



Supplementary Materials for

Silencing mitochondrial gene expression in living cells

Luis Daniel Cruz-Zaragoza, *et al.*

Corresponding authors: Peter Rehling, peter.rehling@medizin.uni-goettingen.de; Luis Daniel Cruz-Zaragoza, luisdaniel.cruzzaragoza@med.uni-goettingen.de

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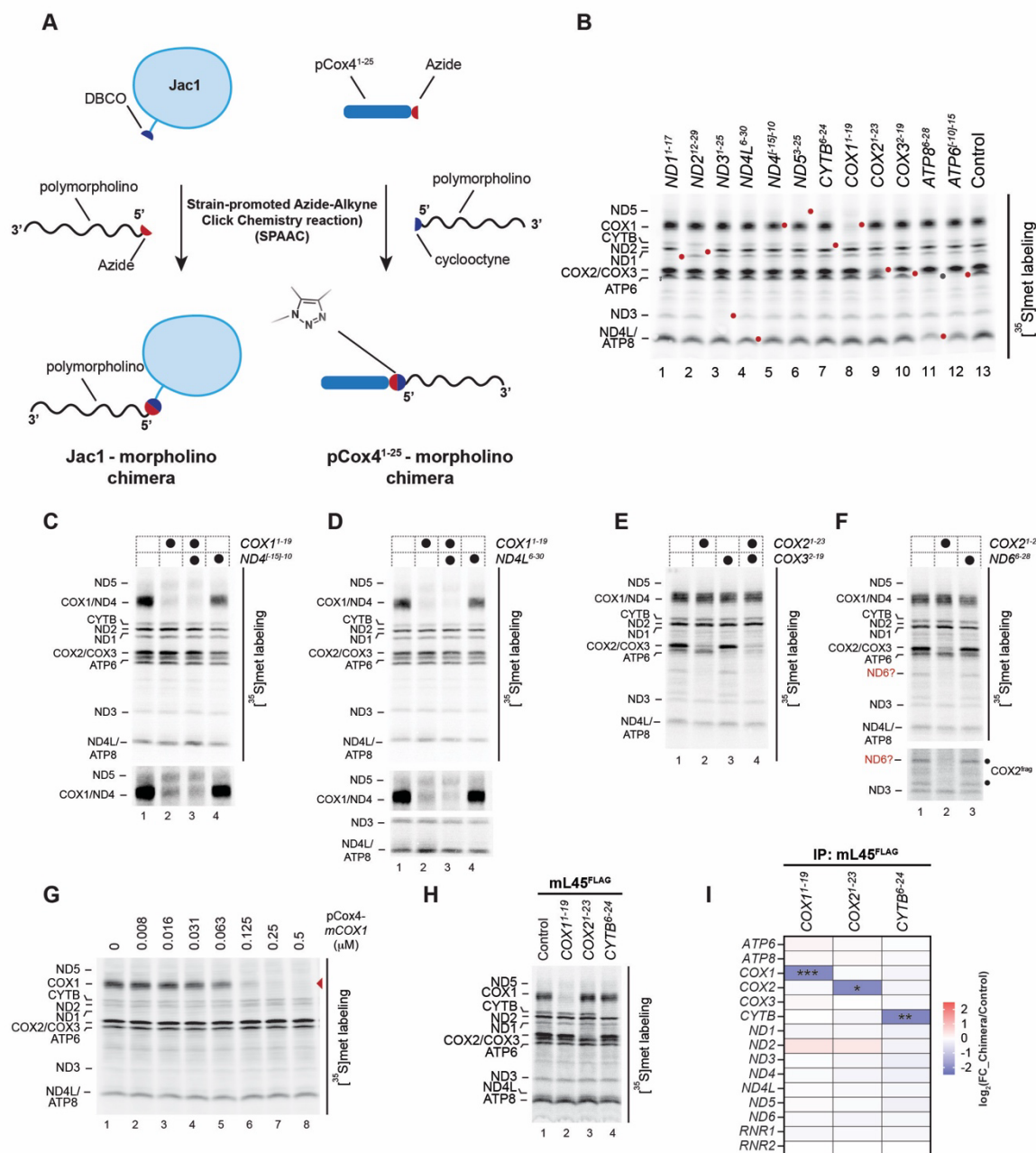


Fig. S1.

