A hypomorphic point mutation in the Wars2 gene was identified

Mutant mice exhibit progressive tissue-specific pathologies

Variable activation of stress response pathways

Demonstrating pleiotropic effects
A Wars2 Mutant Mouse Model Displays OXPHOS Deficiencies and Activation of Tissue-Specific Stress Response Pathways

Thomas Agnew,1 Michelle Goldsworthy,1 Carlos Aguilar,1 Anna Morgan,1 Michelle Simon,1 Helen Hilton,1 Chris Esapa,1 Yixing Wu,1 Heather Cater,1 Liz Bentley,1 Cheryl Scudamore,1 Joanna Poulton,1 Karl J. Morten,2 Kyle Thompson,3 Langping He,3 Steve D.M. Brown,1 Robert W. Taylor,3 Michael R. Bowl,1,4,* and Roger D. Cox1,4,5,*

1MRC Harwell Institute, Mammalian Genetics Unit and Mary Lyon Centre, Harwell Campus, Oxfordshire OX11 0RD, UK
2Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Level 3 The Women’s Centre, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK
3Wellcome Centre for Mitochondrial Research, Institute of Neuroscience, The Medical School, Newcastle University, Newcastle upon Tyne NE2 4HH, UK
4These authors contributed equally
5Lead Contact
*Correspondence: m.bowl@har.mrc.ac.uk (M.R.B.), r.cox@har.mrc.ac.uk (R.D.C.)

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SUMMARY

Mutations in genes essential for mitochondrial function have pleiotropic effects. The mechanisms underlying these traits yield insights into metabolic homeostasis and potential therapies. Here we report the characterization of a mouse model harboring a mutation in the tryptophanyl-tRNA synthetase 2 (Wars2) gene, encoding the mitochondrial-localized WARS2 protein. This hypomorphic allele causes progressive tissue-specific pathologies, including hearing loss, reduced adiposity, adipose tissue dysfunction, and hypertrophic cardiomyopathy. We demonstrate the tissue heterogeneity arises as a result of variable activation of the integrated stress response (ISR) pathway and the ability of certain tissues to respond to impaired mitochondrial translation. Many of the systemic metabolic effects are likely mediated through elevated fibroblast growth factor 21 (FGF21) following activation of the ISR in certain tissues. These findings demonstrate the potential pleiotropy associated with Wars2 mutations in patients.

INTRODUCTION

Mitochondrial diseases are a heterogeneous group of disorders caused by mutations in mitochondrial proteins encoded by either the mitochondrial genome (mtDNA) or the nuclear genome (genomic DNA [gDNA]). The nuclear-encoded mitochondrial aminocyl-tRNA synthetase (mt-aaRS) proteins catalyze the aminoclylation of mitochondrial tRNAs with their cognate amino acid. Mitochondrial tRNA aminoclylation is fundamental to mitochondrial translation and synthesis of mtDNA-encoded respiratory chain subunits and the supply of ATP to the cell. The mt-aaRS proteins are encoded by separate nuclear genes with the exception of glycine- and lysine-tRNA synthetase (GARS and KARS), which function in both the mitochondria and the cytoplasm. With the description of families with compound heterozygous variants in the tryptophanyl-tRNA synthetase 2 (WARS2) gene (Burke et al., 2018; Musante et al., 2017; Theisen et al., 2017; Vantroys et al., 2018; Wortmann et al., 2017), patients have been reported with biallelic, pathogenic mutations in all 19 nuclear-encoded mt-aaRS genes (Oprescu et al., 2017).

Surprisingly, given their common function within mitochondrial translation and ubiquitous expression, mt-aaRS mutations cause distinct tissue-specific pathologies and respiratory chain deficiencies in a gene-dependent manner (Konovalova and Tynismaa, 2013). For example, pathogenic histidyl-tRNA synthetase 2 (HARS2) (Pierce et al., 2011) and leucyl-tRNA synthetase 2 (LARS2) (Soldà et al., 2016) mutations cause Perrault syndrome (sensorineural hearing loss and ovarian dysgenesis), glutamyl-tRNA synthetase 2 (EARS2) (Steenweg et al., 2012) mutations cause leukoencephalopathy with thalamus and brainstem involvement with high lactate (LTBL), and seryl-tRNA synthetase 2 (SARS2) (Belostotsky et al., 2011; Rivera et al., 2013) mutations cause hyperuricemia, pulmonary hypertension, renal failure, and alkalosis (HUPRA) syndrome with hypertrophic cardiomyopathy. The underlying mechanisms dictating the pleiotropic effects and tissue-specific penetrance and variability among individuals with mt-aaRS mutations are unknown and are a major challenge in the understanding and developing therapies for mitochondrial disease (Nunnari and Suomalainen, 2012).

Global mt-aaRS knockout animal models are heterozygous haploinsufficient and homozygous lethal (http://www.mousephenotype.org/) (Dickinson et al., 2016). A heart and skeletal muscle-specific aspartyl-tRNA synthetase 2 (Dars2) knockout (Dars2-KO<sup>SM</sup>) mouse with fatal cardiomyopathy has been reported (Dogan et al., 2014). Complete loss of Dars2 function caused disrupted mitochondrial proteostasis and activating transcription factor 4 (ATF4)-dependent fibroblast growth factor 21 (FGF21) expression specifically in the heart, but not in skeletal muscle, suggesting tissue-specific differences in mitochondrial proteostatic buffering capacity. However, residual mt-aaRS activity is retained in human patients with mt-aaRS...
mutations; thus, animal models with global-hypomorphic mt-aaRS alleles are vital to investigating tissue-specific penetrance.

Hypomorphs from N-ethyl-N-nitrosourea (ENU) mutagenesis screens in the mouse allow the pleiotropic effects of a mutation to be identified. We have incorporated aging as a sensitizing factor to assess recessive pedigrees for late-onset and progressive phenotypes in metabolism and other body systems (Potter et al., 2016). We report here the identification of an ENU-induced mouse mutant harboring a recessive hypomorphic point mutation in the Wars2 gene, Wars2V117L, which causes a complex tissue-specific pathology, including hearing loss, reduced adiposity, adipose tissue dysfunction, and hypertrophic cardiomyopathy. We demonstrated that reduced Wars2 levels causes tissue-specific respiratory chain deficiencies, modeling human mt-aaRS patients. We demonstrate that tissue-specific upregulation of mitochondrial biogenesis is coincident with respiratory chain deficiencies in Wars2V117L/V117L mice, likely contributing to the tissue-specific respiratory chain deficiencies observed. We also show that activation of the integrated stress response (ISR) is a heart-specific response to inhibition of mitochondrial translation that contributes to increased FGF21 levels and systemic changes in metabolism.

RESULTS

We applied high-throughput broad-based phenotyping to pedigrees of mutagenized mice to investigate the pleiotropic effects of the mutations identified (Potter et al., 2016).

