## SUPPLEMENTARY INFORMATION

# The pseudouridine synthase RPUSD4 is an essential component of mitochondrial RNA granules

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**RNA-interactome and Mitocarta 2.0 candidates**

ABCF2
ACAA2
ACLY
AK2
AKAP1
ALAS2
ALDH181
ALDH1L2
ALDH6A1
ALDH7A1
ATP5A1
ATP5B
ATP5C1
ATP5I
ATXN2
C14orf156
C17orf42
C1orf31
**C4orf14**
C7orf30
CAT
**CHCHD3**
**CHCHD7**
CISD1
CISD2
COX4I1
COX5B
CPS1
CYCS
DAP3
DBI
***DDX28***
DECR1
DHCR24
*DHX30*
DLD
DMGDH
DNAJA3
DNM1L
DUT
ECH1
***ELAC2***
**ERAL1**
ETFB
FAM195A
FASN
***FASTK***
**FASTKD1**
***FASTKD2***
FASTKD3
***FASTKD5***
FDPS
FKBP10
FTH1
***FTSJ2***
GADD45GIP1
GAPDH
GARS
GATM
**GFM1**
***GRSF1***
GTPBP10
HADH
HADHB
HARS2
HIBADH
HRSP12
HSDL2
HSPA9
HSPD1
HSPE1
IDH1
IDH2
IMMT
KARS
***KIAA0391***
KIF1B
LAMC1
LDHB
LONP1
LRPPRC
MCAT
MDH1
MDH2
MGST3
MMAB
***MRM1***
MRP63
MRPL1
MRPL11
MRPL13
MRPL14
MRPL15
MRPL17
MRPL19
MRPL2
MRPL21
MRPL22
MRPL24
MRPL27
MRPL28
MRPL3
MRPL32
MRPL33
MRPL34
MRPL37
MRPL38
MRPL39
MRPL4
MRPL40
MRPL41
MRPL42
MRPL43
MRPL44
**MRPL45**
MRPL48
MRPL49
MRPL51
MRPL9
MRPS10
MRPS11
MRPS14
MRPS15
MRPS17
MRPS18A
MRPS18B
MRPS2
MRPS21
MRPS23
MRPS24
MRPS25
MRPS27
MRPS28
MRPS30
MRPS31
MRPS35
MRPS5
MRPS7
MRPS9
MRRF
**MTERF2**
MTERFD1
MTHFD1
MTIF2
*MTPAP*
NDUFS6
NDUFV3
NGRN
OPA1
P4HB
PACSIN2
**PARK7**
PHB
***PNPT1***
***POLRMT***
POR
PPIF
PRDX2
PRDX3
PRDX4
PRDX6
PROSC
PSMA6
**PTCD1**
**PTCD2**
**PTCD3**
PTRH1
**PUS1**
**PUSL1**
RAB35
RARS
***RNMTL1***
RPL10A
RPL34
RPL35A
RPS14
RPS15A
**RPUSD3**
**RPUSD4**
SARS
SDHA
SECISBP2
SHMT2
SLC25A5
**SND1**
SSBP1
SUCLG1
***SUPV3L1***
**TACO1**
**TARS2**
TBRG4
TFAM
**TFB1M**
TFB2M
TIMM44
TKT
TPI1
TRAP1
TRMT1
TRMT61B
**TRUB2**
TSFM
TUFM
TXNRD2
WBSCR16
XPNPEP3
**YARS2**

**Other candidates**

**AFG3L2**
**CH60**
**MRPL47**
**MRPS7**
**MRPS16**
**MRPS9**
**mtIF3**
**NSUN4**
**OXA1L**
**PDE12**
**REXO2**
**YBEY**
**YMEL1**

**Figure S1. Potential mitochondrial RNA binding proteins**

Upper panel: All 207 RNA binding proteins present in Mitocarta 2.0. Lower panel: Other proteins in Mitocarta 2.0 not known to bind RNA but tested as potential components of MRGs. In bold are indicated the proteins that we further investigated for their localization, in italics are indicated the MRG-associated proteins already published.

