**MitoRibo-Tag Mice Provide a Tool for In Vivo Studies of Mitoribosome Composition**

**Graphical Abstract**

1. Generation of MitoRibo-Tag mouse (mL62-Flag knock-in)
2. Isolation of tissues and mitochondria
3. Co-immunoprecipitation
   - Affinity gel
   - anti-FLAG antibody
4. Quantitative MS and interaction proteomics
   - Data analysis
   - Intensity
   - m/z

**Highlights**
- MitoRibo-Tag mice with a tag on mL62 were generated to study mitoribosomes in vivo
- The mitoribosome interactome of different mouse tissues was defined with proteomics
- PUSL1 was identified as a mitoribosome-interacting protein required for efficient mitochondrial translation
- MitoRibo-Tag mice allow mitoribosome analysis under different conditions and setups

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**In Brief**
Busch et al. generated MitoRibo-Tag mice to study mitoribosome composition in vivo in different tissues. Proteomics of MitoRibo-Tag mice with defective assembly identified PUSL1 as a mitoribosome-interacting protein required for efficient mitochondrial translation. MitoRibo-Tag mice provide a tool to study mitoribosomes under different physiological conditions and in disease and aging.
MitoRibo-Tag Mice Provide a Tool for In Vivo Studies of Mitoribosome Composition

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SUMMARY

Mitochondria harbor specialized ribosomes (mitoribosomes) necessary for the synthesis of key membrane proteins of the oxidative phosphorylation (OXPHOS) machinery located in the mitochondrial inner membrane. To date, no animal model exists to study mitoribosome composition and mitochondrial translation coordination in mammals in vivo. Here, we create MitoRibo-Tag mice a tool enabling affinity purification and proteomics analyses of mitoribosomes and their interactome in different tissues. We also define the composition of an assembly intermediate formed in the absence of MTERF4, necessary for a late step in mitoribosomal biogenesis. We identify the orphan protein PUSL1, which interacts with a large subunit assembly intermediate, and demonstrate that it is an inner-membrane-associated mitochondrial matrix protein required for efficient mitochondrial translation. This work establishes MitoRibo-Tag mice as a powerful tool to study mitoribosomes in vivo, enabling future studies on the mitoribosome interactome under different physiological states, as well as in disease and aging.

INTRODUCTION

Mitochondria are semi-autonomous eukaryotic cell organelles with important roles in key cellular processes, for example, iron-sulfur cluster biosynthesis and oxidative phosphorylation (OXPHOS; Westermann, 2010). Mammalian mtDNA is a compact ~16.6 kb circular genome that encodes two rRNAs, 22 tRNAs, and 11 mRNAs necessary for the production of 13 essential OXPHOS proteins (Hällberg and Larsson, 2014). To synthesize mtDNA-encoded proteins, mitochondria harbor specialized ribosomes (mitoribosomes) that are 5S ribonucleoprotein complexes formed by two distinct subunits. The 28S small subunit (SSU) consists of 30 nuclear-encoded proteins and the 12S rRNA, whereas the 39S large subunit (LSU) is composed of 52 proteins, the 16S rRNA, and an integrated tRNA (Amunts et al., 2015; Greber et al., 2015; O’Brien and Kaif, 1967a, 1967b). During evolution, mitoribosomes have acquired 36 organelle-specific proteins not found in bacterial ribosomes (Greber and Ban, 2016). Ribosome assembly has mostly been studied in bacteria and is a complex process occurring through an alternating series of rRNA conformation changes involving sequential binding of >200 proteins (Davis and Williamson, 2017). Mitoribosome assembly occurs co- and post-transcriptionally and depends on rRNA processing and modification and the import of nuclear-encoded proteins from the cytosol (Bogenhagen et al., 2014; Hällberg and Larsson, 2014; Rackham et al., 2016). Mitoribosome assembly and function have mostly been studied in yeast and human cultured cells, while only a few studies have been done in animal models to investigate these processes in differentiated tissues (Câmara et al., 2011; Metodiev et al., 2009, 2014; Richman et al., 2015; Wredenberg et al., 2013). The fact that cultured cells are highly proliferative and display fast protein turnover underlines the need for animal models to obtain insights into regulatory mechanisms of mitochondrial translation in tissues (Bogenhagen et al., 2018; Ruzzenente et al., 2012). Moreover, as mutations in rRNAs and mitoribosomal proteins can cause severe tissue-specific human diseases, animal models will be crucial to understand the molecular consequences of disturbed mitochondrial translation (Hällberg and Larsson, 2014; Jackson et al., 2019). We therefore generated MitoRibo-Tag mice as a versatile tool to study mitoribosome composition and the mitoribosome-interactome in different mouse tissues in vivo. In addition to identifying well-known translation-associated proteins, such as OXA1L, our proteomic analyses reveal a complex network of orphan and known mitochondrial protein interactions with the mitoribosome, called mitoribosome-interacting proteins (MIPs). To investigate mitoribosome biogenesis intermediates and to assess composition changes under defective assembly, we generated MitoRibo-Tag mice lacking the
assembly factor MTERF4. Removal of Mterf4 causes the loss of 55S monosomes and the accumulation of SSU and LSU mitoribosome proteins (Camara et al., 2011). We applied quantitative proteomics in MitoRibo-Tag mice lacking MTERF4 and report that the GTP-binding protein 10 (GTPBP10) and the orphan pseudouridine synthase-like 1 (PUSL1) remain bound to the formed LSU intermediate (Lavdovskaia et al., 2018; Maiti et al., 2018; Zaganelli et al., 2017). MitoRibo-Tag mice are thus powerful tools for in vivo studies of mitoribosome composition during diverse physiological states, disease, and aging.

RESULTS

MitoRibo-Tag Mice Stably Express mL62-FLAG

To study the mitoribosome interactome in vivo, we generated MitoRibo-Tag knockin mice expressing the mitoribosomal protein mL62 with a FLAG-tag sequence at its carboxyl terminus. mL62 is a known LSU constituent, and from recent cryoelectron microscopy (cryo-EM) structures, it is clear that its carboxyl terminus is solvent exposed (Amunts et al., 2015; Greber et al., 2015; Richter et al., 2010). A targeting vector harboring a sequence identical to exons 3 to 6 of the mouse mL62 gene (i.e., Mrpl58, Ensembl: ENSMUSG00000018858) also encoding a carboxy-terminal FLAG-tag sequence was constructed and transfected into mouse embryonic stem cells (ESCs) (Figure 1A). Correctly targeted ESC clones were used to obtain chimeric mice transmitting the mutant mL62 locus through the germline, resulting in heterozygous mice (Figure 1A). The FRT-flanked puromycin cassette was removed by crossing the heterozygous targeted mice to mice expressing the Flp recombinase to obtain heterozygous MitoRibo-Tag (+/T; T for transgenic) mice (Figure 1A). Correct targeting of the mL62-FLAG locus was verified by PCR (Figure 1B). Heterozygous MitoRibo-Tag mice were intercrossed to generate homozygous MitoRibo-Tag mice (T/T).

MitoRibo-Tag mice were viable and indistinguishable from C57BL/6N wild-type (WT) animals and were born at mendelian ratios (data not shown), suggesting that mitochondrial function remains unaffected despite the expression of mL62-FLAG instead of mL62. We isolated mitochondria from WT and MitoRibo-Tag T/T animals to assess the expression of the mL62-FLAG fusion protein by western blots (Figure 1C). FLAG-tagged mL62 is stably expressed in mitochondria isolated from various tissues of MitoRibo-Tag mice and does not affect the steady-state levels of SSU and LSU proteins (Figure 1C). To ensure that the expression of FLAG-tagged mL62 does not alter the mitoribosome composition or the steady-state levels of OXPHOS complexes, we analyzed protein steady-state levels by western blots and label-free quantitative mass spectrometry (LFQ-MS/MS; Figures 1D and S1). All of the mitochondrial proteins in MitoRibo-Tag mice are expressed at levels similar to WT levels, although we detected a slightly reduced expression of the mitochondrial carboxamoyl-phosphate synthase in kidney mitochondria, which was characterized by an adjusted p value of 0.0346, close to the 5% false discovery rate (Figure S1). We therefore conclude that the expression of mL62-FLAG instead of mL62 in homozygous MitoRibo-Tag mice does not affect the steady-state levels of mitoribosome components or other mitochondrial proteins.

MitoRibo-Tag Mice Have a WT Mitoribosome Assembly

To verify that mL62-FLAG incorporation in MitoRibo-Tag mice does not alter mitoribosome assembly, we analyzed mitoribosomes from heart, kidney, and liver mitochondria by sucrose-density gradient centrifugation (Matthews et al., 1982). The sedimentation profile—the assembly status of the 28S, 39S, and 55S (monosomes) mitoribosomal particles—was comparable between MitoRibo-Tag and WT mice in all of the investigated tissues (Figures 2A–2C). The majority of mL62-FLAG is stably integrated into the 55S monosome, as indicated by its co-migration with LSU protein mL37 and the SSU protein bS16m (Figure 2; Akabane et al., 2014; Richter et al., 2010). In general, most mitoribosomes seem to be assembled into 55S complexes in tissues in vivo, which is in contrast to most cultured cells in which 28S and 39S complexes dominate (Figures 2A–2C; Lavdovskaia et al., 2018; Maiti et al., 2018). Based on western blot and density
gradient experiments, we conclude that homozygous MitoRibo-Tag mice have WT mitoribosome levels and a normal assembly state, and thus are a suitable tool to investigate mitoribosomes in vivo.