Gene silencing by peptide-morpholino chimeras in isolated mitochondria and living cells. (A) Jac1-MO and pCox4-MO chimera synthesis diagram. (B-F) *In organello* translation after downregulation of all mtDNA-encoded proteins (except ND6). Peptide-morpholino chimeras were synthesized and imported into isolated mitochondria, mitochondria reisolated, and subjected to [³⁵S]methionine labeling of translation products. Position of targeted proteins indicated by red dot.; position of ATP6 upon ATP8 silencing indicated by a gray dot (B). To visualize ND4 silencing with the chimeras targeting ND4 (C) and ND4L (D) mRNAs, we removed the signal of newly-synthesized COX1 by double knockdown. Since in our system the newly-synthesized COX2, COX3, and ATP6 migrated close together, the COX3 signal is partially hidden (B). Therefore, we

performed double knockdown experiments of COX2 and COX3 translation where the COX3 silencing is easily observed (**E**). The silencing of ND6 expression remained challenging. Strikingly, the newly-synthesized protein commonly referred to as ND6, represents a COX2 translation intermediate (indicated in the cropped section with a dot) (**F**). (**G**) Titration of pCox4-mCOX1¹⁻²⁴ chimera effect on protein translation in mitochondria isolated from AML12 cells (mouse hepatocytes). After chimera import, the newly synthesized mitochondrial proteins were labeled with [³⁵S]methionine. Samples were analyzed by SDS-PAGE followed by digital autoradiography. COX1 signal is indicated by an arrowhead. (**H-I**) Specificity of the silencing effect of chimeras imported into mitochondria is mediated by the impairment for the mitochondrial ribosome to bind to the targeted mRNAs. Mitochondria isolated from mL45^{FLAG}-expressing cells were treated with chimeras targeting the 5' region of the COX1, COX2, and CYTB mRNAs. [³⁵S]methionine labeling of translation products showed the silencing (**H**). Alternatively, upon import and translation, FLAG-IP was performed, RNA purified from the eluate fractions, and analyzed by nanoString. mRNA binding to the ribosome was significantly reduced. As shown in the heatmap (**I**), the amount of the cognate mRNAs, but not the other mtDNA-encoded mRNAs (mt-mRNA) and rRNAs (mt-rRNA, *RNR1* and *RNR2*), changed upon treatment compared to the control (n=3, mean). FC, fold change. Statistical significance was determined by multiple t test using the Holm-Sidak method, with alpha=0.05 (*, padj<0.05; **, padj<0.01; ***, padj<0.001). Non-significant differences are not indicated.