Wars2-V117L ENU-Induced Mutation Causal for Hearing Loss and Reduced Adiposity

Auditory phenotyping of one of these pedigrees (MPC151) identified progressive hearing loss. At 3 months of age, all mice displayed a normal response to a clickbox stimulus. However, at 6 months of age, 2 of 58 mice had a reduced response, increasing to 7 animals (12%) by 9 months of age. At 12 months of age, the pedigree was assessed using auditory brainstem response (ABR) testing, which showed that 5 of the 53 surviving mice exhibited elevated hearing thresholds at all frequencies tested (Figure S1A). In addition, the hearing-impaired mice were found to have reduced body weight (Figure S1B).

A genome scan of G3 mice showed linkage to a ∼73.3 Mb region on chromosome 3 containing 1,298 genes (Figure S1C). DNA from an affected G3 mouse (MPC151/2.10 g) underwent whole-genome sequencing, and analysis of the data indentified only three high-confidence non-synonymous coding changes within the mapped interval. These consisted of Chr3:93446568A–T at nucleotide 3314 of the trichohyalin (Tchh) gene (Ensembl: ENSMUST00000064257), causing an aspartate-to-valine substitution at residue 1105 (TchhD1105V); Chr3:93920453G–T at nucleotide 349 of the Wars2 gene (Ensembl: ENSMUST0000004343), causing a valine-to-leucine substitution at residue 117 (Wars2V117L); and Chr3: 133330454A–T at nucleotide 368 of the pyrophosphatase (inorganic) 2 (Ppa2) gene (Ensembl: ENSMUST00000029644), causing a tyrosine-to-phenylalanine substitution at residue 123 (Ppa2Y123F). The presence of the three lesions was confirmed using Sanger sequencing, and only mice showing hearing impairment were homozygous for these ENU-induced lesions (Figure S1D).

To segregate the mutations, the offspring were backcrossed for three generations to C3H.Pdeeb+ mice. The Ppa2Y123F allele was segregated from the TchhD1105V and Wars2V117L alleles at backcross 2. However, the TchhD1105V and Wars2V117L alleles remained linked due to their proximity. Auditory phenotyping of Ppa2Y123F/V123F mice at 6 months of age showed they had similar ABR thresholds to their wild-type and heterozygous littermates (Figure S1E, A). In addition, the body, fat, and lean mass of animals for each genotype were not significantly different (Figure S1E, B–G). Thus, the Ppa2Y123F lesion was excluded as being causative of the phenotypes.

The Tchh gene encodes a protein for hair shaft formation, and a patient with a homozygous nonsense TCHH mutation and unicorailable syndrome has been described (U Basmanav et al., 2016). We did not observe a hair phenotype in Wars2V117L/V117L mice. However, to determine which of the two lesions, Wars2V117L or TchhD1105V, is causal, we undertook a genetic complementation test, crossing Wars2V117L+/+;TchhD1105V/+ mice with mice heterozygous for a Wars2 knockout (Wars2V117L+/−) allele (Figure S2A). This generated offspring that are compound heterozygotes for Wars2, but heterozygous for Tchh (Wars2V117L+−;TchhD1105V+). These mice displayed elevated ABR thresholds at 4 months of age and reduced weight, total fat, and lean mass compared to their colony mates, which had normal hearing and weight (Wars2+/−;Tchh+−, Wars2V117L+/−; TchhD1105V+−, and Wars2−−;Tchh−−) (Figures S2B–S2E). Failure of the Wars2 alleles to complement confirms the Wars2V117L lesion as the causal mutation underlying the observed phenotypes. Homozygous null (Wars2−−) mice were embryonic lethal, and Wars2−−/− and Wars2V117L/V117L mice were subviable and viable, respectively; thus, the Wars2V117L allele is hypomorphic, rather than a complete loss of function (Table S1).

To further characterize the phenotypes and establish underlying mechanisms, we bred additional cohorts of mice.

Hearing Loss in Wars2V117L/V117L Mice Was Progressive

To investigate progression of the auditory phenotype, ABR was measured at 1, 3, 6, 10, and 12 months of age. The hearing thresholds of Wars2+/− and Wars2V117L/V117L mice were comparable and within the normal range at all ages tested. In contrast, Wars2V117L/V117L mice display an age-related increase in hearing thresholds at all tested frequencies (Figure 1). Investigation of the cochlear sensory epithelia using scanning electron microscopy showed a progressive loss of outer hair cell stereocilia bundles in the homozygous mutants, with an apical-to-basal increase in severity (Figure S3A). In addition, assessment of cochlear histological sections identified a reduced number of spiral ganglion neurons in the cochlear apex of 12-month-old mutant mice (Figure S3B). The mutant mice showed no overt vestibular dysfunction (e.g., circling, head bob, or abnormal swim), and no craniofacial dysmorphology was observed.

Wars2V117L/V117L Mice Failed to Gain Fat Mass

To refine the reduced body weight phenotype, we analyzed body composition at monthly intervals and found reduced total body weight from 2 months of age, reduced fat mass from 2 months...
(female) or 3 months (male) of age, and lean mass from 3 months (male) or 5 months (female, in cohort 1 only) of age (Figures 2A–2C, male; Figures 2D–2F, female cohort 1; Figures S4A–S4C, male; Figures S4D–S4F, female cohort 2). Thus, demonstration of the reduction in total mass was primarily due to decreased adiposity and a failure to increase fat mass. We further investigated whether these differences were the result of specific organ weight changes in \textit{Wars2}^{V117L/V117L} mice dissected at 6 months of age. Visceral gonadal white adipose tissue (gWAT), subcutaneous inguinal WAT (iWAT), and brown adipose tissue (BAT) normalized to body weight were all significantly reduced in \textit{Wars2}^{V117L/V117L} mice compared to wild-type colony mates (Figure 3A), consistent with reduced adiposity. Strikingly, heart weight was increased in \textit{Wars2}^{V117L/V117L} mice (Figure 3A). No significant differences in liver or kidney weight (Figure 3A) were observed, demonstrating organ specificity and that the changes in adipose tissues and heart weight were not because of global growth or development impairment.

\textbf{Wars2}^{V117L/V117L} Mice Showed Hypertrophic Cardiomyopathy

To determine the cause of increased heart weight, \textit{Wars2}^{V117L/V117L} cardiac morphology was assessed by echocardiogram at 5 months of age (Figure 3B). We found significantly increased left ventricular anterior wall (LVAW) diameter and left ventricular (LV) mass in \textit{Wars2}^{V117L/V117L} mice relative to wild-type colony mates, showing that the increase in heart weight was due to hypertrophic cardiomyopathy (Figure 3C). Consistent with this, the LV stroke volume (SV) and cardiac output (CO) were significantly reduced (Figure 3C). These differences were also observed in compound heterozygote \textit{Wars2}^{V117L/V117L} mice, which showed increased LVAW and LV mass and decreased CO relative to \textit{Wars2}^{+/+}, \textit{Wars2}^{+/-V117L}, and \textit{Wars2}^{+/-} mice at the same age, regardless of \textit{Tchh} genotype (Figure 3D), confirming that the \textit{Wars2}^{V117L} allele was the causal mutation for hypertrophic cardiomyopathy.

