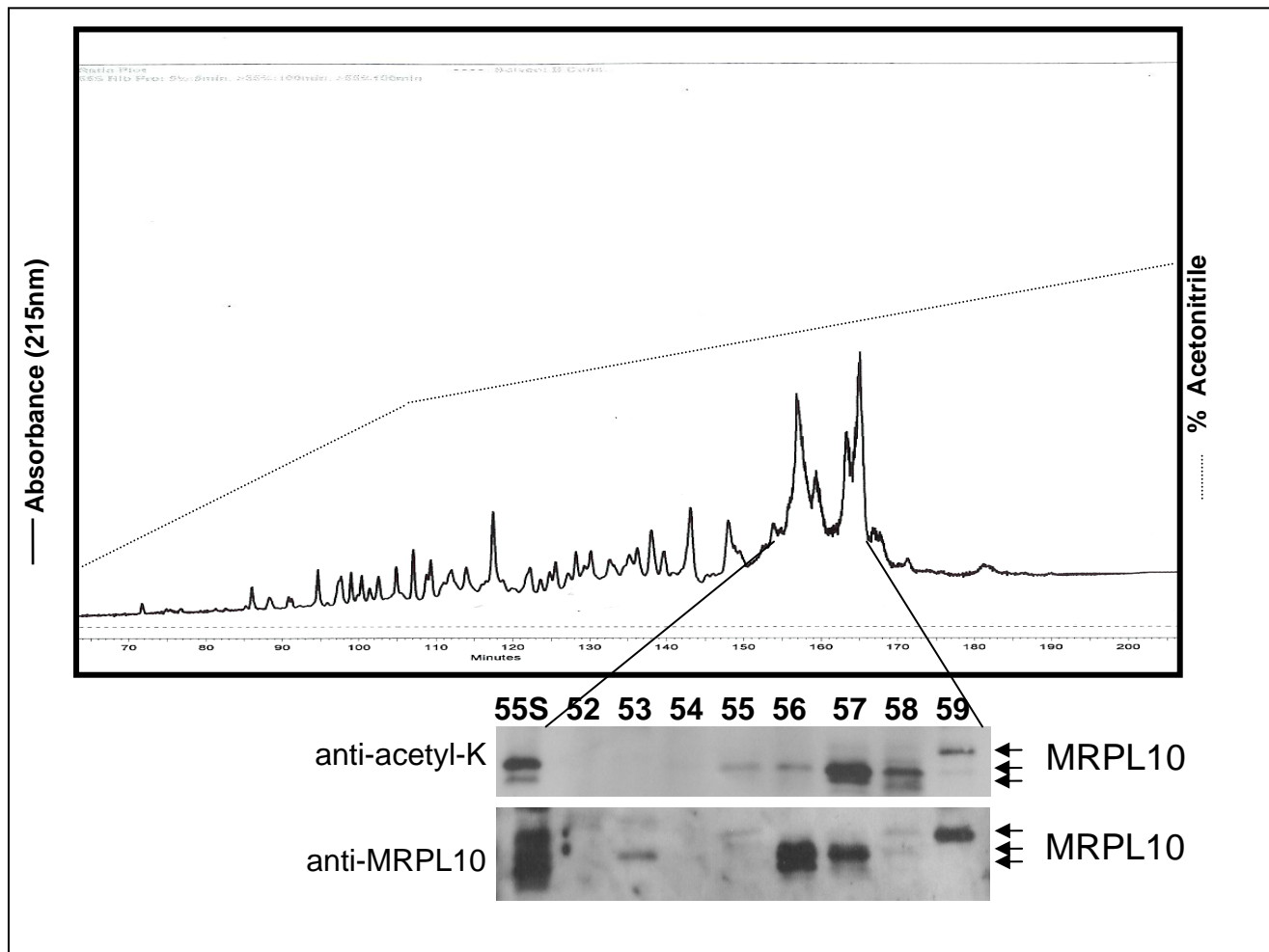


Figure S1. Purification of MRPL10 and its acetylated forms by RP-HPLC. Approximately, 5 A₂₆₀ units of purified 55S ribosome preparation obtained from bovine liver were incubated in the presence of glacial acetic acid as described in Materials and Methods. After centrifugation, supernatant containing the ribosomal proteins was separated with RP-HPLC using 5-55% acetonitrile gradient. Equal volumes of gradient fractions were separated on 12 % SDS-PAGE gels and PVDF blots were probed with anti-N-acetyl lysine and human MRPL10 antibodies. Acetylated protein bands corresponding to MRPL10 protein were shown by arrows.