**MitoRibo-Tag Mice Allow Efficient Mitoribosome Purification**

To investigate mitoribosome composition and associated proteins, we isolated mitoribosomes from mouse liver, kidney, and heart mitochondria with a mild lysis buffer containing digitonin, potassium (K⁺), and magnesium (Mg²⁺) to preserve subunit integrity and interactions with putative soluble or membrane-associated MIPs (Liu and Spremulli, 2000; Metodiev et al., 2009; Rackham et al., 2016; Spremulli and Kraus, 1987). We found that the elution of mitoribosomes from mouse tissues with the FLAG peptide was suboptimal for MS analysis because high levels of this peptide prevented the detection of low abundant proteins in the eluate. Instead, we eluted purified mitoribosomes by a short on-bead digestion with trypsin, obtaining high reproducibility of protein abundances (Figures 3A, 3B, and S2A; Keilhauer et al., 2015). Co-immunoprecipitation (coIP)-coupled LFQ-MS/MS of mitoribosomes from MitoRibo-Tag mice allowed the quantification of 81 of 82 (98.8%) mitoribosomal proteins to very high enrichment and confidence in liver, kidney, and heart mitochondria (Figures 3A and S2A; Suzuki et al., 2001a, 2001b). The only mitoribosomal protein that could not be quantified was bL36m, which is known to assemble very late into the mitoribosome and to be difficult to detect (Bogenhagen et al., 2018; Brown et al., 2017). Only one single peptide of bL36m was detected in two replicates from all of the coIP-experiments from mitochondria of different tissues, which allowed its identification but not quantification (Tables S1 and S2). The method specificity is supported by the absence of MRPS36 in our preparations, a previously falsely annotated mitoribosomal protein, which actually is an α-ketoglutarate dehydrogenase subunit (Heublein et al., 2014). Moreover, the coIP-LFQ-MS/MS results indicate only minor contamination using the partial on-bead trypsin elution, as the majority of proteins are annotated mitochondrial proteins (Figure 3B). Our mitoribosome coIP proteomics demonstrate that MitoRibo-Tag mice are a suitable tool to purify mitoribosomes from tissues. Moreover, this method can be used to analyze rRNA content, as mtRNAs can be detected by northern blot analyses of isolated mitoribosomes (Figure S2B).

**Figure 2. Density Gradient Analysis of MitoRibo-Tag Mitoribosomes**

(A–C) Density gradient analysis of MitoRibo-Tag (T/T) mitoribosomes from liver (A), kidney (B), and heart (C) mitochondria compared to WT (+/+) mitoribosomes. The lysis buffer was supplemented with digitonin, and mitochondrial lysates were loaded onto 10%–30% sucrose density gradients and centrifuged. The inputs correspond to ~30% of the mitochondrial lysate loaded on the gradient. Fractions were taken from the top and analyzed by western blot against mL37, mL62-FLAG, and bS16m. Each panel is representative of at least three biological replicates per genotype.

MitoRibo-Tag Mice Can Be Used to Determine the Mitoribosome Interactome from Differentiated Tissues

We examined the obtained coIP-LFQ-MS/MS data from MitoRibo-Tag mice to detect putative MIPs using high enrichment and confidence values as criteria (Figure 3B). Our data revealed striking tissue-specific compositional differences in the mitoribosome interactomes, with most MIPs identified in heart preparations (Figures 3A, 3B, and S3). These observations are in agreement with studies from Mootha et al. (2003) and (Pagliarini et al., 2008), who found that roughly one-third of the mitochondrial proteome is expressed tissue specifically. We verified the oxidase (cytochrome c oxidase) assembly 1-like (OXA1L) and mitochondrial assembly of ribosomal large subunit 1 (MALSU1) as the most co-enriched MIPs (Figure 3B; Haque et al., 2010; Rorbach et al., 2012; Wanschers et al., 2012). The fact that the mitochondrial translation initiation factors IF2_mt and IF3_mt and the mtDNA-encoded cytochrome c oxidase subunit II (COX2) were co-enriched indicates that our method preserves mitoribosomes in a translationally active state (Figures 3B, S3A, and S3B). Our data are consistent with reported strong interactions of mitoribosomes with the MTERF domain-containing protein 2, the NOP2/Sun RNA methyltransferase family member 4 (MTERF4; NSUN4) complex, and the recently described 16S rRNA module,
composed of neugrin (NGRN), the pseudouridine synthases RNA pseudouridylate synthase domain-containing protein 4 (RPUSD4) and the tRNA pseudouridine synthase B (Trub) family member 2 (TRUB2), and the pentatricopeptide repeat-containing protein 1 (PTCD1; Figure 3B; Antonicka et al., 2017; Arroyo et al., 2016; Câmara et al., 2011; Metodiev et al., 2014; Perks et al., 2018). Several proteins involved in mitochondrial RNA metabolism co-enriched with mitoribosomes, including the mitochondrial RNA methyltransferase MRM3, the DEAD-box RNA helicase 28 (DDX28), the G-rich RNA sequence binding factor 1 (GRSF1), the LRPPRC:SLIRP complex, and the tyrosyl-tRNA synthetase 2 (YARS2; Figure 3B; Antonicka et al., 2013; Jourdain et al., 2013; Rorbach et al., 2014; Ruzzenente et al., 2012; Tu and Barrientos, 2015). Our data corroborate recent findings that several GTP-binding proteins interact with mitoribosomes, including GTPBP5, GTPBP7, GTPBP10, and nitric oxide-associated protein 1 (NOA1; Figure 3B; Kim and Barrientos, 2018; Kolanycz et al., 2011; Lavdovskaia et al., 2018; Malti et al., 2018). These data support recent proposals that RNA metabolism and mitoribosome assembly are coupled and that the machineries responsible for these processes can form large assemblies, similar to the mitochondrial organization of gene expressing (MIOREX) complexes in yeast (Kehrein et al., 2015). Mterf4 knockout mice cannot form 55S monosomes, resulting in increased SSU and LSU steady-state levels (Câmara et al., 2011). In agreement with our previous results, Mterf4 knockout mice show the characteristic SSU and LSU increases compared to control animals. It should be pointed out that the mL62-FLAG fusion protein remained expressed despite the absence of MTERF4, thereby enabling purification of the subassembled 39S intermediate (Figure 4A). Next, we co-immunoprecipitated the putative 39S biogenesis intermediate accumulating in the absence of MTERF4 in Mterf4 knockout mice and determined the protein composition by LFQ-MS/MS, using Mterf4/+; mL62/+ mouse mitochondria as a control (Figure 4B).
As expected, the majority of the significantly enriched proteins purified from Mterf4L/L, +/Cre, mL62T/T mouse mitochondria are 39S mitoribosome constituents, whereas the putative 39S intermediate seems to lack bL32m and bL33m (Figure 4B; Arroyo et al., 2016; Dennerlein et al., 2010; Rorbach et al., 2012; Rozanska et al., 2017; Tu and Barrientos, 2015; Wanschers et al., 2012). The SSU-associated proteins ERAL1, IF2mnt, RBFA, and a few SSU proteins, albeit of lower adjusted p value and enrichment, could also be part of the 39S mitoribosome intermediate complex upon the loss of MTERF4, although the exact molecular nature of these interactions remains to be elucidated (Figure 4B). Moreover, among the highly significant co-enriched proteins were the recently described GTPBP10 and the orphan protein pseudouridine synthase-like 1 (PUSL1; Figure 4B; Lavdovskaia et al., 2018; Maiti et al., 2018; Zaganelli et al., 2017). GTPBP10 and PUSL1 are specifically upregulated in mouse models with defective mitoribosome assembly or poor coordination of mitochondrial translation, such as Mterf4 and Lrpprc knockout mice, but not in mtRNA polymerase (Polrmt), mitochondrial transcription factor A (Tfam), or Twinkle mtDNA helicase (Twink) knockout animals, with a global decrease in tRNAs and rRNAs (Figure 4C).

**GTPBP10 Enables Mitoribosome Assembly in a GTP-Dependent Manner**

The identification of the recently characterized GTPBP10 as a constituent of the 39S intermediate from Mterf4L/L, +/Cre, mL62T/T mice verifies the power of MitoRibo-Tag mice as a tool to study mitoribosome composition and assembly in vivo. During preparation of this manuscript, two independent studies showed that GTPBP10 is exclusively localized to mitochondria and required for biogenesis of the 39S subunit, although the exact mechanism remains unknown (Lavdovskaia et al., 2018; Maiti et al., 2018). To further examine the function of GTPBP10, which remains bound to the 39S intermediate formed in Mterf4 knockout animals, we generated a Flp-In T-REX HEK293 cell line (HEK293T) to express GTPBP10 with a C-terminal FLAG-tag. We co-immunoprecipitated GTPBP10-associated complexes from mitochondria and assessed the interaction with the mitoribosome in the presence of the non-hydrolysable GTP analog guanosine-5’-[(β,γ-imido)triphosphate (GDPNP), which has been shown to inhibit the bacterial GTPBP10 homolog ObgE, using LFQ-MS/MS and western blots (Figures 4A and 4B). We found that GDPNP strongly increased the number of 44 LSU proteins purified with GTPBP10-FLAG, verifying the recently described interaction with the 39S subunit, and additionally showing that GTP hydrolysis enables the dissociation of GTPBP10 from the mitoribosome (Figures 4A and 4B). It should be mentioned that the proteomic analysis of GTPBP10-associated complexes from mitochondrial lysates incubated with GDPNP revealed co-enrichment of a few non-ribosomal proteins (Figure 4A; Tables S1 and S2). This
may result from the GDPNP treatment or GTPBP10-FLAG expression-associated side effects (Lavdovskaia et al., 2018; Maiti et al., 2018). Next, we assessed the effects of GTPBP10 loss on cell growth and mitoribosome assembly by generating GTPBP10 CRISPR-Cas9 knock-out HEK293T cells, confirmed by western blots (Figure S4D) and sequencing (data not shown). We obtained two GTPBP10 knock-out cell lines, both characterized by a severe growth defect in galactose medium, indicating a highly reduced OXPHOS capacity (Figure S4D). The growth phenotype of GTPBP10 knockout cells was completely reversed by the re-expression of GTPBP10 (Figure S4D). In agreement with Maiti et al. (2018), we also observed that the genetic depletion of GTPBP10 in cells decreased LSU (uL3, mL49, and bL28m) and slightly decreased SSU (uS15m, mS22, and uS17m) protein steady-state levels (Figure S4C). Moreover, removal of GTPBP10 almost completely abolished de novo mitochondrial translation (Figure S4E). In summary, these results show that MitoRibo-Tag mice can be used to identify unknown MIPs and that GTP hydrolysis by GTPBP10 is required for mitoribosome assembly and consequently mitochondrial translation (Figure S4).

**PUSL1 Is a Mitochondrial Matrix Protein**

We were intrigued by the identified interaction between the putative mitochondrial tRNA pseudouridine synthase PUSL1 and the mitoribosome. PUSL1 was co-enriched with the 39S mitoribosome intermediate formed in the absence of MTERF4 in mouse mitochondria (Figure 4B). We analyzed the predicted amino acid sequences of human (Ensembl: ENST00000379031.9) and mouse (Ensembl: ENSMUSG00000051557) PUSL1 and found that both variants harbor very similar mitochondrial targeting sequences within their first 38 amino acids (data not shown; Bateman et al., 2017; Claros and Vincens, 1996; Mitchell et al., 2019; Sievers et al., 2011). Human and mouse PUSL1 share two conserved pseudouridine synthase domains at the N- and the C termini, which are homologous to the bacterial tRNA pseudouridine synthase A (TruA) family of pseudouridine synthases. Moreover, PUSL1 has been predicted to localize to human mitochondria in several recent high-throughput studies (Calvo et al., 2016; Pagliarini et al., 2008; Rhee et al., 2013). We found that commercial PUSL1 antibodies are not working reproducibly, being accompanied by many cross-reacting bands, therefore not allowing an adequate localization of PUSL1 by biochemical fractionation. To investigate the submitochondrial localization of PUSL1 and to confirm its interaction with the mitoribosome, we generated a HEK293T cell line to inducibly express FLAG-tagged PUSL1 (Figure 5A). We performed a protease protection assay and found that PUSL1 is protected from degradation by proteinase K added to mitochondria with an intact or a disrupted outer membrane, and thus behaves as the known mitochondrial matrix protein polypeptide K (PK).