\textbf{Wars2}^{V117L/V117L} Mice Did Not Show Gross Brain Pathology

We carried out additional pathology screens to investigate whether there were neurological abnormalities, as reported in patients. On light microscopic examination of the brain (multiple sections of cerebrum and cerebellum) (data not shown) there were no detectable morphological differences between homozygote and wild-type animals (n = 3 of each) of the same age (approximately 7 months) and sex (male). In particular, there was no evidence of myelin deficits. There was also no evidence from visual welfare observation of an \textit{in vivo} neurological phenotype (seizures, tremors, or changes in locomotion), the detection of which often precedes detectable morphological changes at the light-microscopy level. Detection of potential subtle neurological changes would require behavioral or neurophysiological testing, which was beyond the scope of this study.

\textbf{The \textit{Wars2} c.349G>T Mutation Disrupted Exon Splicing and Caused Tissue-Specific \textit{WARS2} Deficiencies}

The \textit{Wars2} c.349G>T lesion causes a missense substitution (p.V117L) in the encoded protein. \textit{In silico} prediction of the functional effects of the p.V117L missense substitution did not
predict that it was deleterious. However, the mutated nucleotide is the first coding nucleotide of exon 3, and the NetGene2 splice site prediction program indicated that substitution of G to T at the first nucleotide of the third exon of \textit{Wars2} would affect the efficiency of exon 3 splicing (Figure 4A) (Hebsgaard et al., 1996).

We modeled the predicted consequence of exon 3 skipping and found that three α helices, required for substrate binding and release, are missing, which would likely lead to a loss of WARS2 function (Figure S1F). To test the prediction of exon skipping \textit{in vivo}, RT-PCR analysis of cochlear RNA derived from wild-type, heterozygous mutant, and homozygous mutant mice was undertaken (Figure 4B). This showed the \textit{Wars2}V117L allele, c.349G \textgreater{} T, caused in-frame skipping of exon 3. However, the mutation does not abolish normal splicing, and some full-length transcript is still produced (Figure 4B). Although the full-length transcript was severely decreased in homozygotes, the small amount still produced would generate mitochondrial \textit{Wars2} tryptophanyl-tRNA synthetase 2 protein (mtTrpRS) (with the p.V117L substitution) and explains why \textit{Wars2}V117L/V117L mutants were viable, unlike \textit{Wars2} \textsuperscript{-/-} nulls (Table S1).

We then investigated changes in RNA splicing using three TaqMan probes targeting \textit{Wars2} 2/3, 4/5, and 5/6 exon-exon boundaries in tissues (Figure 4C). Exon 4/5 and exon 5/6 probes, present in all transcripts, were significantly reduced in the heart, kidney, and BAT and were unchanged in the other tissues (Figure 4C). To determine the effect of these differences on WARS2 steady-state protein levels, tissues from \textit{Wars2}V117L/V117L mice were analyzed by immunoblotting. Consistent with the RNA results, WARS2 protein was significantly decreased in heart, liver, kidney, skeletal muscle, iWAT, and BAT of \textit{Wars2}V117L/V117L mice (Figure 4D; Figure S5A).

The \textit{Wars2} c.349G>T Mutation Caused Tissue-Specific OXPHOS Deficiencies

To determine the functional effects of reduced WARS2 protein in the mitochondria, steady-state levels of mitochondrial oxidative phosphorylation (OXPHOS) components (complex I–CV) were quantified by immunoblotting in each tissue at 12 months of age (Figures 4E–4J; Figure S5A). Consistent with the RNA results, WARS2 protein was significantly decreased in heart, liver, kidney, skeletal muscle, iWAT, and BAT of \textit{Wars2}V117L/V117L mice (Figure 4D; Figure S5A).
oxidoreductase subunit B8 (NDUFB8) and CIV (mitochondrially encoded cytochrome c oxidase I [MTCO1]) protein levels were significantly lower in \( \text{Wars2}^{V117L/V117L} \) heart (Figure 4E; Figure S5A), liver (Figure 4F; Figure S5A), and BAT (Figure 4G; Figure S5A). Furthermore, respiratory complex activity measurements in heart showed decreased CI and CIV activities (Figures S5B and S5C). In addition, CIII (ubiquinol:cytochrome c reductase core protein 2 [UQCRC2]) steady-state protein levels were decreased in \( \text{Wars2}^{V117L/V117L} \) BAT, showing more profound inhibition of mitochondrial translation in BAT compared to heart or liver (Figure 4G; Figure S5A). By comparison, no differences in CI (succinate:ubiquinone oxidoreductase complex flavoprotein subunit A [SDHA]), CIII, and CV (ATP synthase F1 subunit alpha [ATPS vibrant] subunit protein levels were observed in \( \text{Wars2}^{V117L/V117L} \) heart or liver; CV subunit levels were mildly increased in \( \text{Wars2}^{V117L/V117L} \) BAT relative to wild-type controls (Figure S5A).

Despite a severe loss of WARS2 protein, OXPHOS subunit steady-state protein levels remained largely unchanged in the \( \text{Wars2}^{V117L/V117L} \) kidney, with only a significant reduction in CV and a trend to mildly reduce CI observed at 12 months of age (Figure 4H; Figure S5A). Furthermore, immunoblot analysis showed a significant increase in CI and a trend toward increased CII (unadjusted \( p = 0.029 \)) steady-state OXPHOS protein levels in \( \text{Wars2}^{V117L/V117L} \) iWAT, despite decreased WARS2 protein (Figure 4I; Figure S5A). \( \text{Wars2}^{V117L/V117L} \) skeletal muscle showed no consistent respiratory chain deficiencies (Figure 4J; Figure S5A), although CII steady-state protein levels appeared to be increased in skeletal muscle. This was confirmed in skeletal muscle by measurement of respiratory chain complex activities, which also showed a significant increase in CII activity (Figure S5C).

Given the observation of brain pathology in patients, we also determined steady-state WARS2 and OXPHOS components in brains of mice 3–5 months of age and observed clear reduction of WARS2 protein and complex I subunit deficiency and a trend toward CI deficiency (Figures S5D–S5F), indicating that the brain is not spared.
Figure 4. \( \text{Wars}^{2V117L} \) Allele Increases Exon Skipping, Causing Tissue-Specific \( \text{WARS2} \) and Mitochondrial Respiratory Chain Deficiencies

(A) \( \text{Wars}^{2} \) c.349G > T NetGene2 splice site prediction.

(B) RT-PCR of RNA extracted from cochleae of \( \text{Wars}^{2/+} \), \( \text{Wars}^{2V117L/+} \), and \( \text{Wars}^{2V117L/V117L} \) mice using oligonucleotide primer pairs designed to exons 1 and 5. Products were sequenced and contain the exons indicated.