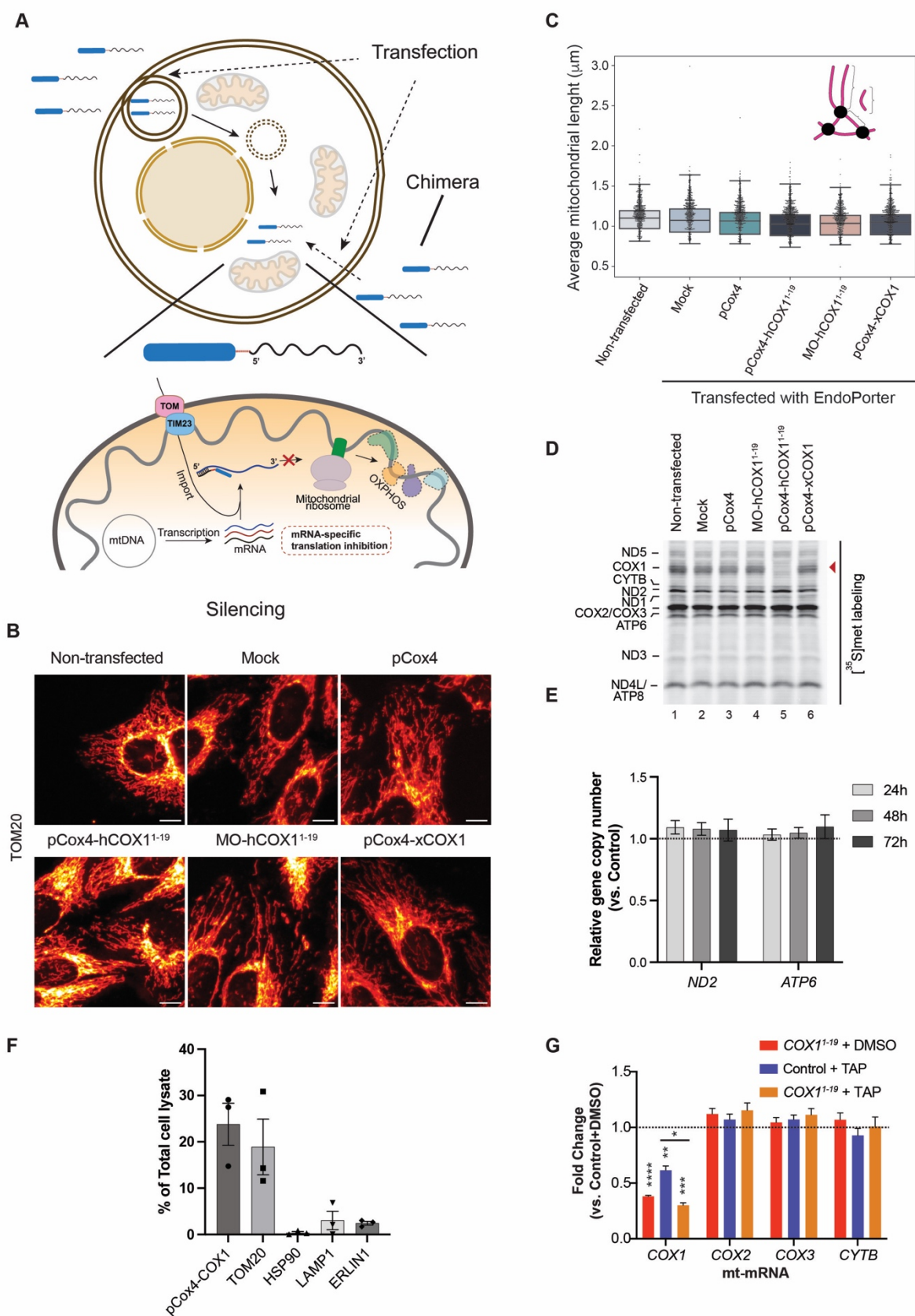


Fig. S2.

Transfection of presequence-morpholino chimera in living cells. (A) Schematic representation of chimera uptake by living cells. After release from lysosomes, the cytosolic chimera is imported into the mitochondrial matrix, a process that is facilitated by the Cox4 presequence (pCox4¹⁻²⁵). Once inside mitochondria, the morpholino binds to the target RNA to block translation. (B) Mitochondrial network analysis after cell transfection with chimeras and controls in HeLa cells. The treatment did not significantly impact mitochondrial morphology. HeLa cells were transfected for 24 hours with chimera targeting human *COX1* mRNA (pCox4-hCOX1¹⁻¹⁹), the unmodified presequence peptide (pCox4), free morpholino (MO-hCOX1¹⁻¹⁹), a chimera targeting *Xenopus laevis* *COX1* mRNA (pCox4-xCOX1), and buffer (Mock). Non-transfected cells were also used as reference. Mitochondria were labeled by anti-TOM20 antibodies. Scale bars, 5 μ m. (C) Analysis of the average mitochondrial length obtained from (B). (D) [³⁵S]methionine labeling of mitochondrial translation products in cells after treatment as in (B). Newly-synthesized COX1 is indicated with an arrowhead. (E) COX1 silencing in cells did not significantly alter mtDNA copy number. (F) Presequence-morpholino chimeras are enriched in mitochondria. Upon transfection of pCox4-COX1¹⁻¹⁹ for 72 hours in HEK293T cells, mitochondria were immunisolated with anti-TOM20 magnetic beads. Immunoisolation efficiency was assessed by Western blotting and quantified. Chimera enrichment is similar to that of TOM20. HSP90 (cytosol), LAMP1 (lysosome), and ERLIN (ER) were used as markers for other cellular compartments. (n=3; mean \pm SEM). (G) *COX1* mRNA reduction is linked to mitochondrial translation. Cells were treated with thiamphenicol (TAP) to inhibit mitochondrial translation or with DMSO (control) for two hours. Then pCox4-COX1¹⁻¹⁹ chimera were transfected for 16 hours in the presence of TAP. Total RNA was purified from isolated mitochondria and analyzed by nanoString to determine the abundance of mRNAs. Only *COX1* mRNA was reduced after TAP treatment, and further decreased with chimera treatment (n=3; mean \pm SEM). Statistical significance was determined by multiple t test using the Holm-Sidak method, with alpha=0.05 (*, padj<0.05; **, padj<0.01; ***, padj<0.001; ****, padj<0.0001).