Figure 5. **PUSL1 Is a Mitochondrial Matrix Protein and Interacts with the Mitoribosome**

(A) Western blot analysis of mitochondrial localization of proteins. Mitochondria (Mito., non-treated) were swollen in hypotonic buffer (Swell.) or lysed with 1% triton X-100 supplemented buffer (triton). Samples were left untreated (−) or treated (+) with 50 μg/mL proteinase K (PK).

(B) Western blot analysis of mitochondrial proteins incubated in HEPES buffer (negative control) or sodium carbonate at indicated pH values. Total (T), pellet (P), and supernatant (S) correspond to fractions obtained before and after extraction and centrifugation.

(C) Western blot analysis of mitochondrial proteins incubated in HEPES buffer (negative control) or sodium carbonate at indicated pH values. Total (T), pellet (P), and supernatant (S) correspond to fractions obtained before and after extraction and centrifugation.

(D) LFO-MS/MS of co-immunoprecipitated PUSL1-FLAG-associated complexes using digitonin to lyse the mitochondria of HEK293T cells expressing PUSL1-FLAG (n = 4) or Mito-GFP (control, n = 3). Mitoribosomal proteins are highlighted in green (SSU) and blue (LSU). Translation-associated proteins are highlighted in orange and other significantly enriched proteins are highlighted in red. The x axis represents the fold change and the y axis indicates the adjusted p value of PUSL1-FLAG versus Mito-GFP. The dashed line represents a 5% false discovery rate. See also Tables S1 and S2.
PUSL1 Interacts with the Mitoribosome

To further examine the nature of the interaction of PUSL1 with the mitoribosome, we isolated mitochondria from HEK293T cells stably expressing PUSL1-FLAG and co-immunoprecipitated PUSL1-associated complexes to determine their composition by LFQ-MS/MS (Figure 5D). We found that PUSL1 interacts with mitoribosomal proteins, including 20 SSU and 36 LSU constituents, as well as the mitoribosome assembly factors L0R8F8, GTPBP10, MALSU1, MTERF4, and NOA1. These results show that PUSL1 is indeed an orphan MIP, with a possible role in mitochondrial translation (Figure 5D; Brown et al., 2017). MitoRibo-Tag mice, combined with conditional knockout of mitoribosome biogenesis factors, thus provide powerful tools to discover novel MIPs, which may not be observed under basal physiological conditions (Figures 3B and 4B).

Loss of PUSL1 Decreases De Novo Mitochondrial Translation

To gain insight into PUSL1 function, we applied CRISPR-Cas9 genome editing with two different guide RNAs (gRNAs) targeting exon 1 and exon 3 of PUSL1 in HEK293T cells (Table S3). Sequencing revealed that the gRNAs induced a single base insertion that led to premature stop codons and the complete loss of PUSL1 on western blots (Figure 6A). We found that PUSL1 interacts with mitoribosomal proteins, including 20 SSU and 36 LSU constituents, as well as the mitoribosome assembly factors L0R8F8, GTPBP10, MALSU1, MTERF4, and NOA1. These results show that PUSL1 is indeed an orphan MIP, with a possible role in mitochondrial translation (Figure 5D; Brown et al., 2017). MitoRibo-Tag mice, combined with conditional knockout of mitoribosome biogenesis factors, thus provide powerful tools to discover novel MIPs, which may not be observed under basal physiological conditions (Figures 3B and 4B).

DISCUSSION

In this study, we present the MitoRibo-Tag knockin mice as a versatile tool to investigate the in vivo composition and interactome of mitoribosomes in different tissues and physiological states. Since the discovery of mitoribosomes in 1967, a number of studies have contributed to our understanding of mitochondrial translation and mitoribosome composition (O’Brien and Kalf, 1967a, 1967b). Many mitoribosome assembly factors modifying essential bases or putatively stabilizing conformations of mitochondrial rRNAs, including MRPP3, MRM1–MRM3, MTERF3, MTERF4, NSUN4, PTCD1, RPUSD4, and TFB1M, have recently been described (Hällberg and Larsson, 2014;
Lee et al., 2018). In addition, a few MIPs are known to specifically regulate the synthesis of mtDNA-encoded proteins mtCOX1 and mtCYTB in yeast and mammals (Ott et al., 2018). Furthermore, auxiliary factors such as Mba1, Mrx15, and Oxa1 in yeast and OXA1L and MTRAC complexes in mammals are known to recruit mitoribosomes to sites of cytochrome c oxidase assembly (Mick et al., 2012; Ott et al., 2006; Richter-Dennerlein et al., 2016; Möller-Hergt et al., 2018). Despite the available mitoribosome structures, we are still lacking the essential knowledge of proteins coordinating translation with OXPHOS biogenesis and mitoribosome assembly (Hallberg and Larsson, 2014; Ott et al., 2016). As mitoribosome assembly and translation coordination in mitochondria strongly diverge from those in bacteria or the eukaryotic cytosol, it is expected that several mitochondrial-specific translation factors and MIPs have been acquired to assist in these processes (Brown et al., 2017). In part, our knowledge of ribosome function and assembly in vivo is limited due to the scarcity of relevant animal models.

We generated MitoRibo-Tag mice to allow the investigation of mitoribosome composition under basal conditions and in response to mitochondrial dysfunction. We used MitoRibo-Tag mice to define the mitoribosome-interactome across different tissues, revealing tissue-specific compositional differences and unknown MIPs. The different interactome compositions may partially explain the variable tissue-specific phenotypes observed in human patients suffering from mitochondrial translation-associated diseases (Hallberg and Larsson, 2014). Therefore, MitoRibo-Tag mice are an important in vivo tool to investigate the tissue specificity of the mitoribosome interactome and the molecular mechanisms underlying the variable outcomes of translation-associated mitochondrial diseases.

In agreement with recently published studies, we verified that GTPBPs are important regulatory mitoribosome assembly factors in vivo, and our experiments provide additional evidence that GTPBP10 controls LSU biogenesis in a GTP-dependent manner. GTPBP10 may allow mitoribosome assembly to proceed only if its substrate GTP can be hydrolyzed. This idea is supported by the presented colP-LFO-MS/MS experiments, which show that GTPBP10 remains bound to mitoribosomal proteins in the presence of the non-hydrolysable GTP analog GDPNP. Hence, one can hypothesize that GTPBP10 prevents the entry of premature LSUs into monosome assembly to avoid diminishing the translational capacity formation of malfunctioning mitoribosomes (Lav dovkskaia et al., 2018; Matti et al., 2018).

We also show that MitoRibo-Tag mice are a suitable tool to discover yet unknown interactions or orphan MIPs and mitoribosome biogenesis intermediates, which are not identified under normal physiological conditions. By colP-coupled proteomics in MitoRibo-Tag mice, we defined the interactome of a mitoribosome biogenesis intermediate formed in mitochondria lacking MTERF4. Similar approaches in bacteria based on a combination of genetics with proteomics or cryo-EM have led to the identification of immature ribosomal particles bound to assembly factors (Davis and Williamson, 2017). In the analysis of mammalian mitoribosomes we present here, PUSL1 and GTPBP10 were found to remain bound to a 39S assembly intermediate formed in the absence of MTERF4. PUSL1 is homologous to Escherichia coli TruA, which is known to pseudouridylate uridines in 17 bacterial tRNAs (Hur and Stroud, 2007; Suzuki et al., 2011). The exact molecular function of each pseudouridine in the different RNA species is not understood, but it is generally accepted that pseudouridines stabilize RNA conformations (Ofengand, 2002). Recent studies have suggested a requirement of pseudouridylation for the stability of mitochondrial tRNAs and the 16S rRNA to enable translation and mitoribosome assembly (Antonicca et al., 2017; Perks et al., 2018).

Using MitoRibo-Tag mice as a tool, we identified PUSL1 as an inner membrane-associated mitochondrial matrix protein interacting with mitoribosomes. Proteomic analysis of PUSL1-FLAG-associated complexes revealed interactions between PUSL1, mitoribosomal proteins, and several assembly factors, including GTPBP10, LOR8F8, MALSU1, MTERF4, and NOA1. One could speculate that PUSL1, together with or independently of GTPBP10, safeguards a quality control checkpoint during mitoribosome assembly as supported by the finding that the removal of these proteins reduces mitochondrial de novo translation. This hypothesis is further strengthened by the demonstration that the mitoribosome interactomes from MTERF4 knockout MitoRibo-Tag mice and PUSL1-associated complexes from human mitochondria partially overlap with the enrichment of GTPBP10, LOR8F8, MALSU1, and, if expressed, MTERF4. Pseudouridines could represent adaptations of the mitochondrial translation system to stabilize RNA conformations or mitoribosomal biogenesis intermediates (Ofengand, 2002; Zaganelli et al., 2017). The recent finding that several pseudouridine synthases are required for mitochondrial translation is intriguing, as there is only one known pseudouridine synthase in E. coli (RluD), which is strictly required for bacterial growth (Arroyo et al., 2016; Del Campo et al., 2004). It is tempting to speculate that PUSL1 chaperones mitoribosome assembly or functions via modification of the structurally integrated central protuberance tRNA. It is known that mitochondrial phenylalanine and valine tRNAs, which serve as structural constituents in the central protuberance in porcine and human mitoribosome LSUs, harbor pseudouridines at base positions 37 (phenylalanine) and 24 and 29 (valine) (Suzuki and Suzuki, 2014). It is possible that PUSL1 pseudouridylates uridines (e.g., at base position 37 [tRNA phenylalanine] or 24 [tRNA valine]), contributing to mitoribosome integrity or regulation of mitochondrial translation (Suzuki et al., 2011). It will be interesting to investigate the exact molecular role of PUSL1 in the regulation of the mitochondrial transcriptome and translatome, for example, by the application of recently developed (pseudouridine) RNA sequencing methods to identify putatively modified bases, and ribosome profiling to investigate the molecular consequences on the translation of certain mRNAs or tRNA codons.