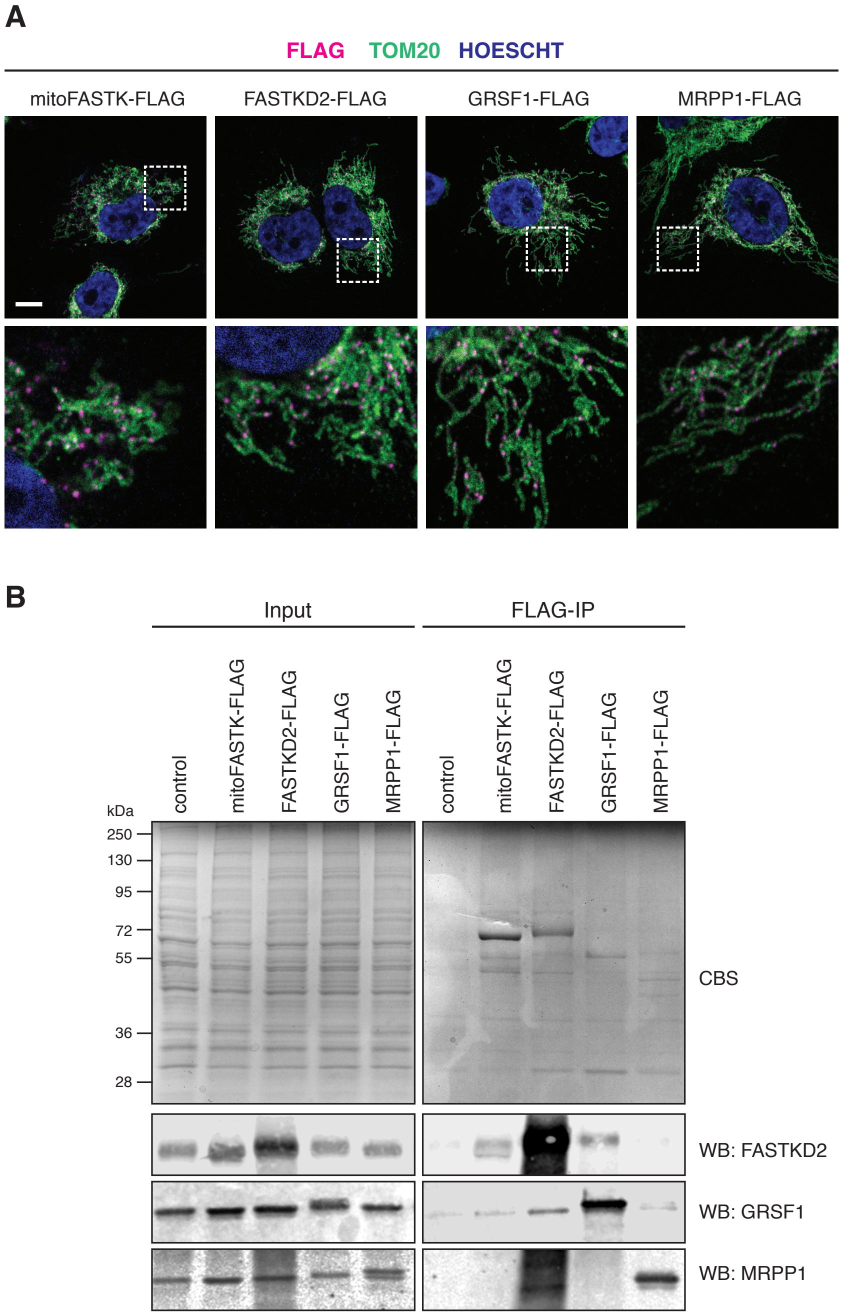

**Figure S2. Co-immunoprecipitation experiments of MRG-associated proteins**
(A) Confocal analysis to verify the expression and the proper localization of FLAG-tagged mitoFASTK, FASTKD2, GRSF1 and MRPP1. 143B cells stably expressing the indicated FLAG-tagged proteins were immunolabelled with anti-FLAG and anti-TOM20, as a mitochondrial marker. White boxes indicate the regions shown at higher magnification. Scale bar is 10 µm. (B) Coomassie gel staining and immunoblot analysis of immunoprecipitated proteins. To visualize proteins, the gels were stained using coomassie blue staining (CBS), and in parallel, to verify the correct co-immunoprecipitation of the indicated proteins, immunoblot analyses of FASTKD2, GRSF1 and MRPP1 were performed.