(C) Relative mRNA Expression

(D) Antibody probe key:
- Complex I (CI): NADH:ubiquinone oxidoreductase Subunit B8 (NDUF8)
- Complex II (CII): Succinate Dehydrogenase Complex Flavoprotein Subunit A (SDHA)
- Complex III (CIII): Ubiquinol-cytochrome c oxidoreductase Core Protein II (UQCRCC2)
- Complex IV (CIV): Mitochondrially-encoded cytochrome c oxidase I (MTCO1)
- Complex V (CV): ATP Synthase, H+ Transporting, Mitochondrial F1 Complex, Alpha Subunit 1, Cardiac Muscle (ATPSA)

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**Wars2V117L/V117L Mice Show Browning of WAT and Dysfunctional BAT Pathology**

To investigate the functional and contrasting differences in iWAT and BAT for CI, CIII, and CIV subunit steady-state levels, we carried out histological analysis at 12 months of age in males. iWAT showed qualitatively higher multi-locular lipid droplet formation indicative of browning, although this was also observed to a lesser extent in wild-type mice. Visceral gWAT appeared relatively normal (Figures 5A and 5B). Similar patterns were seen in females at 3 months of age (data not shown). Gene expression analysis showed significant upregulation of the key browning markers uncoupling protein 1 (Ucp1), iodothyronine deiodinase 2 (Dio2), and cell death-inducing DNA fragmentation factor subunit alpha (DFFA)-like effector a (Cidea) (Figure 5D) and immunoblot analysis showed increased UCP1 protein levels (Figure 5E) in Wars2V117L/V117L-iWAT, showing activation of browning pathways. Furthermore, nuclear-encoded mitochondrial respiratory chain cytochrome c oxidase subunit 7B (Cox7b) and cytochrome c oxidase subunit 8A (Cox8a) mRNA were significantly increased in Wars2V117L/V117L-iWAT (Figure 5D), showing transcriptional upregulation, consistent with the increased respiratory chain subunit protein levels shown earlier (Figure 4I).

Conversely, in BAT, males at 12 months (Figure 5C) and females at 3 months (data not shown) showed strikingly increased unilocular lipid droplet formation, indicative of inhibition of lipolysis and β-oxidation, and reduced BAT thermogenic function. In keeping with these observations, gene expression analysis showed downregulation of browning markers Ucp1, Dio2, and Cidea; and peroxisome proliferator-activated receptor gamma (Pparγ) (Figure 5F) and immunoblot analysis showed reduced UCPI protein (Figure 5G) in males at 12 months, consistent with BAT dysfunction. Nuclear-encoded mitochondrial respiratory chain subunit Cox7b and Cox8a mRNA expression levels were also decreased (Figure 5F), showing transcriptional downregulation, consistent with the respiratory chain dysfunction shown earlier (Figure 4G).

Given the abnormal BAT pathology and tissue-specific respiratory chain dysfunction observed, we carried out indirect calorimetry using a comprehensive laboratory animal monitoring system (CLAMS) at 4 months of age at 22°C (home cage temperature well below ~28°C thermoneutrality). Energy expenditure (EE) was significantly reduced in female Wars2V117L/V117L mice (Figure 5H), consistent with the observed abnormal BAT pathology and tissue-specific respiratory chain dysfunction.

**Upregulation of Mitochondrial Biogenesis Ameliorated Mitochondrial Respiratory Chain Dysfunction in Wars2V117L/V117L MEFs, Skeletal Muscle, and iWAT**

We further examined the effects of Wars2-V117L on mitochondrial dysfunction in Wars2V117L/V117L mouse embryonic fibroblasts (MEFs), which were cultured and assayed using microscale oxygraphy (Figures 6A and 6B). Unexpectedly, Wars2V117L/V117L-MEFs showed significantly increased basal respiration and ATP production compared to wild-type MEFs (Figures 6A and 6C), indicative of increased mitochondrial respiratory chain function. There was no difference in glycolysis as measured by extracellular acidification rate (ECAR) (Figure 6B). We hypothesized that this could be due to increased mitochondrial mass and upregulation of mitochondrial biogenesis. To directly measure mitochondrial mass, MEFs were stained with MitoTracker green, which localizes to mitochondria in live cells independent of mitochondrial membrane potential, and the average fluorescence per cell (30,000 cells per sample) was quantified by fluorescence-activated cell sorting (Figure 6D). We found that the average fluorescence intensity increased 40% in Wars2V117L/V117L-MEFs relative to Wars2+/+ controls showing increased mitochondrial mass (Figure 6D). Consistent with this, the master regulator of mitochondrial biogenesis, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (Pgc1α), was significantly upregulated in Wars2V117L/V117L-MEFs (Figure 6E).

MEFs are derived from the mesenchyme stem cell lineage. We hypothesized that mature tissues composed primarily of cells derived from the mesenchymal stem cell lineage, such as myocytes and adipocytes (skeletal muscle and iWAT, respectively), upregulate Pgc1α and mitochondrial biogenesis to prevent respiratory chain dysfunction in Wars2V117L/V117L mice. Gene expression analysis showed Pgc1α increased, on average, 3.3- and 4.3-fold in Wars2V117L/V117L skeletal muscle and iWAT, respectively, at 12 months of age (Figure 6F), showing transcriptional upregulation of mitochondrial biogenesis, consistent with the increased respiratory chain subunits observed previously (Figures 4I and 4J). No significant differences in Pgc1α expression were observed in other tissues (Figure 6F).

These data show Pgc1α is upregulated in Wars2V117L/V117L tissues displaying increased respiratory chain subunit levels, such as iWAT, indicating upregulation of mitochondrial biogenesis prevented respiratory chain dysfunction. Furthermore, Pgc1α is not upregulated in Wars2V117L/V117L heart or BAT, in which respiratory chain dysfunction and disease pathology were observed, or in kidney, in which respiratory chain subunit levels are comparable with controls. Transcription factor A, mitochondrial (Tfam), required for transcription and associated with mtDNA copy number, was reduced in heart and BAT (Figure 6G).
In addition, peroxisome proliferator-activated receptor alpha (Pparα) expression was increased in skeletal muscle and iWAT of Wars2\(^{V117L/V117L}\) mice, consistent with increased Pgc1α expression (Figure 6H). Conversely, Pparα was significantly decreased in the heart and BAT of Wars2\(^{V117L/V117L}\) mice and was unchanged in liver and kidney (Figure 6H). Overall, these data show that the tissue-specific respiratory chain dysfunction observed is partly because of the tissue-specific capacity for upregulation of Pgc1α and compensatory mitochondrial biogenesis.

Heart-Specific Activation of the ISR Caused Increased Plasma FGF21 and Systemic Changes in Metabolism

Fasted plasma FGF21 protein levels showed a trend toward elevation in male Wars2\(^{V117L/V117L}\) mice relative to controls at 12 months (Figure 7A) and similarly, but reaching significance, at 4 months of age in males only (Figures S6A and S6B). Plasma clinical chemistry analysis showed unchanged plasma free fatty acid levels and plasma glucose levels (Figures S6C and S6D), trends for reduced plasma triglycerides (Figure S6E), and markedly increased plasma ketone bodies (\(\beta\)-hydroxybutyrate) (Figure S6F) in Wars2\(^{V117L/V117L}\) mice. Furthermore, intraperitoneal glucose tolerance tests (IPGTTs) demonstrated increased glucose tolerance relative to wild-type controls (Figure S6G). FGF21 has previously been shown to reduce body weight by stimulating WAT lipolysis, induce temperature-dependent browning of WAT (Fisher et al., 2012), increase glucose tolerance by increasing insulin-independent glucose uptake in WAT and skeletal muscle (Kharitonenkov et al., 2005; Mashili et al., 2011), and increase hepatic ketogenesis (Inagaki et al., 2007). Thus, our findings, together with the reduced adiposity phenotype (Figure 2) and increased WAT browning observed previously (Figures 5A and 5B), align with the known effects of FGF21 on systemic metabolism and implicate FGF21 as the cause of metabolic phenotypes observed in Wars2\(^{V117L/V117L}\) mice.