In summary, we have generated MitoRibo-Tag mice and show that they are a valuable and versatile tool to molecularly define the mitoribosome composition and interacting proteins that are necessary for regulating mitochondrial translation in vivo. The technological advantages of MitoRibo-Tag mice include the possibility of analyzing mitoribosomes from different tissues and analyzing the composition of assembly intermediates obtained by the disruption of genes encoding biogenesis factors. We also envision that MitoRibo-Tag mice will be important tools to study mitoribosomes and their interactomes in different conditions.
tissues, when animals are subjected to varying physiological conditions (e.g., starvation, exercise, caloric restriction or high-fat diet), or in disease states (e.g., diabetes), as well as during the normally occurring aging process.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- LEAD CONTACT AND MATERIALS AVAILABILITY
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Cell Lines
  - Generation of Knock-out Cell Lines
  - Generation of Stable Cell Lines
  - Animal Models
  - Ethics Statement and Animal Housing
- METHOD DETAILS
  - Isolation of Mitochondria from Mouse Tissues
  - Mitoribosome Co-immunoprecipitation
  - Mitoribosome Gradient Analysis
  - Preparation of Mitochondria for Proteomics
  - Liquid-Chromatography Mass Spectrometry
  - Mass Spectrometry Data Analysis
  - SDS-Polyacrylamide Gel Electrophoresis
  - Western Blot
  - Isolation of Genomic DNA from Murine Tissue
  - RNA Isolation
  - Northern Blot
  - Cell Growth Assay
  - RNA Interference using siRNAs
  - In Cellulo Translation Assay
  - Isolation of Mitochondria from Cultured Cells
  - Protein Co-immunoprecipitation
  - Immunocytochemistry and Microscopy
  - Sodium Carbonate Extraction of Proteins
  - Protease Protection Assay
- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND CODE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.09.080.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.D.B., J.R., and N.-G.L.; Methodology, J.D.B., M.C., A.B., E.S.R., and T.S.; Formal Analysis, I.A. and X.L.; Investigation, J.D.B., M.C., I.A., A.B., E.S.R., T.S., X.L., S.F.P., and D.M.; Data Curation, J.D.B. and I.A.; Writing – Original Draft, J.D.B. and N.-G.L.; Writing – Review & Editing, all authors; Visualization, J.D.B. and I.A.; Supervision, J.R. and N.-G.L.; Funding Acquisition, J.R. and N.-G.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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### STAR★METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| bL12m / MRPL12      | Sigma-Aldrich | RRID: AB_1854099; Cat#HPA022853 |
| bL28m / MRPL28      | Sigma-Aldrich | RRID: AB_10601136; Cat#HPA030594 |
| bS16m / MRPS16      | Proteintech Group | RRID: AB_2180166; Cat#16735-1-AP |
| Cytochrome c        | Abcam | RRID: AB_2107448; Cat#ab8254 |
| DDDDK tag (FLAG-tag) | Abcam | RRID: AB_299216; Cat#ab1257 |
| DYKDDDDK tag (9A3) Mouse mAb | Cell Signaling | RRID: AB_10950495; Cat#8146S |
| GAPDH               | Abcam | RRID: AB_2107448; Cat#ab8254 |
| GTPBP10             | Sigma-Aldrich | RRID: AB_1850411; Cat#HPA021076 |
| mL37 / MRPL37       | Proteintech Group | RRID: AB_2282151; Cat#14677-1-AP |
| mL48 / MRPL48       | Proteintech Group | RRID: AB_2146189; Cat#15542-1-AP |
| mL49 / MRPL49       | Proteintech Group | RRID: AB_2146189; Cat#15542-1-AP |
| mL51 / MRPL51       | Abcam | Cat#ab235828 |
| mS22 / MRPS22       | Proteintech Group | RRID: AB_2146488; Cat#10984-1-AP |
| mS35 / MRPS35       | Proteintech Group | RRID: AB_2146521; Cat#16457-1-AP |
| mHSP60              | Santa Cruz | RRID: AB_631683; Cat#sc-1052 |
| mHSP60 (for mouse samples) | Cell Signaling | Cat#ab470S |
| mHSP70              | Abcam | RRID: AB_881520; Cat#ab4755 |
| OXPHOS cocktail human | Abcam | Cat#ab110411 |
| OXPHOS cocktail mouse | Abcam | RRID: AB_2629281; Cat#ab110413 |
| PUSL1               | Sigma-Aldrich | RRID: AB_10601251; Cat#HPA032057 |
| SDHA                | Invitrogen | RRID: AB_459200 |
| TIM22               | Proteintech Group | RRID: AB_11183050; Cat#14927-1-AP |
| TIM23               | Abcam | RRID: AB_10903878; Cat#ab116329 |
| TIM44               | Abcam | Cat#ab194829 |
| TOM20               | Cell Signaling | RRID: AB_2687663; Cat#D8T4N |
| TOM20               | Santa Cruz Biotechnology | RRID: AB_2207533; Cat#sc-11415 |
| TOM22               | Abcam | RRID: AB_945897; ab57523 |
| uL3m / MPRL3        | Sigma-Aldrich | RRID: AB_2678606; Cat#HPA043665 |
| uS15m / MRPS15      | Proteintech Group | RRID: AB_2301068; Cat#17006-1-AP |
| uS17m / MRPS17      | Proteintech Group | RRID: AB_10597844; Cat#18881-1-AP |
| VDAC                | Abcam | RRID: AB_443084; Cat#ab14734 |
| α-Tubulin           | Sigma-Aldrich | RRID: AB_477583; Cat#T6199 |
| Anti-rabbit IgG F(ab')2-HPR | GE Healthcare | RRID: AB_772191; Cat#A16005 |
| Sheep Anti-Mouse IgG, Whole Ab ECL Antibody, HRP conjugated | GE Healthcare | RRID: AB_772209; Cat#A16005 |
| Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP conjugated | Thermo Fisher | RRID: AB_2534679; Cat#A16005 |
| Alexa Fluor 488 donkey anti-mouse antibody | Jackson Immuno | RRID: AB_2340850; Cat#715-546-151 |
| Cy3-AffiniPure Donkey Anti-Rabbit IgG (H+L) antibody | Jackson Immuno | RRID: AB_2307443; Cat#711-165-152 |
| F(ab')2-Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 | Thermo Fisher | RRID: AB_2534084; Cat#A-11017 |
| Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 | Thermo Fisher | RRID: AB_10563566; Cat#A-11036 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| 2-chloroacetamid     | Merck  | Cat#8024120100 |
| Anti-FLAG M2 beads  | Sigma-Aldrich | Cat#A2220 |
| Complete EDTA-free protease inhibitor cocktail | Roche | Cat#05056489001 |
| Digitonin           | Calbiochem | Cat#300410 |
| Doxycycline         | Sigma-Aldrich | Cat#D9891 |
| Empore Octadecyl C18 Extraction Disks | 3M | N/A |
| FLAG peptide        | Sigma-Aldrich | Cat#F3290 |
| FLAG® Immunoprecipitation Kit lysis buffer | Sigma-Aldrich | Cat#L3412 |
| Guanidinium chloride | Sigma-Aldrich | Cat#G3272 |
| Guanosine-5’-[(β,γ-imido)triphosphate (GDPNP) | Jena Bioscience | Cat#NU-401-50 |
| L-[³⁵S]-methionine  | Hartmann Analytic | Cat#SCM-01 |
| Lipopectamine RNAiMAX transfection reagent | Invitrogen/Thermo Fisher | Cat#1377815 |
| Lipopectamine2000 Transfection Reagent | Invitrogen/Thermo Fisher | Cat#11668027 |
| Lipopectamine3000 Transfection Reagent | Invitrogen/Thermo Fisher | Cat#L3000008 |
| Phenylmethylsulfonyl fluoride | Sigma-Aldrich | Cat#P7626 |
| RNase inhibitor     | New England Biolabs | Cat#M0307L |
| Tetracycline        | Sigma-Aldrich | Cat#T7760 |
| Tris(2-carboxyethyl)phosphine | Thermo Fisher | Cat#T2556 |
| Triton X-100        | Sigma-Aldrich | Cat#T8787 |
| Trypsin Gold        | Promega | Cat#V5280 |
| x-[³²P]-cytidine triphosphate | Hartmann Analytic | Cat#SRP-209 |
| x-[³²P]-deoxyctydine triphosphate | PerkinElmer | Cat#NEG513H |
| x-[³²P]-uridine triphosphate | PerkinElmer | Cat#NEG007H |
| γ-[³²P]-adenosine triphosphate | Hartmann Analytic | Cat#SRP-301 |

**Critical Commercial Assays**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| FLAG-immunoprecipitation kit | Sigma-Aldrich | Cat#FLAGIPT1 |
| Prime-It II Random Primer Labeling Kit | Agilent | Cat#300385 |
| Qubit Protein Assay Kit | Thermo Fisher | Cat#Q33211 |
| Riboprobe® System - SP6/T7 | Promega | Cat#P1460 |

**Experimental Models: Cell Lines**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| HeLa Cell Line Homo sapiens | ATCC Inc. | RRID: CVCL_0030 |
| Flip-In T-Rex HEK293 Cell Line Homo sapiens | Invitrogen/Thermo Fisher | RRID: CVCL_U427 |
| Flip-In T-Rex HEK293 Cell Line GTPBP10 knock-out Homo sapiens | This study | N/A |
| Flip-In T-Rex HEK293 Cell Line PUSL1 knock-out Homo sapiens | This study | N/A |
| Flip-In T-Rex HEK293 GTPBP10-Flag expression Cell Line Homo sapiens | This study | N/A |
| Flip-In T-Rex HEK293 PUSL1-Flag expression Cell Line Homo sapiens | This study | N/A |