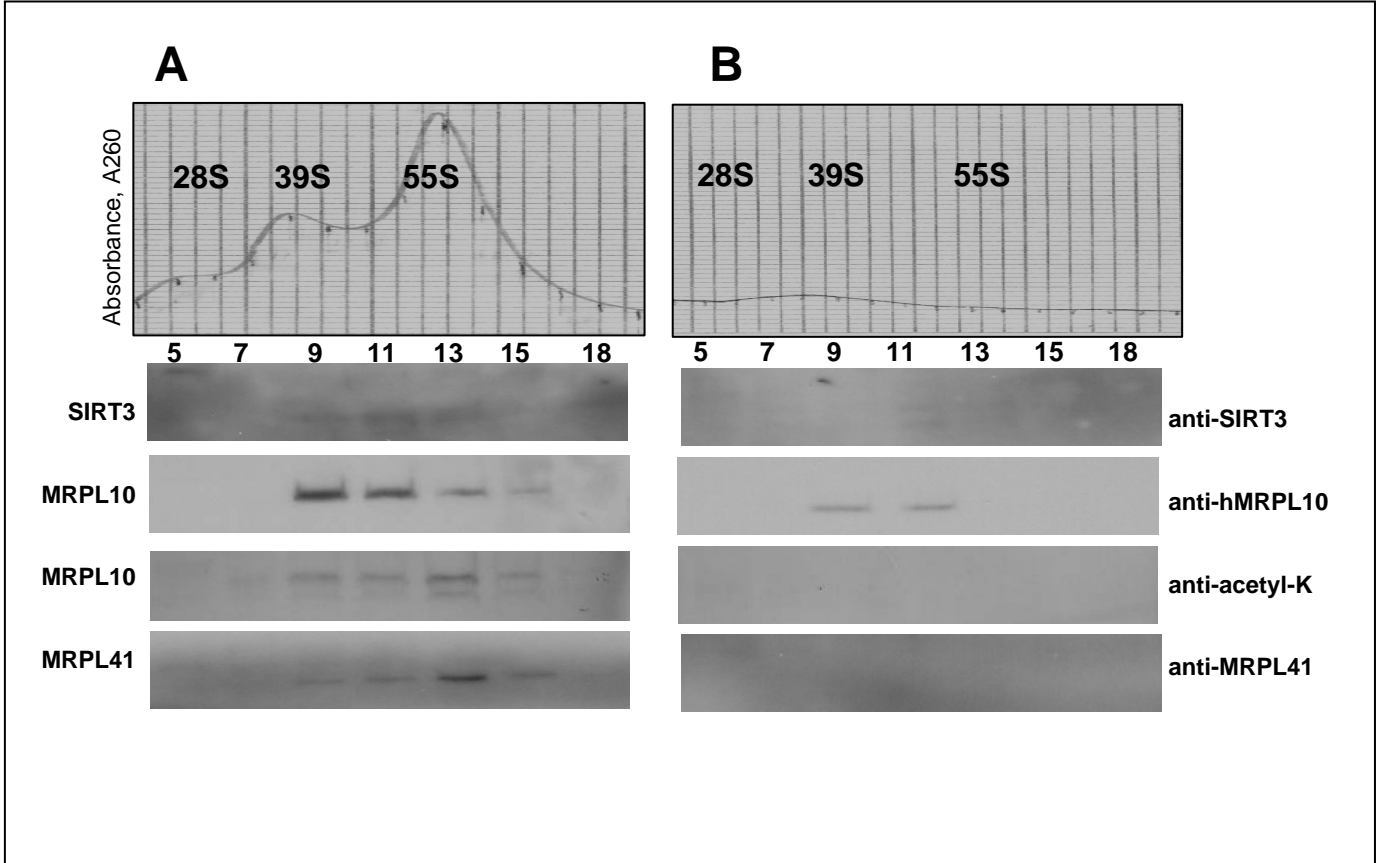


Figure S2. Dissociation of SIRT3 from the large subunit of mitochondrial ribosomes by RNase A treatment. Approximately, 20 A₂₆₀ units of crude ribosome preparation obtained from bovine liver were incubated in the absence (A) and presence of RNase A (B) and loaded onto two 10-30% linear sucrose gradients. After centrifugation, equal volumes of gradient fractions were separated on 12 % SDS-PAGE gels and PVDF blots were probed with SIRT3, MRPL10, acetyl-Lys and MRPL41 antibodies.