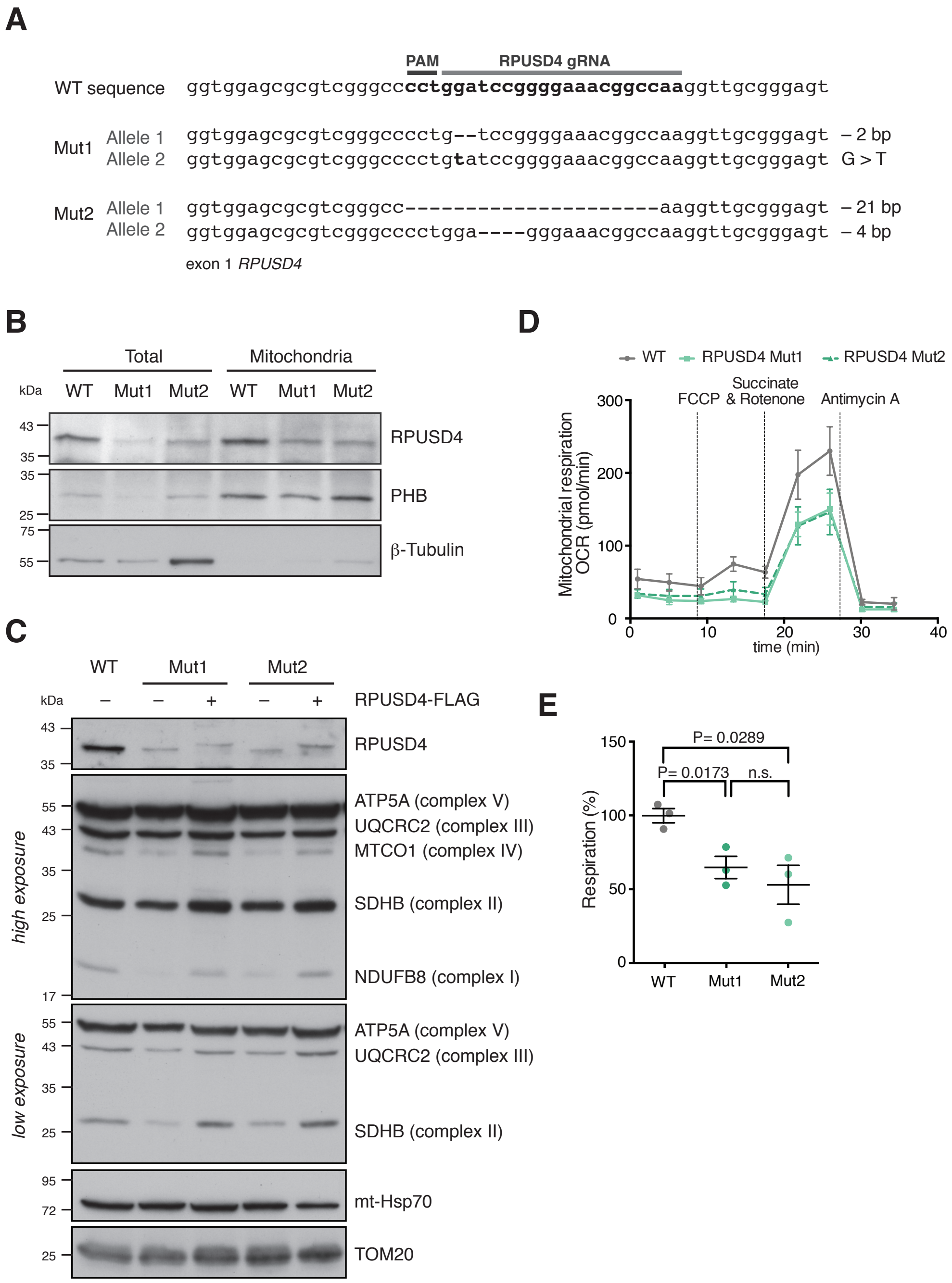

**Figure S3. CRISPR/Cas9-derived RPUSD4 heterozygous cells show defective mitochondrial respiration**

(A) Sequence analysis of *RPUSD4* exon 1 showing the mutations present in the 2 viable clones (Mut1 and Mut2) obtained following CRISPR/Cas9-mediated gene disruption. In both cases, the targeted regions in *RPUSD4* gene lie within the sequence encoding the mitochondrial targeting signal. (B) RPUSD4 mutations present in Mut1 or Mut2 do not impair mitochondrial import. Western blotting of mitochondrial fractions of the 2 mutant cell lines shows only ~50% depletion of the endogenous RPUSD4, consistent with their genotypes. The purity of the mitochondrial fractions was assessed using β-Tubulin and PHB as cytosolic and mitochondrial markers respectively. (C) SDS-PAGE analysis shows reduced steady-state levels of respiratory chain components in RPUSD4 Mut1 and Mut2 heterozygous cells. Western blot membranes were probed with an antibody cocktail specific for components of each of the OXPHOS complexes. mtHSP70 and TOM20 were used as loading controls. Due to differences in the signals for the respiratory chain subunits, high and low exposures of the blot are presented. (D) Partially reduced levels of RPUSD4 cause decreased mitochondrial respiration. Representative profiles of oxygen consumption rates (OCR) in the XF-PMP permeabilized cell lines are indicated. Assays were performed in the presence of pyruvate (10 mM) and malate (1 mM) as carbon sources, and the following compounds were injected at the times indicated by vertical lines. FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (2 μM); succinate (10 mM) and rotenone (1 μM); antimycin A (1 μM). All measurements were made in the presence of oligomycin (1 μM) to inhibit ATP-linked respiration. Data are represented as means ± SD. (E) Oligomycin-sensitive respiration was normalized to antimycin A-driven respiration and expressed as % of respiration relative to wild-type (WT) cells. Data from 3 independent experiments are represented as means ± SEM. *P* values were obtained using the Student's t-test.