**Experimental Models: Organisms/Strains**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mus musculus C57BL/6N Genotype: WT/+ | The Jackson Laboratory | RRID: MGI:5795896 |
| Heterozygous MitoRibo-Tag mice (mL62-Flag knock-in) | This study | N/A |
| Mus musculus C57BL/6N Genotype: mL62-Flag+/T | This study | N/A |
| Heterozygous MitoRibo-Tag mice (mL62-Flag knock-in) | This study | N/A |
| Mus musculus C57BL/6N Genotype: mL62-Flag+/T | This study | N/A |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **MitoRibo-Tag Mterf4 control mice** | This study | N/A |
| Mus musculus C57BL/6N | | |
| Genotype: ml62-Flag<sup>T/T</sup> Mterf4 loxP<sup>P</sup>P Ckmm Cre<sup>+/+</sup> | | |
| **MitoRibo-Tag Mterf4 knock-out mice** | This study | N/A |
| Mus musculus C57BL/6N | | |
| Genotype: ml62-Flag<sup>T/T</sup> Mterf4 loxP<sup>P</sup>P Ckmm Cre<sup>+</sup>Cre | | |
| **Oligonucleotides** | | |
| See Table S3 | See Table S3 | See Table S3 |
| **Recombinant DNA** | | |
| pcDNA5/FRT/TO | Invitrogen/Thermo Fisher | Cat#V652020 |
| pcDNA5/FRT/TO-GTPBP10-FLAG | This study | N/A |
| pcDNA5/FRT/TO-PUSL1-FLAG | This study | N/A |
| pcDNA5/FRT/TO-Mito-GFP | This study | N/A |
| pOG44 Fip-Recombinase Expression Vector | Invitrogen/Thermo Fisher | Cat#V600520 |
| pSpCas9(BB)-2A-Puro (PX459) V2.0 | Ran et al., 2013 | RRID: Addgene_62988 |
| **Software and Algorithms** | | |
| cowplot version 0.9.2 | R project | https://cran.r-project.org/web/packages/cowplot/index.html |
| CRISPOR | Haeussler et al., 2016 | RRID: SCR_015935; http://www.crispor.tefor.net/ |
| GraphPad Prism version 5 | GraphPad Software | RRID: SCR_002798; https://www.graphpad.com/scientific-software/prism/ |
| limma version 3.34.5 | Ritchie et al., 2015 | https://bioconductor.org/packages/3.3/bioc/html/limma.html |
| MaxQuant version 1.6.1.0 | Cox and Mann, 2008 | RRID: SCR_014485; http://www.coxdocs.org/doku.php?id=maxquant:start |
| MitoProt | Claros and Vincens, 1996 | https://ihg.gsf.de/ihg/mitoprot.html |
| R: A language and environment for statistical computing version 3.4.3 | R Development Core Team, 2008 | RRID: SCR_001905; http://www.R-project.org/ |
| readxl version 1.1.0 | | https://cran.r-project.org/web/packages/readxl/index.html |
| Rstudio version 1.1.383 | RStudio | RRID: SCR_004322; https://rstudio.com/ |
| tidyverse version 1.2.1 | | https://cran.r-project.org/web/packages/tidyverse/index.html |
| TMHMM Server version 2.0 | Sonnhammer et al., 1998 | RRID: SCR_014935; http://www.cbs.dtu.dk/services/TMHMM/ |
| Uniprot | Bateman et al., 2017 | RRID: SCR_002380; http://www.uniprot.org |
| vsn version 3.46.0 | Huber et al., 2002 | RRID: SCR_001459; http://www.bioconductor.org/packages/release/bioc/html/vsn.html |
| **Other** | | |
| Human reference proteome | Bateman et al., 2017 | Uniprot: UP000005640 |
| Mouse reference proteome | Bateman et al., 2017 | Uniprot: UP000000589 |
| Silencer select<sup>©</sup> negative control siRNA | Invitrogen/Thermo Fisher | Cat#4390844 |
| Silencer select<sup>©</sup> siRNA PUSL1-siRNA1 | Invitrogen/Thermo Fisher | Cat#43084 |
| Silencer select<sup>©</sup> siRNA PUSL1-siRNA2 | Invitrogen/Thermo Fisher | Cat#225579 |
| ssniff M-Z Low-Phytoestrogen (mouse food newly weaned animals) | Ssniff Spezialdiaeten GmbH | N/A |
| ssniff RM-H Low-Phytoestrogen (mouse food) | Ssniff Spezialdiaeten GmbH | N/A |
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents, which may require a completed Materials Transfer Agreement, should be directed to and will be fulfilled by the Lead Contact, Nils-Göran Larsson (nils-goran.larsson@ki.se).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell Lines

HeLa (RRID: CVCL_0030, female, ATCC), Flp-In T-REx HEK293 (HEK293T, RRID: CVCL_U427, female) and derivative cell lines (listed in the Key Resources Table) were grown in DMEM+GlutaMAX culture media (Thermo Fisher, cat. no. 31966-021) supplemented with 10% fetal bovine serum (Thermo Fisher, cat. no. 10270-106), 1x non-essential amino acids (Thermo Fisher, GIBCO, cat. no. 11140050), 100 U/ml Penicillin / 100 μg/ml Streptomycin (Thermo Fisher, GIBCO, cat. no. 15140122) and maintained at 37°C and 5% CO₂. One day after thawing, culture media was refreshed to remove residual DMSO. Cells were split every second to fourth day and maintained at least one week before experiments were performed.

Generation of Knock-out Cell Lines

HEK293T GTPBP10 and PUSL1 knock-out cell lines were generated according to Ran et al. (2013) using the pSpCas9(BB)-2A-Puro (pX459) V2.0 vector (Ran et al., 2013), whereby gRNAs to edit PUSL1 were designed using the CRISPOR software (Haeussler et al., 2016). Essentially, the gRNAs were cloned into the pX459 vector which was transfected into cultured cells followed by puromycin selection (Ran et al., 2013). Two pairs of gRNAs targeting exon two were generated to induce an out-of-frame deletion within the GTPBP10 coding sequence. To generate PUSL1 knock-out HEK293T cells, gRNAs targeting exon 1 or exon 3 were cloned separately into the pX459 vector. Cells were transfected with pX459 using Lipofectamine 3000 according to the manufacturer’s instructions. Transfected clones were selected with puromycin at 1.5 to 2 μg/ml (final concentration) for 48 h. Subsequently, cells were diluted to single cell suspension and plated on 96 well plates. Single colonies cells were sequenced with Sanger sequencing to confirm the predicted out-of-frame deletion or frameshift mutation. Knock-out of GTPBP10 and PUSL1 in selected cloned was confirmed by western blot and sequencing.

Generation of Stable Cell Lines

Flp-In T-REx HEK293 expression cell lines were generated according to manufacturer’s instructions with some modifications (Thermo Fisher, cat. no. K6500-01). Essentially, Flp-In T-REx HEK293 host cells were split and 300,000 cells were seeded into a 6-well dish with a final volume of 1 ml DMEM+GlutaMAX culture media, supplemented with 10% Tet-approved FBS (Clontech, cat. no. 631106) and non-essential amino acids but without antibiotics. On the next day, the pcDNAs/FRT/TO vector harboring, the gene of interest, and the pOG44 vector, harboring the FLP recombinase, were co-transfected with Lipofectamine2000 or Lipo- fectamine3000 (Thermo Fisher, cat. no. 11668027 and L3000008) Transfection Reagent to generate HEK293T expression cell lines. Transfected cells were selected adding 15 μg/ml Blasticidin S and 100 μg/ml Hygromycin B to culture media. Approximately two to three weeks post transfection positive colonies appeared and single colonies were picked and expanded. Protein expression was induced adding doxycycline (Sigma-Aldrich, cat. no. D9891) or tetracycline (Sigma-Aldrich, cat. no. T7760) to a final concentration of 50 ng/ml to culture media 24 h prior experimental analysis according to manufacturer’s instructions.

Animal Models

The mouse models used in this study belong to the C57BL/6N strain (RRID: MGI:5795896, The Jackson Laboratory). To generate mL62-Flag knock-in mice, the mL62-Flag knock-in targeting vector was constructed using BAC clones from the C57BL/6J RPCIB-731 BAC library by Taconic Biosciences (Cologne, Germany). The targeting vector harbored exons three to six of mL62, the FLAG-tag DNA sequence (introduced after the last amino acid of exon six: …MTMD-DYKDDDDK), a puromycin resistance selection marker (PuroR) flanked by F3 recombination sites (downstream of the mL62 3’UTR) and a thymidine kinase (Tk) cassette. After transfection of a C57BL/6N embryonic stem cell line, homologous recombinant clones were isolated using positive PuroR and negative Tk selections. The PuroR cassette was removed by crossing heterozygous-targeted knock-in mice with transgenic mice ubiquitously expressing the Flp recombinase. The remaining F3 recombination site remained in a non-conserved region of the genome. Heterozygous mL62-Flag+/T knock-in mice were backcrossed with C57BL/6N wild-type mice for several generations. The backcrossed heterozygous mL62-Flag+/T knock-in mice were intercrossed to generate homozygous mL62-Flag+/T knock-in mice, denoted MitoRibo-Tag mice (T/T), constitutively expressing the mL62-FLAG fusion protein controlled by the endogenous promoter. Approved animal experiments (see ethics statement below) were performed with female and male homozygous MitoRibo-Tag+/T mice at an age of 16 to 20 weeks and corresponding age and sex C57BL/6N wild-type mice as controls. Furthermore, MitoRibo-Tag mice were intercrossed with homozygous carriers of the Mterf4 loxP allele to generate Mterf4+/L, mL62+/T mice (Câmara et al., 2011). In subsequent crossings, Mterf4+/L, mL62+/T mice were intercrossed with heterozygous C57BL/6N mice expressing the Cre recombinase under the ckm promoter (genotype: +/-Cre). Ultimately, intercrossed animals with the genotypes Mterf4+/L, +/-, mL62+/T (control genotype) and Mterf4+/L, +/-Cre, mL62+/T (knock-out genotype), which lack the...
assembly factor MTERF4 in heart and skeletal muscle, were used for approved animal experiments at an age of 18 to 20 weeks, or for colony propagation. No female Mterf4−/−, +/Cre, mL62T/T knock-out animals were used for matings.

**Ethics Statement and Animal Housing**

The study, including all animal experiments, was approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (reference numbers 84-02.04.2015.A103 and 84-02.50.15.004) and performed in accordance with the recommendations and guidelines of the Federation of European Laboratory Animal Science Associations (FELASA). The health status of the animals is specific pathogen-free according to the FELASA recommendations. Wild-type C57BL/6N and transgenic mice were housed in individually ventilated cages (45 × 29 × 12 cm) with a 12 h light/dark cycle and controlled environmental conditions of 22 ± 2°C and 50 ± 10% relative humidity. The animals were fed *ad libitum* on a standard mouse food (ssniff RM-H Low-Phytoestrogen, Ssniff Spezialdiaeten GmbH) or an enhanced diet during breeding and for newly weaned mice (ssniff M-Z Low-Phytoestrogen, Ssniff Spezialdiaeten GmbH).