FGF21 has previously been shown to be transcriptionally regulated via independent pathways governed by ATF4 and PPARα (Inagaki et al., 2007; Kim et al., 2013). To determine the mechanism of increased plasma FGF21, gene expression analysis was performed in Wars2\(^{V117L/V117L}\) mice at 12 months of age. Fgf21 expression was significantly increased in heart, skeletal muscle, and iWAT (Figure 7B). No difference was observed in other tissues (Figure 7B). A significant reduction in Atf4 expression was observed in BAT, but not in other tissues (Figure 7B). However, regulation of ATF4 at the protein level is key in Fgf21 regulation. In other tissues, such as skeletal muscle and iWAT, an alternate
mechanism governed by Pparα could contribute to the increased plasma FGF21 observed in Wars2V117L/V117L mice (Figure 6H).

Upon various cellular stresses, the ISR is activated by phosphorylation of eukaryotic translation initiation factor 2A (eIF2α), resulting in reduced cytoplasmic 5′ cap-dependent protein synthesis and preferential translation of mRNAs that contain upstream open reading frames in their 5′ UTR, such as ATF4 (Lu et al., 2004). ATF4 has been shown to transcriptionally regulate stress response genes, including Atf5, DNA damage-inducible
Figure 7. Heart-Specific Activation of the Integrated Stress Response Causes Increased Plasma FGF21 and Systemic Changes in Metabolism

(A) Relative plasma FGF21 protein levels in male mice at 12 months of age. Wars2^{V117L/V117L} and Wars2^{+/+} animal numbers were 5 and 9, respectively; mean ± SD. Unpaired t test. 
(B and C) Relative mRNA expression levels of (B) Fgf21 and (C) Atf4 in tissues from male mice at 12 months of age. Wars2^{V117L/V117L} and Wars2^{+/+} animal numbers were 5 and 5, respectively; mean ± SD. Data were log transformed and analyzed using t tests or a Mann-Whitney test (Fgf21 in heart and Atf4 in skeletal muscle and iWAT). Fgf21 RNA expression is very low in kidney and in wild-type skeletal muscle: mean CT > 33. 
(D and E) Immunoblot analysis (D) and quantification (E) of p-eIF2a and total EIF2a protein levels in heart, liver, kidney, and skeletal muscle from female mice at 12 months of age. Wars2^{V117L/V117L} and Wars2^{+/+} animal numbers were 3 and 3, respectively; bands were normalized to tubulin and expressed relative to wild-type as the mean ± SD. Significance was determined using an unpaired t test with Welch’s correction.

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transcript 3 (C/EBP homologous protein [Chop]), and Fgf21 (De Sousa-Coelho et al., 2012). We hypothesized that the Wars2-V117L allele caused cardiac-specific activation of the ISR pathway, leading to increased Fgf21 expression and activation of stress response pathways. To assess activation of the ISR, we performed immunoblot analysis of phosphorylated eukaryotic translation initiation factor 2α (p-eIF2α) levels in Wars2V117L/V117L heart, liver, kidney, and skeletal muscle at 12 months of age (Figures 7D and 7E). We found that p-eIF2α levels were significantly increased in heart (average of 6.1-fold) compared to controls (Figure 7E). Conversely, no significant differences in p-eIF2α levels were observed in kidney, liver, or skeletal muscle (Figure 7E). Furthermore, ISR pathway genes such as Atf5 and Chop were significantly increased at the mRNA level in the heart of Wars2V117L/V117L mice (Figures 7F and 7G). However, no significant differences in Atf5 or Chop were observed in liver, kidney, skeletal muscle, or IWAT (Figures 7F and 7G). Altogether, these data show robust tissue-specific activation of the ISR in the heart of Wars2V117L/V117L mice.

**Progressive Activation of the ISR Is Coincident with CI Deficiency and Independent of Disrupted Mitochondrial Proteostasis in the Heart of Wars2V117L/V117L Mice**

Disrupted mitochondrial proteostasis, rather than respiratory chain dysfunction, was the primary stress caused by inhibition of mitochondrial translation, leading to ATF4-dependent FGF21 expression and systemic changes in metabolism in the heart of Wars2-KOChmm mice (Dogan et al., 2014). To characterize activation of the ISR in the heart, we performed time course immunoblot analysis of the ISR marker p-eIF2α, CI and CIV OXPHOS subunits, and mitochondrial proteostasis markers caseinolytic mitochondrial matrix peptidase proteolytic subunit (CLPP), Lon peptidase 1, mitochondrial (LONP1), heat shock protein family D (Hsp60) member 1 (HSP60), and mitochondrial heat shock protein family A (Hsp70) member 9 (mHSP70) at 1, 3, and 12 months of age (Figures S7A–S7C). At 1 month, no differences in p-eIF2α, CI (NDUF8B), or CIV (COX1) steady-state protein levels were observed (Figure S7A). At 3 and 12 months of age, p-eIF2α levels were increased (Figure 7E; Figures S7B and S7C). At 3 and 12 months of age, CI and CIV levels were decreased (Figures S7B and S7C). Altogether, these data show that activation of the ISR and respiratory chain dysfunction occurred after 1 month of age in the heart of Wars2V117L/V117L mice and that activation of the ISR was progressive, with age from 3 to 12 months, and coincident with progressive CI deficiency. CI deficiencies were comparable between 3 and 12 months of age, showing no progressive further deficiency.

Finally, we found no significant differences in the steady-state protein levels of LONP1, CLPP, HSP60, or mHSP70 at any time point measured in the heart of Wars2V117L/V117L mice with the exception of a mild reduction in LONP1 protein at only 1 month of age (Figures S7A–S7C).

**DISCUSSION**

Sensorineural hearing loss is a common feature of human mitochondrial disease, and mutations in LARS2, HARS2, and NARS2 have been shown to cause sensorineural hearing loss (Pierce et al., 2011, 2013; Simon et al., 2015). Reduced body mass is also associated with mitochondrial diseases (Wolny et al., 2009). Furthermore, mutations in genes encoding critical proteins of the mitochondrial translation system, including MTO1, GTBP3, and ELAC2 (Baruffini et al., 2013; Haack et al., 2013; Koppjich et al., 2014) and several mt-aaRS mutations in AARS2, PARS2, SARS2, and YARS2, have been shown to cause hypertrophic cardiomyopathy (Belostotsky et al., 2011; Götz et al., 2011; Riley et al., 2010; Rivera et al., 2013; Shahni et al., 2013; Sofu et al., 2015). Individuals with compound heterozygous WARS2 mutations showing neurological problems have been reported (Burke et al., 2018; Musante et al., 2017; Theisen et al., 2017; Vantroys et al., 2018; Wortmann et al., 2017). We demonstrate that hypomorphic Wars2 alleles, Wars2V117L/C0 and Wars2V117L/V117L, which do not have direct genocopies in humans, cause sensorineural hearing loss, reduced adiposity, and hypertrophic cardiomyopathy in mice. However, we have not observed gross neurological effects during welfare observations or morphological differences using light microscopy of brain sections. This may reflect the severity of the alleles described so far in patients, in comparison with this mouse hypomorphic allele, and possible species differences.