## Table S1: Mass spectrometry results of co-immunoprecipitation experiments

| | **Mass spectrometry results of co-immunoprecipitation experiments** | **RELATIVE PEPTIDE ABUNDANCE** | | | | | **ENRICHEMENT OVER CONTROL (FOLD)** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **SYMBOL** | **GENE NAME** | **CONTROL** | **mitoFASTK** | **FASTKD2** | **GRSF1** | **MRPP1** | **mitoFASTK** | **FASTKD2** | **GRSF1** | **MRPP1** | **NUMBER OF IPs** |
| GRSF1 | G-rich sequence factor 1 | 0 | 9,1642 | 7,9257 | 52,37 | 4,8591 | ∞ | ∞ | ∞ | ∞ | 4 |
| L2HGDH | L-2-hydroxyglutarate dehydrogenase | 0 | 8,0187 | 5,2838 | 3,4341 | 3,8873 | ∞ | ∞ | ∞ | ∞ | 4 |
| PNPT1 | Polyribonucleotide nucleotidyltransferase 1 | 0 | 5,7276 | 6,1645 | 2,5756 | 2,9155 | ∞ | ∞ | ∞ | ∞ | 4 |
| FASTKD2 | FAST kinase domain-containing protein 2 | 5,1888 | 14,892 | 126,81 | 23,18 | 7,7746 | 2,870027752 | 24,43917669 | 4,467314215 | | 3 |
| GCAT | 2-amino-3-ketobutyrate coenzyme A ligase | 0 | 5,7276 | 7,9257 | 3,4341 | 0 | ∞ | ∞ | ∞ | | 3 |
| GCDH | Glutaryl-CoA dehydrogenase | 0 | 5,7276 | 2,6419 | 0 | 2,9155 | ∞ | ∞ | | ∞ | 3 |
| KIAA0391 | Mitochondrial ribonuclease P protein 3 | 0 | 4,5821 | 4,4032 | 0 | 2,9155 | ∞ | ∞ | | ∞ | 3 |
| MRPL22 | 39S ribosomal protein L22 | 0 | 0 | 4,4032 | 3,4341 | 4,8591 | | ∞ | ∞ | ∞ | 3 |
| MRPS35 | 28S ribosomal protein S35 | 0 | 0 | 3,5226 | 3,4341 | 2,9155 | | ∞ | ∞ | ∞ | 3 |
| PDPR | Pyruvate dehydrogenase phosphatase regulatory subunit | 0 | 8,0187 | 5,2838 | 0 | 3,8873 | ∞ | ∞ | | ∞ | 3 |
| STOML2 | Stomatin-like protein 2 | 0 | 4,5821 | 5,2838 | 2,5756 | 0 | ∞ | ∞ | ∞ | | 3 |
| YARS2 | Tyrosine--tRNA ligase | 0 | 4,5821 | 7,9257 | 5,1511 | 0 | ∞ | ∞ | ∞ | | 3 |
| ATP5O | ATP synthase subunit | 3,1133 | 9,1642 | 7,0451 | 6,0096 | 5,8309 | 2,943564706 | 2,262904314 | | | 2 |
| BCS1L | Mitochondrial chaperone BCS1 | 0 | 0 | 3,5226 | 0 | 2,9155 | | ∞ | | ∞ | 2 |
| CLPB | Caseinolytic peptidase B protein homolog | 0 | 3,4366 | 2,6419 | 0 | 0 | ∞ | ∞ | | | 2 |
| DHX30 | Putative ATP-dependent RNA helicase DHX30 | 7,2643 | 30,929 | 15,851 | 12,878 | 11,662 | 4,257671076 | 2,18204094 | | | 2 |