**METHOD DETAILS**

**Isolation of Mitochondria from Mouse Tissues**

Heart, liver and kidney were dissected from mice and washed two times with ice cold phosphate-buffered saline (PBS, Thermo Fisher, cat. no. 14190-094). Hearts were additionally washed once with mitochondrial isolation buffer (MIB: 310 mM sucrose, 10 mM Tris-HCl (Merck, cat. no. 108382) and 0.05% fatty acid-free BSA (w/v; Sigma-Aldrich, cat. no. A8806)). Tissues were cut and gently homogenized within 5 to 15 mL of MIB with a Potter S (Sartorius). The volume of heart and kidney was brought to 15 mL and of liver samples to 50 mL total volume prior centrifugation for 10 min/1000 g/4°C to remove cell debris. The obtained mitochondria-containing supernatants from heart were centrifuged for 15 min/4500 g/4°C and from kidney and liver for 10 min/10000 g/4°C to isolate mitochondria. The crude mitochondrial pellets were resuspended in MIB supplemented with 1x complete EDTA-free protease inhibitor cocktail (PIC; Roche, cat. no. 05056489001), aliquoted and snap frozen. The protein concentration was determined by means of Bradford assay using BSA as standard.

**Mitoribosome Co-immunoprecipitation**

Crude mitochondrial protein of 1 mg was pelletted (5 min/9200 g/4°C) and resuspended with 200 μl purification-lysis buffer (10 mM Tris-HCl pH 7.5, 100 mM potassium chloride (KCl, Merck, cat. no. 1.04936.1000), 20 mM magnesium dichloride (MgCl₂, Merck, cat. no. M2670), 1x PIC), 1% digitonin (Calbiochem, cat. no. 300410, 2 g detergent/g protein)). The lysates were incubated for 20 min on ice and clarified by centrifugation for 45 min/9200 g/4°C. The resulting supernatants were diluted 1:10 with dilution buffer (10 mM Tris-HCl pH 7.5, 100 mM KCl, 20 mM MgCl₂) and incubated for 2 h at 4°C with 100 μl anti-FLAG M2 beads (Sigma-Aldrich, cat. no. A2220, equilibrated twice with 15 volumes TBS and wash buffer ( = dilution buffer supplemented with 0.1% digitonin)) while rotating at 10 rpm. Following binding, beads were washed three times with 15 volumes of wash buffer and two times with dilution buffer. Proteins were eluted with 300 μl elution buffer (50 mM Tris-HCl pH 7.5, 1 mM Tris(2-carboxyethyl)phosphine (TCEP; Thermo Fisher, cat. no. T2556), 5 mM 2-chloroacetamid (Merck, cat. no. 8024120100) and 1 ng/μl trypsin Gold (Promega, cat. no. V5280, resuspended in 50 mM acetic acid) rotating for 30 min/RT for protein analysis or 60 min/4°C for RNA analysis rotating at 5 rpm. Eluates were transferred to ice and subsequently prepared for mass spectrometry or mixed 3:1 with TRIzol LS reagent (Thermo Fisher, cat. no. 10296028) for RNA analysis.

**Mitoribosome Gradient Analysis**

Sucrose density gradient fractionation of mitochondrial ribosomes was performed according to Matthews et al. (1982) and Rackham et al. (2016). Mitochondria (1 mg from mouse tissue or 750 μg from cultured cells) were re-isolated by centrifugation for 5 min/9200 g/4°C. Mitochondria were resuspended at protein concentrations of 7.5 mg/ml (from cultured cells) or 10 mg/ml (from mouse tissues) in lysis buffer (260 mM sucrose, 10 mM Tris-HCl pH 7.5, 100 mM KCl, 20 mM MgCl₂, 2% digitonin for mouse mitochondria or 6% digitonin for human mitochondria, 40 U/ml RNase inhibitor (New England Biolabs, cat. no. M0307L) and 1x PIC) and incubated for 20 min on ice. Samples were clarified by centrifugation for 45 min/9200 g/4°C. Sucrose density gradients of 10% to 30% were cast as described by Metodiev et al. (2009) using a Gradient Master (BioComp Instruments, Inc.). The sucrose gradient solutions were prepared with 10 mM Tris-HCl pH 7.5, 100 mM KCl and 20 mM MgCl₂. Ten percent of the cleared mitochondrial lysates were saved as input control. The remaining lysate was loaded on top of the 10% - 30% sucrose density gradient and centrifuged for 15 h/71092 g/4°C. Fractions were collected from the top (first fraction: 750 μl and loaded lysate volume, fraction two to 15: 750 μl and fraction 16: residual volume). The input samples and one third of each fraction were precipitated with 0.02% sodium deoxycholate and 12% trichloroacetic acid (final concentrations) for SDS-PAGE and western blot analysis. The loaded input fractions of mitochondrial lysate from mouse correspond to ~30% of total and the input fraction of mitochondrial lysates from human cultured cells to ~16% of total. Mitoribosomal proteins were detected using antibodies listed in the STAR methods table. Chemiluminescent detection was carried out with Amersham ECL Western Blotting Detection reagent (GE Healthcare, cat. no. RPN2106).
Preparation of Mitochondria for Proteomics
Mitochondria were resuspended with an appropriate volume of guanidinium hydrochloride lysis buffer (6 M guanidinium chloride (Sigma-Aldrich, cat. no. G3272), 10 mM TCEP, 40 mM chloroacetamide and 100 mM Tris-HCl) as previously described by Kulak et al., 2014 with some modifications. Lysates were diluted 1:10 with 20 mM Tris pH 8.3 and digested overnight at 37°C with at least 300 ng or 1:200 (protein:trypsin) of trypsin Gold (Promega). Peptides were desalted using home-made StageTips (Empore Octadeacyl C18, 3M; Rappsilber et al., 2003) and eluted with 80 to 100 μl of 60% acetonitrile / 0.1% formic acid buffer. The peptides were dried with a vacuum concentrator plus (Eppendorf) and resuspended with 0.1% formic acid for mass spectrometry.

Liquid-Chromatography Mass Spectrometry
Peptides obtained from co-immunoprecipitations were separated on a 25 cm, 75 μm internal diameter PicoFrit analytical column (New Objective) packed with 1.9 μm ReproSil-Pur 120 C18-AQ media (Dr. Maisch) using an EASY-nLC 1000 or EASY-nLC 1200 (Thermo Fisher Scientific). The column was maintained at 50°C. Buffer A and B were 0.1% formic acid in water and 0.1% formic acid in acetonitrile or 0.1% formic acid in 80% acetonitrile. Peptides were separated on a segmented gradient from 6% to 25% or 31% buffer B. Eluting peptides were analyzed on an Orbitrap Fusion or QExactive HF mass spectrometer (Thermo Fisher Scientific). Peptide precursor m/z measurements were carried out at 60000 resolution in the 300 to 1500 or 1800 m/z range. The ten most intense precursors with charge state from two to seven only were selected for HCD fragmentation using 27% or 25% normalized collision energy. The m/z values of the peptide fragments were measured in the orbitrap at a resolution of 30000 using an AGC target of 2e5 and 55, 80 or 100 ms maximum injection time. Upon fragmentation, precursors were put on a dynamic exclusion list for 45 s.

Mass Spectrometry Data Analysis
The raw data were analyzed with MaxQuant version 1.6.1.0 using the integrated Andromeda search engine (Cox and Mann, 2008; Cox et al., 2011). Peptide fragmentation spectra were searched against the canonical and isoform sequences of the mouse (Uniprot proteome ID: UP000000589, downloaded September 2018 from UniProt) or human (Uniprot proteome: ID UP000005640, downloaded September 2018 from UniProt) reference proteome (Bateman et al., 2017). Methionine oxidation and protein N-terminal acetylation were set as variable modifications; cysteine carbamidomethylation was set as fixed modification. The digestion parameters were set to “specific” and “Trypsin/P.” The minimum number of peptides and razor peptides for protein identification was one and the minimum number of unique peptides was zero. Protein identification was performed at a peptide spectrum matches and protein false discovery rate of 0.01. The “second peptide” option was on. Successful identifications were transferred between the different raw files using the “Match between runs” option. Label-free quantification (LFQ) was performed using an LFQ minimum ratio count of two. LFQ intensities were filtered for a number of valid values that was equal to the minimum number of replicates in an experimental group minus one. Missing values were imputed from a normal distribution with a width of 0.3 and down shift of 1.8. For the analysis of the label-free data from the MitoRibo-Tag CoIPs in heart, liver, and kidney mitochondria, the median enrichment value was added to the label-free data from the MitoRibo-Tag CoIPs in heart, liver, and kidney mitochondria, the median enrichment value was added to

SDS-Polyacrylamide Gel Electrophoresis
Protein samples were resuspended with 2x NuPAGE® LDS sample buffer (Thermo Fisher, cat. no. NP0007) supplemented with 100 mM dithiothreitol and separated on NuPAGE 4 – 12% or 10% Bis-Tris midi gels (Thermo Fisher, cat. no. WG1202BOX and WG1402BOX) using NuPAGE 1x MOPS (cat. no. NP0001) or 1x MES (cat. no. NP0002) running buffer for appropriate separation.

Western Blot
Proteins were blotted onto PVDF membrane using 1x wet western blot buffer (38.63 mM glycine, 47.87 mM Tris and 10% methanol) at 4°C for 2 h at 400 mA or overnight at 80 mA. Membranes were blocked for 1 h at RT with blocking buffer (Rockland, cat. no. MB-070) or 5% milk-Tris buffer saline (TBS; 50 mM Tris-HCl pH 7.4 and 150 mM NaCl). The blocked membranes were incubated overnight with primary antibodies at indicated dilutions. Following overnight incubation, membranes were washed three times with 1x TBS-Tween20 (TBST; TBS with 0.1% Tween20) and incubated with HRP-conjugated secondary antibodies for 1.5 to 2 h at RT. Membranes were washed two times with 1x TBST and 1x TBS. Detection was performed using Amersham ECL western blotting detection reagent (GE Healthcare, Amersham, cat. no. RPN2106).

Isolation of Genomic DNA from Murine Tissue
400 μl tissue lysis buffer (0.5% SDS (Merck, cat. no. 112533), 100 mM NaCl, 20 mM Tris pH 8, 2.5 mM EDTA pH 8 (Merck, cat. no. 108418)) was supplemented with 80 μg proteinase K and added to each ear clip or tail cut of a mouse. The samples were lysed for 5 to 24 h at 56°C. Afterwards, 40 μl 8 M potassium acetate and subsequently 500 μl chloroform were added to each sample and DNA was pelleted by centrifugation for 10 min/13000 rpm/RT. The resulting upper aqueous phase was carefully transferred to a new tube and
mixed with 1 mL of 95% ethanol. DNA was precipitated by incubation at ~80 °C for 30 min and pelleted by centrifugation for 15 min/13000 rpm/4 °C. The resulting supernatant was discarded, and the DNA pellets were washed once with 500 µl of 70% ethanol. The DNA was subsequently dried for 15 min at 60 °C using a vacuum concentrator plus (Eppendorf). Finally, the DNA was dissolved with 125 µl water and stored at 4 °C for further use.