Genetic mapping in the rat for coronary flow and capillary density traits in the heart identified a causal missense variant in Wars2 that reduced WARS2 activity by ~40% (Wang et al., 2016). It was also shown that the mutation reduced endothelial cell proliferation and activated pro-apoptotic pathways, as well as impairing BAT function (Praveneck et al., 2017; Wang et al., 2016). Finally, Wang et al. (2016) demonstrated that Wars2 is a critical pro-angiogenic factor in zebrafish. We have not carried out an analysis of coronary vasculature in the Wars2V117L/V117L model, although we observed BAT dysfunction.

As in our model, human hypomorphic mt-aaRS mutations cause tissue-specific pathology and respiratory chain dysfunction in humans, although the tissue-specific mechanisms remain unknown. However, increased respiratory chain subunits observed in IWAT of Wars2V117L/V117L were associated with upregulation of Pgc1α mRNA expression. Furthermore, we showed increased Pgc1α expression, mitochondrial mass, and function in Wars2V117L/V117L MEFs. We suggest that Pgc1α is upregulating mitochondria mass, preventing impaired respiratory chain function. In support of this, targeting Pgc1α to upregulate mitochondrial biogenesis, via therapeutic administration or genetic manipulation, can alleviate disease traits and increase mitochondrial respiratory capacity in human patient cell lines and mouse models (Basin et al., 2008; Khan et al., 2014). Altogether, these data indicate that tissue-specific upregulation of
possibility that we showed that activation of the ISR was independent of UPRmt (chel et al., 2015). Furthermore, activation of the ISR due to doxycycline treatment depended on the eIF2α chelation (Wall et al., 2015). Some effects of FGF21, such as in fat, could also be through an autocrine or paracrine mechanism, as reported in thermogenic recruitment of WAT (Fisher et al., 2012). FGF21, a biomarker of mitochondrial translation defects in human, likely has a beneficial role in tissues such as WAT by upregulating browning and mitochondrial biogenesis, providing some explanation for the tissue-specific respiratory chain deficiencies observed in \( \text{Wars}^{V117L/V117L} \) mice.

Common single-nucleotide polymorphisms, such as rs984222, with an effect allele frequency of 0.635, are associated with a 45% reduction in \( \text{WARS2} \) RNA expression in multiple tissues, including adipose (GTExPortal, http://www.gtexportal.org/home/). These single-nucleotide polymorphisms (SNPs) are associated with the waist-hip ratio in human or mouse association studies, which are explained by changes in adipose tissue distribution (Heid et al., 2010). Our studies support the possibility that \( \text{WARS2} \) is one of the effector genes in this association locus (Pravenec et al., 2017).

Oxidative stress, mitochondrial unfolded protein response (UPR\( ^{\text{mt}} \)), inhibition of mitochondrial translation, and respiratory chain dysfunction are linked to activation of the ISR (Baker et al., 2012; Kim et al., 2013; Michel et al., 2015; Rath et al., 2012). Dogan et al. (2014) showed that knocking out Dars2 caused tissue-specific activation of the UPR\( ^{\text{mt}} \), leading to ATF4-dependent Fgf21 expression in the heart of \( \text{Dars}^{2\text{Kocmm}} \) mice before respiratory chain dysfunction and concluding that mitochondrial proteostasis was the primary stress. We also demonstrated that activation of the ISR was a cardiac-specific response to inhibition of mitochondrial translation in \( \text{Wars}^{V117L/V117L} \) mice, resulting in increased Fgf21 gene expression. However, in contrast with the Dogan et al. (2014) findings, we showed that activation of the ISR was independent of UPR\( ^{\text{mt}} \) activation, was progressive with age, and was coincident with progressive CI respiratory chain deficiency. Several studies have demonstrated activation of the ISR upon progressive respiratory chain deficiency independent of activation of the UPR\( ^{\text{mt}} \). Inhibition of expression of the mitochondrial genome via mtDNA depletion or inhibition of mitochondrial translation through doxycycline treatment caused respiratory complex deficiencies and activation of the ISR independent of UPR\( ^{\text{mt}} \) activation \( \text{in vitro} \) (Michel et al., 2015). Furthermore, activation of the ISR due to doxycycline treatment depended on the elf2α kinase GCN2 (Michel et al., 2015). Activation of the ISR in the heart of \( \text{Wars}^{V117L/V117L} \) mice is thus due to progressive respiratory chain dysfunction, is independent of the UPR\( ^{\text{mt}} \), and may occur via GCN2-dependent phosphorylation of elf2α.

We conclude that inhibition of mitochondrial translation can cause ISR activation via alternate mechanisms that depend upon the degree of mitochondrial translation inhibition. We speculate that complete inhibition of mitochondrial translation, e.g., via \( \text{Wars}^{V117L/V117L} \) heart, causes activation of the ISR due to respiratory chain dysfunction and loss of mitochondrial membrane potential. The failed ability of the ISR to attenuate mitochondrial proteostatic stress likely explains the increased severity of the cardiac phenotype observed in \( \text{Dars}^{2\text{Kocmm}} \) mice that cannot survive beyond 6 weeks of age compared to \( \text{Wars}^{V117L/V117L} \) mice.

In summary, we have generated a key mouse model for studying tissue-specific deficits in mitochondrial protein translation, linking phenotypes and mechanisms and offering the potential for therapeutic testing.
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AUTHOR CONTRIBUTIONS
Conceptualization, T.A., M.G., J.P., K.J.M., S.D.M.B., R.W.T., M.R.B., and
H.H., C.E., and Y.W.; Validation, Y.W.; Formal Analysis, T.A., M.S., R.D.C.,
and M.R.B.; Writing, Reviewing, and Editing, T.A., R.W.T., M.R.B., and
R.D.C.

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Mouse Phenotyping Consortium; Jackson Laboratory; Infrastructure Natio-
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# STAR METHODS

## KEY RESOURCES TABLE

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**Chemicals, Peptides, and Recombinant Proteins**

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### Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Roger Cox (r.cox@har.mrc.ac.uk).