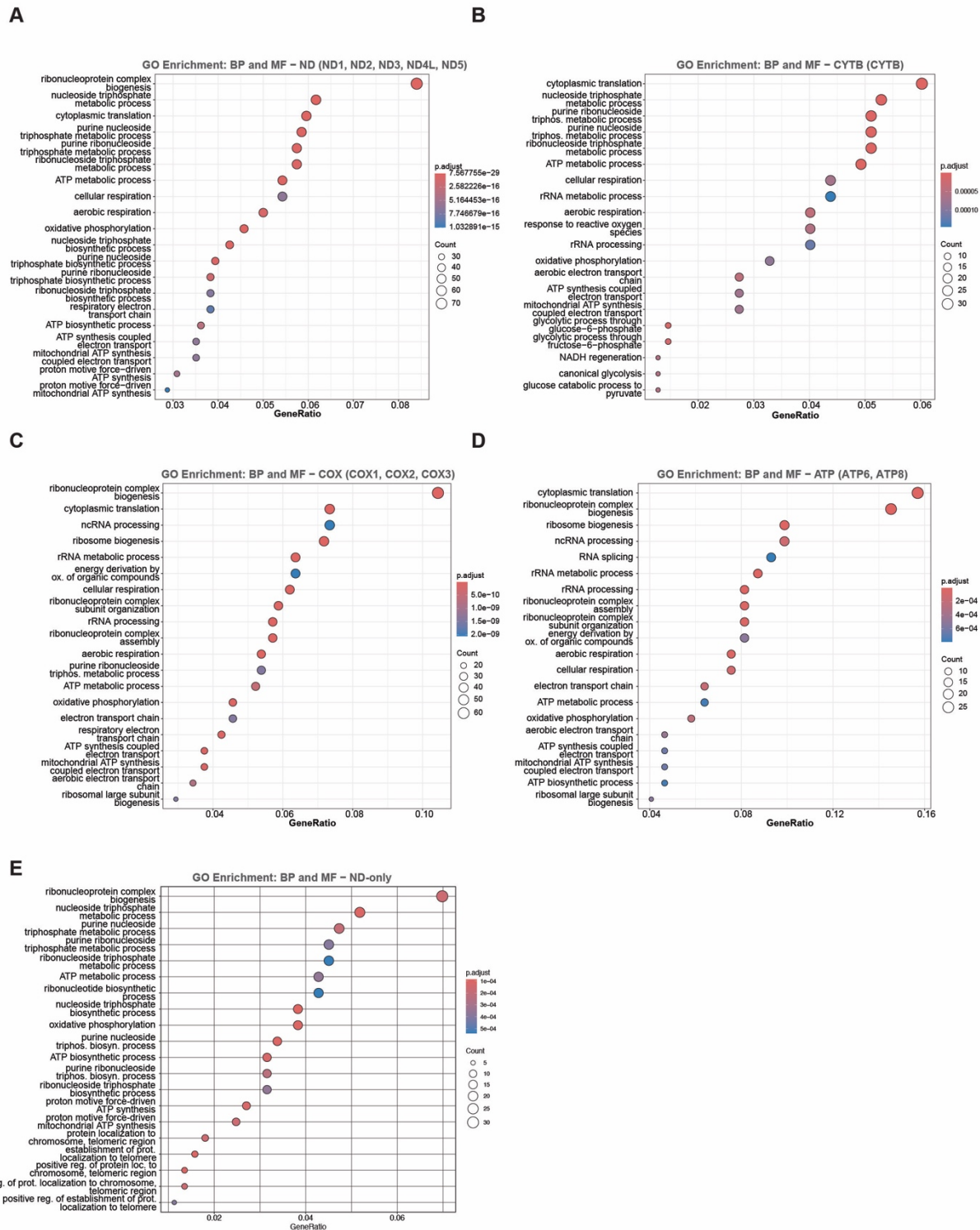


Fig. S3.