RNA Isolation
RNA was isolated using the Trizol (for RNA of cultured cells) or Trizol LS (for RNA obtained from co-immunoprecipitations of mouse mitochondria) reagents (Thermo Fisher, cat. no. (15596026 and 10296028) according to manufacturer’s instructions. RNA was precipitated overnight in the presence of glycogen.

Northern Blot
Isolated RNA of 1 to 4 µg were solubilized in NorthernMax-Gly Sample loading dye (Thermo Fisher, cat. no. AM8551) and loaded onto a 1.2% LE agarose gels. Following separation, gels were blotted overnight onto Hybond-N+ membrane (GE Healthcare, cat. no. 45-000-927) according to standard procedures. The probes for labeling of mouse and human mitochondrial 12S and 16S rRNA were prepared using the Prime-It II Random Primer labeling kit (Agilent) and 50 µCi z-[32P]-dCTP (Hartmann Analytic, cat. no. SRP-209) for mouse rRNAs or z-[32P]-dCTP (PerkinElmer, cat. no. NEG513H). tRNA probes against mouse mitochondrial tRNA were prepared by T4 polynucleotide kinase end labeling using with 50 µCi γ-[32P]-ATP (Hartmann Analytic, cat. no. SRP-301). For labeling of human tRNAs 4 µg total RNA were loaded onto a 15% TBE-urea gel in 1x TBE buffer and wet transferred at 30 V for 1 hour in 0.5x TBE buffer. The tRNA probes against human mitochondrial tRNAs were prepared using the Riboprobe in vitro Transcription System (Promega) with 50 µCi z-[32P]-UTP (PerkinElmer, cat. no. NEG007H).

Cell Growth Assay
For growth measurements, 20,000 cells were seeded into 6-well plates and grown in the presence or absence of 50 ng/ml of doxycycline in glucose-free DMEM containing 0.9 g/l galactose, 10% (v/v) FBS, 2 mM Glutamax and 1 x Penicillin/Streptomycin. Cell confluence was determined every second day with EVETM Automated Cell Counter (NanoEnTek, cat. no. E1000).

RNA Interference using siRNAs
HEK293T were cultured for several days and seeded 24 h before first transfection in DMEM+GlutaMAX culture media supplemented with 50 µg/ml uridine (Sigma-Aldrich, cat. no. U3003). For in cellulo translation experiments 300.000 control and 400.000 knock-down cells were seeded, whereas 1.5 million control and 3 million cells were seeded onto 500 cm^2 square dishes for isolation of mitochondria (Thermo Fisher, cat. no. 10489282). On the next day, 2.875 µg (for 10 cm-dishes) or 18 µg siRNA (for square dishes) of silencer select® siRNA (Thermofisher, negative control: cat. no. 4390844, PUSLsiRNA1: cat. no. s43084, PUSLsiRNA2 cat. no. s25579) were transfected using 15 µl or 90 µl Lipofectamine RNAiMAX transfection reagent (Thermo Fisher, cat. no. 13778150) according to manufacturer’s instructions. On the third day, the siRNA transfection was repeated and cells were incubated for two to three additional days.

In Cellulo Translation Assay
HEK293T knock-out or knock-down and control cells were carefully washed with 3 mL PBS and incubated for 30 min in labeling media (DMEM, high glucose, no glutamine, no methionine, no cysteine, supplemented with 10% dialyzed fetal bovine serum (Thermo Fisher, cat. no. A3382001), 1x GlutaMax (Thermo Fisher, cat. no. 3505003), sodium pyruvate (Thermo Fisher, cat. no. 11360039) and 50 µg/ml uridine). Meanwhile, emetine (Sigma-Aldrich, cat. no. E2375) was solubilized in PBS, sterile filtered and added to a final concentration of 100 µg/ml, followed by 5 min incubation, to inhibit cytosolic translation. Subsequently, 1000 µCi L-[35S]-methionine (Hartmann Analytic, cat. no. SCM-01) were added to each dish and incubated for 30 min or 1 h at 37 °C / 5% CO₂. Following labeling, cells were washed three times with 5 mL PBS, transferred into 1.5 mL Eppendorf tubes, resuspended with 2x RIPA buffer (150 mM sodium chloride, 1% triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate) and 50 mM Tris, pH 8 supplemented with 1x PICI) and snap frozen. Cell lysates were thawed and sonicated three times for 20 s at 90% amplitude with a sonoplus mini20 (Bandelin, cat. no. 3665) equipped with a MS 1.5 sonotrode (Bandelin, cat. no. 3639). Insoluble material was removed by centrifugation for 5 min/2000 rpm/4 °C. The resulting supernatants were saved and quantified using Qubit Protein Assay Kit (Thermo Fisher, cat. no. Q33211). An appropriate amount of protein lysate was mixed with 2x SDS sample buffer and 20 µg of lysate from each approach were loaded onto NuPAGE 12% Bis-Tris proteins gel (ran with 1x MES buffer). Gels were subsequently incubated for 30 min in protein fixation buffer (20% ethanol and 10% acetic acid) supplemented with PhastGel Blue R (Sigma-Aldrich, cat. no. B4921) and scanned. Afterwards, gels were destained with fixation buffer and incubated for 30 min in Amersham Amplify (GE Healthcare, cat. no. NAMP100) to increase sensitivity of the radioactive signal. Detection was performed using a Typhoon FLA7000 (GE Healthcare).

Isolation of Mitochondria from Cultured Cells
Dishes with ~80 to 90% confluent cells were harvested (from two to three 500 cm² dishes). The cells were subsequently pelleted by centrifugation for 5 min/800 g/4 °C and washed two times with cold PBS. The cell pellet was initially resuspended with 2 mL isolation
buffer (20 mM HEPES pH 7.6, 220 mM mannitol, 20 mM sucrose, 1 mM EDTA, 2 mg/ml fatty acid-free BSA and 1x PIC). Thereafter, 6 mL isolation buffer was added to the samples followed by 15 min incubation on ice to facilitate cell swelling. Swollen cells were gently homogenized with 20x strokes at 500 rpm using a Schuett homogenizer (Schuett Biotec, cat. no. 3.201-011). The cell homogenates were centrifuged for 5 min at 800 g/4 °C to obtain a crude mitochondrial pellet, which was washed twice with isolation buffer without BSA prior protein quantification. For some experiments mitochondria from cultured cells were isolated according Lee and Bogenhagen (2016) with some modifications as described below. All buffers were prepared with DEPC-treated water. The mitochondrial pellet obtained after the nuclease treatment (step 12 of the medium/large-scale preparation from Lee and Bogenhagen [2016]) was washed twice with 1 mL of cell mitochondrial isolation buffer, aliquoted, snap frozen and stored at –80 °C.

**Protein Co-immunoprecipitation**

Co-immunoprecipitations of putative MIPs were performed with mitochondria isolated from HEK293T cells. Essentially, 750 μg of mitochondria were lysed with 150 μl purification buffer supplemented with 3% digitonin (for purification of PUSL1-FLAG-associated complexes) or FLAG® immunoprecipitation kit lysis buffer (Sigma-Aldrich, cat. no. L3412) supplemented with 1% triton X-100 (for purification of GTPBP10-FLAG-associated complexes) and 5 mM MgCl₂. All lysis buffers were additionally supplemented with 1x PIC. At indicated experiments guanosine-5′-[β,γ-imido]triphosphate (Jena Bioscience, cat. no. NU-401-50) was added to a final concentration of 20 mM to the lysis buffer. Protein complexes were subsequently purified as described in “Mitoribosome co-immunoprecipitation from mouse mitochondria” or using the FLAG-immunoprecipitation kit (Sigma-Aldrich, cat. no. FLAGIPT1) with buffers freshly supplemented with 5 mM MgCl₂. Elution of GTPBP10-FLAG co-immunoprecipitations carried out using the FLAG peptide at 300 ng/μl final concentration (Sigma-Aldrich, cat. no. F3290) according to manufacturer’s instructions.

**Immunocytochemistry and Microscopy**

30,000 HeLa cells were seeded onto coverslips in 12 well dishes. One day after seeding, cells were transiently transfected with 1 μg of vector DNA, harboring the gene-of-interest, and 0.5 μl Lipofectamine2000 per well according to manufacturer’s instructions. Following 24 h incubation, cells were fixed with 4% PFA (EM sciences, cat. no. 15710) in PBS for 10 min at RT. After several washes with PBS, cells were permeabilized with 0.1% triton X-100 in PBS for 5 min at RT. Unspecific sites were blocked with 3% BSA (Sigma-Aldrich, cat. no. A9898) in PBS. Primary and secondary antibodies were also prepared with 3% BSA. The following primary antibodies were used and incubated at 4 °C overnight, mouse anti-Flag (1:500, Cell Signaling, cat. no. 8146) and rabbit anti-TOM20 (1:1000, Santa Cruz Biotechnology, cat. no. sc-11415). After several washes with 3% BSA, the following secondary antibodies were applied for two hours at RT: donkey anti-mouse conjugated to Alexa Fluor 488 nm (Jackson Immuno, cat. no. 715-546-151) and donkey anti-rabbit conjugated to Cy3 (Jackson Immuno, cat. no. 711-165-152). Coverslips were washed with PBS and nuclei counterstained with DAPI before mounting with Aqua-Poly/ Mount (Polysciences, cat. no. 18606). A laser-scanning confocal microscope (Leica, TCS SP8-X) equipped with a white light laser, a 405-diode UV laser, a 100x objective lens (HCX Plan-Apochromat CS 100x oil, 1.46 NA), and hybrid detectors was used to acquire fluorescence images. The following parameters were used for acquisition: image size of 1,024 × 1,024 pixels, bi-directional X, scan speed 200 Hz, and z-step size 0.2 μm.

**Sodium Carbonate Extraction of Proteins**

For each approach, 200 μg of crude mitochondria were initially resuspended in 100 μl mitochondrial isolation buffer and sonicated on ice, whereas one aliquot of mitochondria was kept on ice as total protein control. The sonicated mitochondria were pelleted and subsequently resuspended in 200 μl of 20 mM HEPES-KOH pH 7.4 supplemented with 1x PIC. After the initial resuspension, additional 200 μl of HEPES buffer or 200 μl of 200 mM sodium carbonate buffer (Na₂CO₃; Sigma-Aldrich, cat. no. S7795) at pH 10.5, 11.5 or 12.5, were added to the samples, followed by gently mixing and 30 min incubation on ice. The samples were then centrifuged for 30 min/45000 rpm/4 °C to separate soluble and membrane (-associated) proteins. The supernatants were precipitated in the presence of 0.02% sodium deoxycholate with 12% trichloroacetic acid and further analyzed by SDS-PAGE and western blot.