### Deposited Data

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### Experimental Models: Cell Lines

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### Experimental Models: Organisms/Strains

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### Software and Algorithms

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Animal Models
All mice used in this study were housed in the Mary Lyon Centre at MRC Harwell. Mice were kept and studied in accordance with UK Home Office legislation and local ethical guidelines issued by the Medical Research Council (Responsibility in the Use of Animals for Medical Research, July 1993; Home Office license 30/3146 and 30/3070). Procedures were approved by the MRC Harwell Animal Welfare and Ethical Review Board (AWERB). Mice were kept under controlled light (light 7am–7pm, dark 7pm–7am), temperature (21 ± 2 °C) and humidity (55 ± 10%) conditions. They had free access to water (9–13 ppm chlorine) and were fed ad libitum on a commercial diet (SDS Rat and Mouse No. 3 Breeding diet, RM3, 3.6 kcal/g).

MPC-151 pedigree was generated from The Harwell Aging ENU-mutagenesis Screen as documented previously (Potter et al., 2016). These mice are C57BL/6J mutagenized mice crossed with C3H/Pde (Pde6b+ repaired mice) and subsequently maintained by backcrossing to C3H/Pde mice. Age and sex of mice is indicated in the figure legends. Estimates for required cohort sizes were made using GraphPad Statmate using trait data from previous experiments.

Cohorts of male and female mice were bred for longitudinal blood and body composition-based phenotyping tests. Four cohorts of mice Wars2V117L/+ mice were generated from Wars2+/V117L x Wars2+/V117L matings and were aged to 1- (20 mice total), 3- (58 mice total), 9- (74 mice total) and 12 months (58 mice total) of age before being humanely killed in accordance with Home Office schedule 1 regulations. Wars2V117L/- mice were generated from Wars2+/– x Wars2+/V117L/- matings (78 mice total). Ppa2Y123F/Y123F mice were generated from Ppa2+/V123F x Ppa2+/V123F matings (47 mice total). Mice were randomly assigned to cages at weaning before subsequent genotyping of individual mice. Downstream phenotyping experiments were performed blinded to the genotype of the mice.

For body composition three cohorts were analyzed, two with multiple time points and one at 1 month only. In the first cohort one wild-type mouse was humanely killed because it was sick and one found dead and all data from these animals was excluded. Final cohort sizes were Wars2+/+ n = 11 and 3, Wars2+/V117L n = 13 and 17, Wars2+/V117L/+ n = 7 and 8, male and female respectively. In the second cohort data from one homozygous mouse was excluded after being found dead before the 6-month time-point and two heterozygous mice humanely killed to reduce cage numbers prior to starting phenotyping. Final cohort sizes were Wars2+/+ n = 13 and 18, Wars2+/V117L+ n = 19 and 19, Wars2+/V117L/V117L n = 11 and 7, male and female respectively. In cohort 3 there were Wars2+/+ n = 4, Wars2+/V117L n = 15, Wars2+/V117L/+ n = 9 and none of the differences for body weight, fat mass or lean mass were significant (tested at one month only).

An additional fifth intercross cohort, congenic on C3H/Pde, was generated for additional replication experiments including OXPHOS blots and FGF21 measurements in plasma at 3-4 months.

The NIH KOMP Wars2-KO allele (Wars2^{gml(KOMP)Vlcg}) obtained from the KOMP repository (https://www.komp.org/) comprises a targeting construct integrated into the C57BL/6N ES cell genome by homologous recombination, deleting 46632bp of the Wars2 gene locus, including coding regions of both Wars2-Exon1 and Wars2-Exon2, leading to a frameshift and a premature stop codon. Wars2^{gml(KOMP)Vlcg} ES cells were micro-injected into C57BL/6N blastocysts generating mosaic C57BL/6N-Wars2^{gml(KOMP)Vlcg} offspring. Germ-line transmission (GLT) of the Wars2^{gml(KOMP)Vlcg} construct was determined by genotyping C57BL/6N-Wars2^{gml(KOMP)Vlcg} x C57BL/6N offspring for the neomycin selection cassette.

Primary Cultures
MEFs were harvested from E12.5-14 (dpc) embryos from timed Wars2+/V117L x Wars2+/V117L matings and dissected on ice in Dulbecco’s PBS (ThermoFischer 14190094). The sex of the embryos was unknown. Head, liver, heart and limbs were placed in 3ml GIBCO 0.25% trypsin (EDTA) (ThermoFischer 25200056) and minced using surgical scissors and then pipetted 10x using a P1000 pipette and sterile filter tip, before transfer to a 15ml Falcon tube and incubated at 37°C for 10 minutes. The trypsin was neutralized with 7ml of culture medium, DMEM (ThermoFischer 31966021) supplemented with 1 X NEAA (Sigma-Aldrich M7145), 1 X Penicillin/Streptomycin (ThermoFischer 15070-063), 50 μM 2-mercaptopethanol (2-mercaptopethanol (ThermoFischer 31350010) and 10% GIBCO FBS (ThermoFischer 10500064). Cells were then plated on a 10cm dish and incubated at 37°C and 5% CO2.

METHOD DETAILS
SNP Mapping and Whole Genome Sequencing
SNP mapping and NGS were performed as described previously (Potter et al., 2016). Briefly individual mutations were mapped using the Illumina GoldenGate Mouse Medium Density Linkage Panel (Gen-Probe Life Sciences Ltd, UK) that utilizes over 900 SNPs for the C3H/Pde (Pde6b+ repaired mice) and C57BL/6J strains. The genotypes of G3 ‘affected’ (elevated ABR thresholds) MPC-151 mice were compared to ‘non-affected’ (‘normal’ ABR thresholds) littermate controls. This allowed us to identify a 75Mb region within which all ‘affected’ MPC-151 mice were homozygous for C57BL/6J SNPs and ‘non-affected’ MPC-151 littermates were either heterozygous or homozygous for C3H.Pde6b+ SNPs. To identify candidate causal ENU-induced mutations within the mapped region, WGS was performed using DNA from an ‘affected’ G3 MPC-151 mouse. WGS was performed as previously described (Potter et al., 2016). Briefly, following DNA extraction a library was generated and a single lane or paired-end sequencing (100nt) was performed using the Illumina HiSeq platform (Oxford Genomics Centre, Wellcome Centre for Human Genetics). The 100nt paired-end
reads were aligned to the reference mouse genome (NCBIM38/mm10) using Burrows-Wheeler Aligner software (Li and Durbin, 2009). Single-nucleotide variants (SNVs) were identified for each alignment using the unified GenotypeCaller tool in the Genome Analysis Toolkit (GATK) as previously described (Potter et al., 2016). Here the mouse dbSNP version 137 was used as the background SNP set using default parameters. Identified SNVs were then given a quality score (Phred scaled quality score, $-10 \times \log(1-p)$, $p$ is the probability of a SNV being called incorrectly). SNVs with a quality score of < 100 or with a read depth of < 3 reads were removed from all further analysis. All remaining SNVs were termed ‘high-confidence’ mutations and were compared to previously identified SNPs from 17 inbred strains from the Mouse Genome Project (Keane et al., 2011) as well as an in-house library of SNVs. Any overlapping sites were removed leaving the final list of novel ENU-induced SNVs for the ‘affected’ MPC-151 G3 mouse. SNVs were annotated using NGS-SNP to give an indication of the nature of the SNV (e.g., Missense, splice-site variant or intronic).