| FASTKD5 | FAST kinase domain-containing protein 5 | 0 | 4,5821 | 6,1645 | 0 | 0 | ∞ | ∞ | | | 2 |
| FOXRED1 | FAD-dependent oxidoreductase domain-containing protein 1 | 0 | 3,4366 | 2,6419 | 0 | 0 | ∞ | ∞ | | | 2 |
| GFM2 | Ribosome-releasing factor 2 | 0 | 5,7276 | 2,6419 | 0 | 0 | ∞ | ∞ | | | 2 |
| GRPEL2 | GrpE protein homolog 2 | 0 | 0 | 0 | 2,5756 | 2,9155 | | | ∞ | ∞ | 2 |
| HK2 | Hexokinase-2 | 0 | 6,8731 | 3,5226 | 0 | 0 | ∞ | ∞ | | | 2 |
| MRPL53 | 39S ribosomal protein L53 | 0 | 4,5821 | 2,6419 | 0 | 0 | ∞ | ∞ | | | 2 |
| NDUFS8 | NADH dehydrogenase [ubiquinone] iron-sulfur protein 8 | 0 | 5,7276 | 2,6419 | 0 | 0 | ∞ | ∞ | | | 2 |
| NDUFV2 | NADH dehydrogenase [ubiquinone] flavoprotein 2 | 0 | 0 | 0 | 2,5756 | 3,8873 | | | ∞ | ∞ | 2 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| NSUN4 | 5-methylcytosine rRNA methyltransferase NSUN4 | 0 | 0 | 3,5226 | 0 | 3,8873 | | ∞ | | ∞ | 2 |
| PARS2 | Probable proline--tRNA ligase | 0 | 0 | 0 | 4,2926 | 3,8873 | | | ∞ | ∞ | 2 |
| PDK1 | [Pyruvate dehydrogenase (acetyl-transferring)] kinase isozyme 1 | 0 | 4,5821 | 4,4032 | 0 | 0 | ∞ | ∞ | | | 2 |
| POLRMT | DNA-directed RNA polymerase | 0 | 0 | 3,5226 | 0 | 3,8873 | | ∞ | | ∞ | 2 |
| SLC25A22 | Mitochondrial glutamate carrier 1 | 0 | 0 | 2,6419 | 2,5756 | 0 | | ∞ | ∞ | | 2 |
| SLIRP | SRA stem-loop-interacting RNA-binding protein | 0 | 5,7276 | 4,4032 | 0 | 0 | ∞ | ∞ | | | 2 |
| SND1 | Staphylococcal nuclease domain-containing protein 1 | 0 | 0 | 0 | 4,2926 | 5,8309 | | | ∞ | ∞ | 2 |
| TIMM50 | Mitochondrial import inner membrane translocase subunit TIM50 | 0 | 0 | 4,4032 | 3,4341 | 0 | | ∞ | ∞ | | 2 |
| AFG3L2 | AFG3-like protein 2 | 3,1133 | 0 | 8,8064 | 0 | 4,8591 | | 2,828638422 | | | 1 |
| AGK | Acylglycerol kinase | 0 | 0 | 2,6419 | 0 | 0 | | ∞ | | | 1 |
| BDH1 | D-beta-hydroxybutyrate dehydrogenase | 0 | 0 | 2,6419 | 0 | 0 | | ∞ | | | 1 |
| C2orf47 | Uncharacterized protein C2orf47 | 0 | 0 | 2,6419 | 0 | 0 | | ∞ | | | 1 |
| CPS1 | Carbamoyl-phosphate synthase [ammonia] | 0 | 0 | 2,6419 | 0 | 0 | | ∞ | | | 1 |
| DDX28 | Probable ATP-dependent RNA helicase DDX28 | 0 | 0 | 5,2838 | 0 | 0 | | ∞ | | | 1 |
| DLAT | Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex | 0 | 3,4366 | 0 | 0 | 0 | ∞ | | | | 1 |