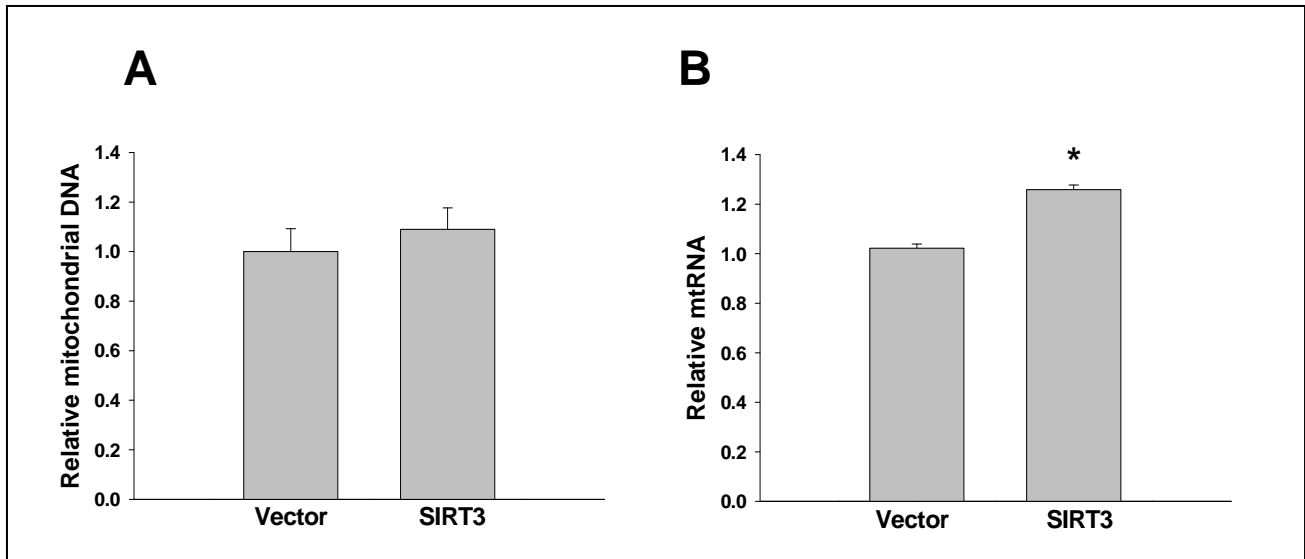


Figure S3. Determination of mtDNA and mtRNA levels in SIRT3 over-expression cells.

Relative mtDNA copy number **A**) and its transcription product mtRNA **B**) were determined by the quantitative real-time PCR as described in materials and methods. The efficiency of the real-time PCR amplification was established from 20 ng to 0.4441 pg (4-fold repeated dilutions) cellular DNA from A9 cells, which were allowed to react with primers specific to mtDNA (COX III gene) and nDNA (β -actin gene), respectively. The correlation coefficient $R^2 > 0.99$ for both nDNA and mtDNA. Each analysis was quadruplicated and mean \pm S.D. of the results was used for data presentation. Asterisk denotes $p < 0.001$.