Changes in mRNA abundance upon silencing of mitochondrial mRNAs. (A) (A-D) Gene functional enrichment analysis by gene ontology (GO) annotation of mRNAs. mRNAs with significantly altered abundance ($\text{padj} < 0.05$) from each treatment were grouped based on the

OXPHOS complex to which the silenced protein belongs: ND (complex I: ND1, ND2, ND3, ND4L, and ND5) (**A**), CYTB (complex III: CYTB) (**B**), COX (complex IV: COX1, COX2, and COX3) (**C**), and ATP (complex V: ATP6 and ATP8) (**D**). GO enrichment analysis was performed focusing on biological process (BP) and molecular function (MF). (**E**) Gene functional enrichment analysis by gene ontology annotation of mRNAs that significantly varied only upon silencing of complex I subunits (ND: ND1 \cup ND2 \cup ND3 \cup ND4L \cup ND5).

Fig. S4.

Interaction network of proteins encoded by mRNAs in COX and ND_CYTB_COX_ATP exclusive intersections. The group of mRNAs forming the exclusive intersections COX-only (**A**) and ND_CYTB_COX_ATP (**B**) were analyzed using the STRING algorithm (39) for functional protein association networks. The nodes related to particular pathways are represented with different colors. For COX-only subset, ribonucleoproteins (red), gene expression (blue), nucleic acid binding (green) and ubiquitin binding (yellow) processes or pathways are indicated (**A**). For ND_CYTB_COX_ATP intersection, translation (red), gene expression (blue), and aerobic respiration processes or pathways are indicated (**B**).

Fig. S5.

Characteristic response to silencing of mtDNA-encoded core component proteins complexes I, III, and IV. (A) Gene functional enrichment analysis by gene ontology annotation of mRNAs that significantly varied ($p_{adj} < 0.05$) only in the exclusive intersection ND_CYTB_COX. (B) mRNAs forming the exclusive intersections ND_CYTB_COX subset were analyzed using the STRING algorithm for functional protein association networks. The nodes related to particular pathways are represented with different colors: OXPHOS activity (red), cellular metabolic process (yellow), gene expression (blue), and organelle organization (green).