| DNAJA3 | DnaJ homolog subfamily A member 3 | 5,1888 | 16,037 | 9,687 | 6,0096 | 6,8028 | 3,090695344 | | | | 1 |
| EARS2 | Probable glutamate--tRNA ligase | 0 | 3,4366 | 0 | 0 | 0 | ∞ | | | | 1 |
| ELAC2 | Zinc phosphodiesterase ELAC protein 2 | 4,151 | 5,7276 | 3,5226 | 0 | 16,521 | | | | 3,980004818 | 1 |
| ERAL1 | GTPase Era | 3,1133 | 3,4366 | 7,0451 | 2,5756 | 4,8591 | | 2,262904314 | | | 1 |
| FASTK | Fas-activated serine/threonine kinase | 0 | 57,276 | 0 | 0 | 0 | ∞ | | | | 1 |
| GFM1 | Elongation factor G | 8,302 | 17,183 | 15,851 | 6,8682 | 9,7182 | 2,069742231 | | | | 1 |
| HADHB | Trifunctional enzyme subunit beta | 4,151 | 8,0187 | 5,2838 | 8,5852 | 5,8309 | | | 2,068224524 | | 1 |
| HSD17B10 | 3-hydroxyacyl-CoA dehydrogenase type-2 | 11,415 | 12,601 | 14,09 | 11,161 | 46,648 | | | | 4,086552781 | 1 |
| HSD17B4 | Peroxisomal multifunctional enzyme type 2 | 0 | 0 | 0 | 0 | 2,9155 | | | | ∞ | 1 |
| HSDL2 | Hydroxysteroid dehydrogenase-like protein 2 | 0 | 0 | 0 | 0 | 2,9155 | | | | ∞ | 1 |
| HSPD1 | 60 kDa heat shock protein | 39,435 | 307 | 55,48 | 40,35 | 42,76 | 7,784962597 | | | | 1 |
| MPST | 3-mercaptopyruvate sulfurtransferase | 0 | 0 | 3,5226 | 0 | 0 | | ∞ | | | 1 |
| MRPL4 | 39S ribosomal protein L4 | 0 | 0 | 3,5226 | 0 | 0 | | ∞ | | | 1 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| MRPS16 | 28S ribosomal protein S16 | 0 | 0 | 2,6419 | 0 | 0 | | ∞ | | | 1 |
| MRPS17 | 28S ribosomal protein S17 | 0 | 0 | 0 | 0 | 2,9155 | | | | ∞ | 1 |
| MRPS18A | 28S ribosomal protein S18a | 0 | 0 | 2,6419 | 0 | 0 | | ∞ | | | 1 |
| MRPS24 | 28S ribosomal protein S24 | 0 | 5,7276 | 0 | 0 | 0 | ∞ | | | | 1 |
| MRPS25 | 28S ribosomal protein S25 | 0 | 5,7276 | 0 | 0 | 0 | ∞ | | | | 1 |
| MRPS31 | 28S ribosomal protein S31 | 0 | 0 | 0 | 2,5756 | 0 | | | ∞ | | 1 |
| MTERFD1 | mTERF domain-containing protein 1 | 0 | 0 | 4,4032 | 0 | 0 | | ∞ | | | 1 |
| MTERFD2 | mTERF domain-containing protein 2 | 0 | 0 | 0 | 0 | 2,9155 | | | | ∞ | 1 |
| MTG2 | Mitochondrial ribosome-associated GTPase 2 | 3,1133 | 8,0187 | 3,5226 | 0 | 0 | 2,575627148 | | | | 1 |