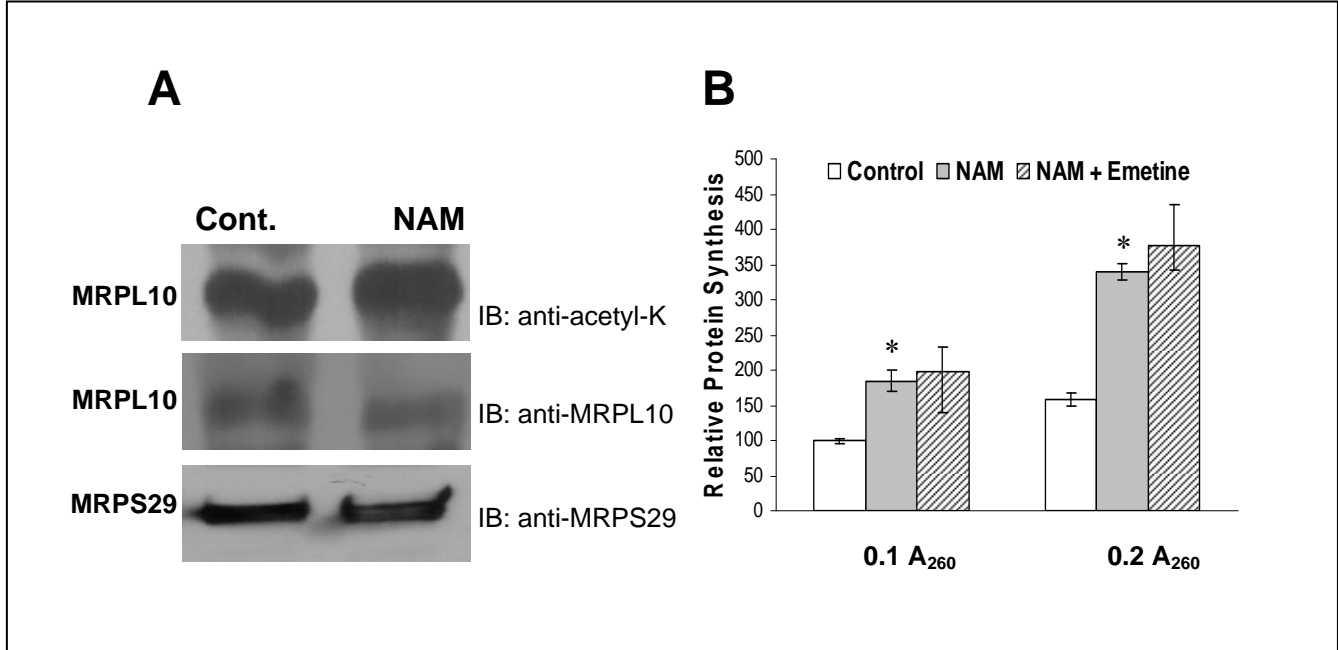


Figure S4. Role of nicotinamide (NAM) and emetine on mitochondrial protein synthesis.

A) Immunoblotting analysis of mitochondrial ribosomes (0.2 A₂₆₀) prepared in the absence (Cont.) and presence of 10 mM nicotinamide (NAM) from bovine liver mitochondria probed with anti-N-acetyl lysine, MRPL10 and MRPS29 antibodies. **B)** Mitochondrial ribosomes (0.1-0.2 A₂₆₀ units) isolated from control and NAM treated bovine liver mitochondria were used in the poly(U)-directed *in vitro* translation assays described in Materials and Methods below. *In vitro* translation assays using NAM treated ribosomes were also repeated by adding 1mM emetine. Asterisks denote p<0.05.

Supplemental Materials and Methods

Mass spectrometric analysis of bovine mitochondrial ribosomal proteins. Identification of mitochondrial ribosomal proteins and mapping of post-translational modification (PTM) sites was achieved by database searching of tandem mass spectra of proteolytic peptides searched against protein databases (Miller et al., 2008). Tandem MS spectra obtained by fragmenting a peptide by collision-induced dissociation (CID) were acquired using a capillary liquid chromatography - nanoelectrospray ionization - tandem mass spectrometry (LC/MS/MS) system that consisted of a Surveyor HPLC pump, a Surveyor Micro AS autosampler, and an LTQ linear ion trap mass spectrometer (ThermoFinnigan). The raw CID tandem MS spectra were converted to Mascot generic files (.mgf) using the extract msn software (ThermoFinnigan). Both, the .mgf and .raw files were submitted to site-licensed Mascot (version 2.2) and Sequest search engines, respectively, to search against in-house generated sequences of 55S proteins, all known human and bovine mitochondrial proteins, and proteins in the Swiss-Prot database. The variable modifications were methionine oxidation (+16 Da), acetylation of lysine residues (+42) and phosphorylation (+80 Da) of Ser, Thr, and Tyr residues. Up to 2 missed cleavages were allowed for the protease of choice. Peptide mass tolerance and fragment mass tolerance were set to 3 and 2 Da, respectively. Tandem MS spectra were manually evaluated at the raw data level with the consideration of overall data quality, signal-to-noise of matched peaks, and the presence of dominant peaks that did not match to any theoretical m/z value.