**Protease Protection Assay**

Freshly prepared mitochondria (200 μg) for each approach were pelleted and resuspended in 200 μl isolation buffer, hypotonic swelling buffer (10 mM HEPES-KOH, pH 7.4) or isolation buffer supplemented with 1% triton X-100. The aliquots were split into two, whereby to one aliquot proteinase K (Roche, cat. no. 03450384103) was added to a final concentration of 25 μg/ml. Samples were incubated on ice for 10 min and subsequently inhibited with 2 μl of 0.1 M phenylmethylsulfonyl fluoride (PMFSF, Sigma-Aldrich, cat. no. P7626) added to each sample. Proteins were precipitated with 0.02% sodium deoxycholate and 12% trichloroacetic acid and further analyzed by SDS-PAGE and western blot.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Quantitative proteomics data (Related to Figures 3, 4, 5, S1, S2, S3, S4, and S6) were analyzed using limma (Ritchie et al., 2015). The statistical significance of differential expression or protein enrichment analysis was calculated using limma’s moderated t test. The number of experiments or (biological) replicates (n) used for the statistical evaluation of each experiment are indicated...
in corresponding figure legends, whereby the significance is represented by the adjusted p value using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). The adjusted p values, in addition to the log fold change values and the 95% confidence interval values for the log fold change are reported in the supplemental data Tables S1 and S2. In Figure S4D, the mean growth of each cell line was determined based on three replicates and analyzed using GraphPad Prism version 5 (GraphPad Software) applying a two-way analysis of variance test (ANOVA) and the Bonferroni correction (p > 0.05 not significant; p < 0.0001 significant). The error bars represent the standard error of the mean (from three replicate experiments).

DATA AND CODE AVAILABILITY

The published article includes the mass spectrometry datasets generated and analyzed during this study, which can be found in the supplemental data tables Table S1 – Label-free quantitative mass spectrometry data and Table S2 – Quantification of label-free quantitative mass spectrometry data.
Supplemental Information

MitoRibo-Tag Mice Provide a Tool for In Vivo Studies of Mitoribosome Composition

Jakob D. Busch, Miriam Cipullo, Ilian Atanassov, Ana Bratic, Eduardo Silva Ramos, Thomas Schöndorf, Xinping Li, Sarah F. Pearce, Dusanka Milenkovic, Joanna Rorbach, and Nils-Göran Larsson
Figure S1 – Mass spectrometry of protein steady-state levels in mitochondria of MitoRibo-Tag mice (related to Figure 1)

Comparison of protein steady-state levels in heart, liver and kidney mitochondria of MitoRibo-Tag (T/T) versus wild-type (+/+, WT) mice as determined by LFQ-MS/MS. The x-axis represents the fold change, and the y-axis indicates the adjusted p-value (upper panel) or the average expression value (lower panel) of MitoRibo-Tag vs. WT. The dashed line represents a 5 % false discovery rate. The red dot in the panels of kidney mitochondria represents the mitochondrial carbamoyl phosphate synthase. The results represent five biological replicates per genotype and tissue. See also supplemental data Tables S1 and S2.
Figure S2 – Overview of mitoribosome components purified from heart, liver and kidney mitochondria of MitoRibo-Tag mice (related to Figure 3)

(A) LFQ-MS/MS of mL62-FLAG co-immunoprecipitated mitoribosomal proteins from liver, kidney and heart mitochondria of MitoRibo-Tag (T/T) versus wild-type (+/+, WT) mice. The lysis buffer was supplemented with digitonin. The log2 (fold change; dot size), −log10 (adjusted p-value; blue to yellow color) as well as significant enrichment (red circle) are highlighted. Proteins with an adjusted p-value of less than 0.05 (5 % false discovery rate) were termed significant. The depicted result is representative of five biological replicates per genotype. See also supplemental data Tables S1 and S2. (B) Representative northern blot of mitochondrial rRNAs and tRNAs co-purified with mitoribosomes from liver mitochondria of MitoRibo-Tag (T/T) or WT mice. Non-lyzed and lyzed samples correspond to one µg total mitochondrial RNA prior the co-immunoprecipitations. Full-length (FL), one prime and two prime indicate full length or cleaved 12S and 16S rRNAs, respectively.
Figure S3
**Figure S3 – The mitoribosome-interactomes from liver and kidney mitochondria of MitoRibo-Tag mice (related to Figure 3)**

LFQ-MS/MS analysis of the mitoribosome-interactome in liver (A) and kidney (B) mitochondria of MitoRibo-Tag (T/T) versus wild-type (+/+, WT) mice. The lysis buffer was supplemented with digitonin. Mitoribosomal proteins are colored in green (SSU) and blue (LSU). Translation-associated proteins are highlighted in orange and other significantly enriched proteins in red. The x-axis represents the fold change and the y-axis the adjusted p-value. The dashed line represents a 5 % false discovery rate. The results are representative for five biological replicates per genotype and tissue. See also supplemental data Tables S1 and S2.
Figure S4 – GTPBP10 interacts with the mitoribosome in a GTP-dependent manner (related to Figure 4)

(A) LFQ-MS/MS of GTPBP10-FLAG-associated complexes in mitochondria of HEK293T cells in the absence or presence of GDPNP (n = 3). The lysis buffer was supplemented with triton X-100. Mitoribosomal proteins are colored in green (SSU) and blue (LSU). Translation-associated proteins are highlighted in orange and other significantly enriched proteins in red. The x-axis represents the fold change and the y-axis the adjusted p-value. The dashed line represents a 5 % false discovery rate. See also supplemental data Tables S1 and S2. (B) Representative western blots of GTPBP10-FLAG co-immunoprecipitation complexes in the presence (+) or absence (-) of non-hydrolyzable GDPNP. The lysis buffer was supplemented with triton X-100. (C) Western blot analysis of protein steady-state levels in cell lysates of GTPBP10 knock-out versus HEK293T control (Cntrl.) cells (n = 2). (D) Western blot of GTPBP10 levels in relation to VDAC (loading control; n = 2) and cell growth in knock-out and control HEK293T cells (n = 3). The depicted error bars represent the standard error of the mean from three replicate experiments. (E) In cellulo [35S]-methionine pulse labeling of HEK293T cells to assess mitochondrial translation in control, GTPBP10 knock-out and GTPBP10 knock-out cells with re-expression of GTPBP10 (n = 3).
Figure S5 – PUSL1 localizes to mitochondria (related to Figure 5)
Representative confocal images of human HeLa cells transiently transfected with a pcDNA5/FRT/TO vector encoding PUSL1-FLAG or mitochondrial-targeted GFP (Mito-GFP). Cells were immunostained using anti-TOM20 (mitochondrial marker) and anti-FLAG antibodies. Nuclear DNA was stained with DAPI. Scale bars correspond to 20 µm. Staining patterns were observed in three biological replicates.
**Figure S6** – siRNA-mediated knock-down of PUSL1 decreases mitochondrial de novo translation and mildly affects translation-associated proteins (related to Figure 6)

(A) Western blot analysis of PUSL1 steady-state levels in mitochondria after siRNA treatment with negative control (NC), *PUSL1*-targeting siRNA1 (1) or siRNA2 (2), showing two of three replicate experiments. Asterisk (*) and number sign (#) indicate cross-reacting bands using the commercial PUSL1 antibody. (B) Representative in cellulo [³⁵S]-methionine pulse-labeling experiment in HEK293T cells treated with negative control or *PUSL1*-siRNA1 (n = 3). (C, D) LFQ-MS/MS analysis of protein steady-state levels in mitochondria upon knock-down of *PUSL1* using the denoted siRNA1 (C) or siRNA2 (D) versus the negative control (NC) in HEK293T cells. Mitoribosome proteins are colored in green (SSU) and blue (LSU). Translation-associated proteins are highlighted in orange and other significantly enriched proteins in red. The x-axis represents the fold change and the y-axis indicates the adjusted p-value of *PUSL1* siRNA1 or siRNA2 vs. NC. The dashed line represents a 5 % false discovery rate. The results are representative for three replicates per genotype. See also supplemental data Tables S1 and S2.
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Oligonucleotides** |        |            |
| MitoRibo-Tag (mL62-Flag knock-in) forward primer: 5’-AGCAACAGAGGATTGTGAGTCCC-3’ | This study | N/A |
| MitoRibo-Tag (mL62-Flag knock-in) reverse primer: 5’-TGGGAATGAGCTCAATG G-3’ | This study | N/A |
| Mterf4 loxP forward primer: 5’-CTCCCGGCTGCGTGTG-3’ | Cámar et al., 2011 | N/A |
| Mterf4 loxP reverse primer: 5’-TTTGGGGAAGGAATCTGTG-3’ | Cámar et al., 2011 | N/A |
| Ckmm Cre forward primer: 5’-CAGGACCAAGTGCACAGCAAT-3’ | This study | N/A |
| Ckmm Cre reverse primer: 5’-AGAGACGGAAATCCATCG | This study | N/A |
| CRISPR/Cas9 gRNA1_top_GTPBP10: 5’-CACCGCAGCCAACAAACCGTTTCG-3’ | This study | N/A |
| CRISPR/Cas9 gRNA1_bottom_GTPBP10: 5’-AAAACGGAAACGTGCTGTGCTGC-3’ | This study | N/A |
| CRISPR/Cas9 gRNA2_top_GTPBP10: 5’-CAGCGAGGAATCCAGGATCC-3’ | This study | N/A |
| CRISPR/Cas9 gRNA2_bottom_GTPBP10: 5’-AAAGGGAATCCAGCTGGAATCC-3’ | This study | N/A |
| CRISPR/Cas9 gRNA exon1_sense_PUSL1: 5’-CACCGCGAGGCTATCCATC-3’ | Haeussler et al., 2016 | N/A |
| CRISPR/Cas9 gRNA exon1_antisense_PUSL1: 5’-AAACTGAGGCTATCCATC-3’ | Haeussler et al., 2016 | N/A |
| CRISPR/Cas9 gRNA exon3_sense_PUSL1: 5’-CACCGCGAGGCTATCCATC-3’ | Haeussler et al., 2016 | N/A |
| CRISPR/Cas9 gRNA exon3_antisense_PUSL1: 5’-AAACTGAGGCTATCCATC-3’ | Haeussler et al., 2016 | N/A |