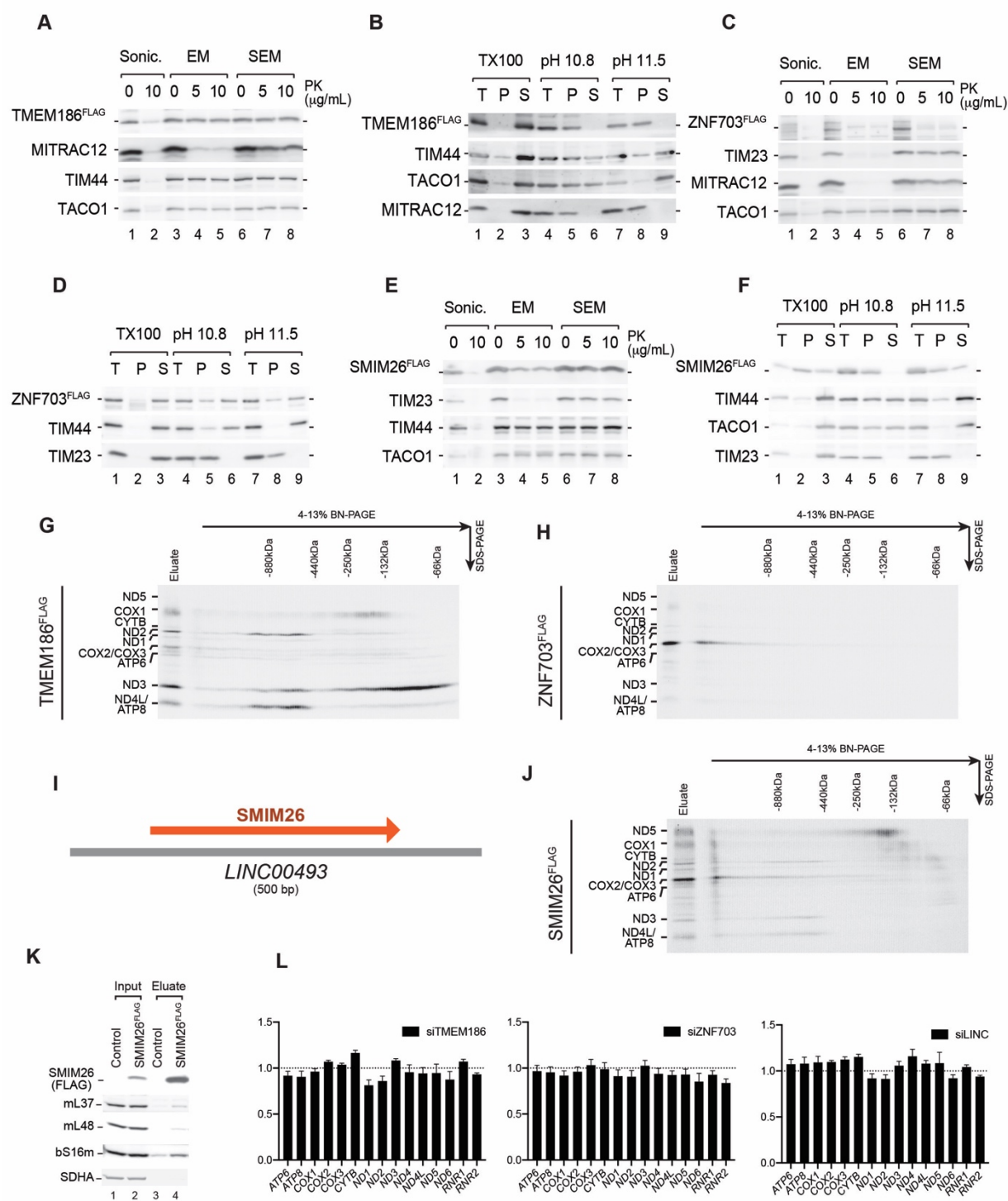


Fig. S7.

Characterization of identified mitochondrial biogenesis factors. (A-F) Proteinase K accessibility assay and organellar fractionation experiments to define the sub-mitochondrial localization of TMEM186 (A and B, respectively), ZNF703 (C and D, respectively), and SMIM26 (E and F, respectively). (G-H) Newly-synthesized mitochondrial proteins associated to TMEM186 (G) and ZNF703 (H). Mitochondria were purified from TMEM186^{FLAG}- and ZNF703^{FLAG}-expressing HEK293T cells, and subjected to [³⁵S]methionine labeling of mitochondrial translation products. Upon FLAG-immunoprecipitation, the eluates were analyzed by two-dimension (2D) BN-/SDS-PAGE and digital autoradiography. (I) Diagram of the long non-coding RNA LINC00493, and the open reading frame coding for SMIM26. (J) Newly-synthesized mitochondrial proteins associated to SMIM26 (determined as in G-H). (K) Mitochondria were purified from SMIM26^{FLAG}-expressing HEK293T cells. Upon FLAG-immunoprecipitation, the samples were analyzed by SDS-PAGE and western blotting. SMIM26 interacts with the mitochondrial ribosome. Total, 3%; eluate 100%. (L) RNA abundance was determined by nanoString technology in mitochondria isolated from HEK293T cells after siRNA-mediated downregulation of TMEM186, ZNF703, and *LINC00493* and compared to siNT control (dashed line)(n=4, mean ± SEM).

Table S1. Transcriptomic analyses of HEK293T cells upon treatment with peptide-morpholino chimeras for 48 hours by RNA-seq.

See also Fig. 3; fig. S3, S4, and S5

Table S2. Subsets obtained by analysis of the significantly altered transcript abundance with the UpSet intersection algorithm.

See also Fig. 3; fig. S3, S4, and S5

Table S3. Quantitative Mass Spectrometry analysis of isolated mitochondria upon ND2 and CYTB silencing in HEK293T cells for 48 hours.

See also Fig. 4

Table S4. Quantitative Mass Spectrometry analysis of isolated mitochondria upon COX1 silencing in HEK293T cells for 8, 16, 24, 48, and 72 hours.

See also Fig. 4 and fig. S6

Table S5. Quantitative Mass Spectrometry analysis of FLAG-immunoprecipitation eluates of mitochondria isolated from TMEM186^{FLAG}, ZNF703^{FLAG}, and SMIM26^{FLAG} expressing cells.

See also Fig. 5 and fig. S7

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