RP-HPLC purification. Approximately 5A₂₆₀ units of purified 55S ribosomes from bovine mitochondria was incubated in 67% glacial acetic acid for 16 h at 4°C to precipitate the rRNA.

After centrifuging the precipitate 18,000 g for 15 min, supernatant containing ribosomal proteins was dialyzed in 6% glacial acetic acid for 16 h at 4°C. HPLC analysis of ribosomal proteins was conducted using a Shimadzu Model SCL-10A_{vp} equipped with an SCL-10A diode array detector (Shimadzu, Kyoto, Japan). The separation was performed on a 300 Å pore RP4 (5µm) column (250 x 4.6 mm) (Eprogen Inc.). Solvent A was 0.1% trifluoroacetic acid and solvent B consisted of 0.1% trifluoroacetic acid in acetonitrile. The gradient ranged from 5% to 35% B in 100 min, from 35% to 55% 100 min. The flow rate was 1.0 ml/min and the column effluent was monitored at 215 nm.

Determination of mtDNA and mtRNA levels. Total DNA and RNA were isolated from cells using Wizard SV Genomic DNA Purification System (Madison, New Jersey, USA) and Aurum Total RNA Mini Kit (BIO-RAD, Hercules, California, USA), respectively. cDNA is generated the purified RNA with iScript cDNA Synthesisi Kit (BIO-RAD, Hercules, California, USA). The relative mtDNA and mtRNA levels were measured by a real-time PCR and normalized by simultaneous measurement of the nuclear DNA. The primers and probes information is as follows: MusCOXIIIF: TCTGACTCCCCCAAATAAATCTG, MusCOXIIIR: GGGTCGAATCCGCATTCA, MusCoxIII probe: FAMTCAGAAAAAGCAAATCCMGBNFQ; MusActinF: AAATCGTGCGTGACATCAAAGA, MusActinR: GGCCATCTCCTGCTCGAA, MusActin probe: VICAGCTGTGCTATGTTGCTMGBNFQ. The The PCR was performed in a ABI PRISM 7700 Sequence Detection System (PE Biosystems, California, USA), using the SYBR[®] GREEN PCR MASTER MIX kit (Applied Biosystems, New Jersey, USA). Genomic DNA or cDNA (10 ng) was mixed with 12.5 µl SYBR[®] GREEN PCR MASTER MIX containing 50 nmol of forward and reverse primers, in a final volume of 25 µl. The PCR conditions were

15 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and primer extension at 60 °C for 60 s. The threshold cycle number (Ct) values of the β -actin gene and the mitochondrial COXIII gene were determined. Each measurement was carried out four times and normalized against a serial dilution of a control DNA sample. Ct values can be used as a measure of the input DNA contents and Ct value differences were used to quantify mtDNA and mtRNA levels relative to the β -actin gene with the following equation: Relative copy number (R_c) = $2^{\Delta Ct}$, where ΔCt is the $Ct_{\beta\text{-actin}} - Ct_{\text{COXIII}}$.

***In vitro* translation assays.** Varying amounts of mitochondrial ribosomes obtained from bovine mitochondria prepared in presence and absence of 10 mM nicotinamide were assayed in reactions contained 50 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, 0.1 mM spermine, 40 mM KCl, 7.5 mM MgCl₂, 2.5 mM phosphoenolpyruvate, 0.18 U pyruvate kinase, 0.5 mM GTP, 50 U RNasin Plus, 12.5 μ g/mL poly(U), 20 pmol [¹⁴C]-Phe-tRNA, 0.15 μ M mtEF-Tu, 1 μ g mtEF-G and incubated at 37°C for 15 min followed by addition of cold 5% trichloroacetic acid and incubation at 90°C for 10 min. EF-Tu_{mt} and EF-G_{mt} were prepared from recombinant proteins as described previously (1, 2). The *in vitro* translated [¹⁴C] labeled-poly(Phe) was collected on nitrocellulose filter membranes and quantified using a liquid scintillation counter.

References.

1. Worriax, V., Burkhart, W., Spremulli, L.L. (1995) *Biochim. Biophys. Acta* **1264**, 347–56
2. Bhargava, K., Templeton, P., Spremulli, L.L. (2004) *Protein Expr. Purif.* **37**(2), 368-76