| MTHFD1 | C-1-tetrahydrofolate synthase | 0 | 0 | 0 | 3,4341 | 0 | | | ∞ | | 1 |
| NDUFAF7 | NADH dehydrogenase [ubiquinone] complex I | 4,151 | 3,4366 | 5,2838 | 5,1511 | 8,7464 | | | | 2,10705854 | 1 |
| NDUFB10 | NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10 | 0 | 0 | 0 | 2,5756 | 0 | | | ∞ | | 1 |
| NDUFS2 | NADH dehydrogenase [ubiquinone] iron-sulfur protein 2 | 4,151 | 9,1642 | 7,9257 | 2,5756 | 4,8591 | 2,207708986 | | | | 1 |
| OAT | Ornithine aminotransferase | 6,2265 | 13,746 | 7,0451 | 6,8682 | 5,8309 | 2,207660805 | | | | 1 |
| OCIAD1 | OCIA domain-containing protein 1 | 0 | 0 | 6,1645 | 0 | 0 | | ∞ | | | 1 |
| PRDX5 | Peroxiredoxin-5 | 0 | 3,4366 | 0 | 0 | 0 | ∞ | | | | 1 |
| PTCD1 | Pentatricopeptide repeat-containing protein 1 | 0 | 0 | 2,6419 | 0 | 0 | | ∞ | | | 1 |
| RARS2 | Probable arginine--tRNA ligase | 0 | 0 | 2,6419 | 0 | 0 | | ∞ | | | 1 |
| RPS14 | 40S ribosomal protein S14 | 0 | 0 | 0 | 3,4341 | 0 | | | ∞ | | 1 |
| RPUSD3 | RNA pseudouridylate synthase domain-containing protein 3 | 0 | 0 | 3,5226 | 0 | 0 | | ∞ | | | 1 |
| RSAD1 | Radical S-adenosyl methionine domain-containing protein 1 | 0 | 4,5821 | 0 | 0 | 0 | ∞ | | | | 1 |
| SLC16A1 | Monocarboxylate transporter 1 | 0 | 0 | 4,4032 | 0 | 0 | | ∞ | | | 1 |
| SUCLG1 | Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha | 0 | 0 | 2,6419 | 0 | 0 | | ∞ | | | 1 |
| TFB2M | Dimethyladenosine transferase 2 | 0 | 0 | 4,4032 | 0 | 0 | | ∞ | | | 1 |
| TOMM40 | Mitochondrial import receptor subunit TOM40 homolog | 0 | 0 | 3,5226 | 0 | 0 | | ∞ | | | 1 |
| TRMT10C | Mitochondrial ribonuclease P protein 1 | 3,1133 | 5,7276 | 6,1645 | 3,4341 | 63,169 | | | | 20,29004593 | 1 |
| TRNT1 | CCA tRNA nucleotidyltransferase 1 | 0 | 0 | 0 | 0 | 3,8873 | | | | ∞ | 1 |
| TRUB2 | Probable tRNA pseudouridine synthase 2 | 0 | 0 | 4,4032 | 0 | 0 | | ∞ | | | 1 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| TST | Thiosulfate sulfurtransferase | 4,151 | 11,455 | 7,0451 | 3,4341 | 3,8873 | ∞ | | | | 1 |
| WBSCR16 | Williams-Beuren syndrome chromosomal region 16 protein | 0 | 0 | 2,6419 | 0 | 0 | | ∞ | | | 1 |
| YME1L1 | ATP-dependent zinc metalloprotease YME1L1 | 0 | 0 | 4,4032 | 0 | 0 | | ∞ | | | 1 |

## Table S2. List of primers

| | FWD 5’-3’ | REV 5’-3’ |
|---|---|---|
| *ND1* | CTA CGC CCT GAT CGG CGC AC | CGG TAA TAC GAC TCA CTA TAG GGA GAG GTC GTA GCG GAA TCG GGG G |
| *ND2* | CAC CCC TCT GAC ATC CGG CCT | CGG TAA TAC GAC TCA CTA TAG GGA GAG GCC TCC TAG GGA GAG GAG GGT |
| *ND3* | CCA CCC CTT ACG AGT GCG GC | CGG TAA TAC GAC TCA CTA TAG GGA GAA GGC CAG ACT TAG GGC TAG GAT GA |
| *ND5* | ACG CCC GAG CAG ATG CCA AC | CGG TAA TAC GAC TCA CTA TAG GGA GAG GCG CAG ACT GCT GCG AAC A |
| *CO1* | TGG AGG CCG GAG CAG GAA CA | CGG TAA TAC GAC TCA CTA TAG GGA GAC GGC GGG GTC GAA GAA GGT G |
| *CO2* | CGC CCT CCC ATC CCT ACG CA | CGG TAA TAC GAC TCA CTA TAG GGA GAT ACC CCC GGT CGT GTA GCG G |
| *ATP6/8* | TGG CCC ACC ATA ATT ACC CCC A | CGG TAA TAC GAC TCA CTA TAG GGA GAG GGG GCA ATG AAT GAA GCG AAC AG |
| *CYB* | CTC ACT CCT TGG CGC CTG CC | CGG TAA TAC GAC TCA CTA TAG GGA GAG CCT CAC GGG AGG ACA TAG CC |
| *12S* | ACT CAA AGG ACC TGG CGG TGC | CGG TAA TAC GAC TCA CTA TAG GGA GAG GTG ACG GGC GGT GTG TAC G |
| *16S* | CCG TGA AGA GGC GGG CAT AAC AC | CGG TAA TAC GAC TCA CTA TAG GGA GAA TCC AAC ATC GAG GTC GTA AAC CCT |
| *Met-tRNA* | AGT AAG GTC AGC T | CGG TAA TAC GAC TCA CTA TAG GGA GAT AGT ACG GGA AGG |
| *Val-tRNA* | CAG AGT GTA GCT TAA CAC | CGG TAA TAC GAC TCA CTA TAG GGA GAT CAG AGC GGT CAA GTT AAG |
| *Phe-tRNA* | GTT TAT GTA GCT TAC CTC C | CGG TAA TAC GAC TCA CTA TAG GGA GAT GTT TAT GGG GTG ATG TGA G |
| *7SL* | GCC GGG CGC GGT GGC GCG TG | CGG TAA TAC GAC TCA CTA TAG GGA GAA GAG ACG GGG TCT CGC TAT G |
| *5S* | GTC TAC GGC CAT ACC ACC CTG | AAA GCC TAC AGC ACC CGG TAT |