3 high-confidence, ENU-induced, missense mutations were identified for the MPC-151 G3 ‘affected’ mouse that were located within the 75Mb mapped region as previously identified.

**Genotyping**

Mice were assayed for the presence or absence of ENU-induced mutations $Ppa2^{A398T}$ and $Wars2^{G349T}$ by pyrosequencing (Potter et al., 2016). PCR primers were designed to amplify the regions of interest using a biotinylated primer for the Pyrosequencing template strand. $Ppa2^{A398T}$ primers: biotinylated forward (5'-CTCAATCCCCATTAGAAGAT-3') and sequencing reverse (5'-GGGAAGGTGTGTTGAGT-3'). $Wars2^{G349T}$ primers: forward (5'-GGGAGAGCTTCTCTCCTT-3') and biotinylated reverse (5'-CCAGGTAGATCCCACTTT-3') and forward sequencing (5'-TCTCCCTCTCTCTTTAG-3'). Mice generated from $Wars2^{V117L/-} \times Wars2^{/-}$ matings were genotyped using two strategies. The $Wars2^{V117L}$ allele was genotyped using the Idaho Technology LightScanner System (Idaho Technology Inc, Utah, USA) and was used in accordance with the manufacturers standard protocols. $Wars2^{V117L}$ Primers: forward (5'-TGCGGCTATCCTGTTGCTA-3') and reverse (5'-TGTTGAATGGTTCAATCT-3') and probe (5'-CTCTCCTTCTTTCTGATTGAACACACTCAG-3'). The $Wars2$-KO allele was genotyped for the presence of the LacZ reporter cassette using a RT-PCR copy number assay using FAM-labeled tagman probes. Assays were performed using FAM-labeled TaqMan probes for LacZ and $Wars2$-WT DNA sequences as controls. Each assay was performed along with an additional VIC-labeled TaqMan probe designed to Dot1l that acted as an internal controls. $Wars2^{WT}$ primers: forward (5'-GGCCAGACTTGGATGTG-3') and reverse (5'-GAGGCGCCTCACCAGT-3'), FAM labeled probe (5'-TCCCTTCCACCTTCTCGTCTC-3'). LacZ primers: forward (5'-CTCGGCAACTCAACAGTAC-3'), reverse (5'-TTAT CAGCAGGAAAACCTC-3'), FAM labeled probe (5'-TCGCCATTTGGCCACACTTAC-3'), Dot1l primers: forward (5'-GGCCAGACGACCATT-3'), reverse (5'-TAGTTGGCATCCTTATGTTTCAT-3') and VIC labeled probe (5'-CCAGCTCT CAAGTCG-3').

**Auditory phenotyping**

Click box protocol as previously described (Hardisty-Hughes et al., 2010). Briefly, mice were placed on the operator’s palm and hearing was tested with a purpose built frequency calibrated click box (CB) that emits a 90 dB SPL tone at 20 kHz (CB apparatus was obtained from MRC Institute of Hearing Research, Nottingham, UK). The CB emits a tone that elicits a Preyer reflex from the mouse as seen by a visible flick of the pinna or a startle response if the mouse can hear. The presence or absence of a Preyer reflex is then obtained from MRC Institute of Hearing Research, Nottingham, UK). The CB emits a tone that elicits a Preyer reflex from the mouse as seen by a visible flick of the pinna or a startle response if the mouse can hear. The presence or absence of a Preyer reflex is then obtained from MRC Institute of Hearing Research, Nottingham, UK). The CB emits a tone that elicits a Preyer reflex from the mouse as seen by a visible flick of the pinna or a startle response if the mouse can hear. The presence or absence of a Preyer reflex is then obtained from MRC Institute of Hearing Research, Nottingham, UK). The CB emits a tone that elicits a Preyer reflex from the mouse as seen by a visible flick of the pinna or a startle response if the mouse can hear. The presence or absence of a Preyer reflex is then obtained from MRC Institute of Hearing Research, Nottingham, UK). The CB emits a tone that elicits a Preyer reflex from the mouse as seen by a visible flick of the pinna or a startle response if the mouse can hear. The presence or absence of a Preyer reflex is then obtained from MRC Institute of Hearing Research, Nottingham, UK). The CB emits a tone that elicits a Preyer reflex from the mouse as seen by a visible flick of the pinna or a startle response if the mouse can hear. The presence or absence of a Preyer reflex is then obtained from MRC Institute of Hearing Research, Nottingham, UK). The CB emits a tone that elicits a Preyer reflex from the mouse as seen by a visible flick of the pinna or a startle response if the mouse can hear. The presence or absence of a Preyer reflex is then obtained from MRC Institute of Hearing Research, Nottingham, UK). The CB emits a tone that elicits a Preyer reflex from the mouse as seen by a visible flick of the pinna or a startle response if the mouse can hear. The presence or absence of a Preyer reflex is then obtained from MRC Institute of Hearing Research, Nottingham, UK). The CB emits a tone that elicits a Preyer reflex from the mouse as seen by a visible flick of the pinna or a startle response if the mouse can hear. The presence or absence of a Preyer reflex is then obtained from MRC Institute of Hearing Research, Nottingham, UK). The CB emits a tone that elicits a Preyer reflex from the mouse as seen by a visible flick of the pinna or a startle response if the mouse can hear. The presence or absence of a Preyer reflex is then obtained from MRC Institute of Hearing Research, Nottingham, UK).

**Body weight and composition analysis**

Body mass was measured monthly on scales calibrated to 0.01 g. Body composition was measured monthly using an Echo-MRI quantitative NMR machine (Echo-MRI-100, Echo-MRI, Texas, U.S.A.).

**Echocardiograms**

Mice were placed under general anesthetic using 4% isoflurane using an anesthetic chamber. Once unconscious the mouse was placed on an ECG platform (Visualsonics heatpad / ECG platform) and mouse limbs are taped to ECG probes to allow heart rate monitoring. Anesthesia was maintained using a nose cone and 1.5% (or as appropriate to maintain a heart rate < 400 bpm) isoflurane. Hair was removed from the mouse chest using hair clippers followed by hair removal cream. A rectal thermometer was inserted and used to monitor core body temperature throughout the procedure. Contact gel was applied to the shaven mouse chest and a 707B probe was lowered to the mouse chest locating the mouse heart left ventricle until contractions of the left ventricle could be monitored.
on the Visualsonics Vevo 770 high resolution in vivo micro imaging system. Several images of mouse heart were taken in M-mode and were analyzed using the Vevo 770 software. Following successful data capture, the rectal probe, contact gel and limb tape was removed and the mouse was placed in a heat box to recover from the anesthetic.

**Comprehensive Laboratory Animal Monitoring System**

The Comprehensive lab animal monitoring system (CLAMS) was used to measure mice energy expenditure at home cage temperature (22°C) according to standard protocols. Briefly mice were placed in individual cages for a total of 72 hours. Measurements of oxygen (O₂) and carbon-dioxide (CO₂) in-flow and out-flow concentrations were automatically monitored and recorded along with food consumption and water intake throughout the 72-hour period. Data from the first 24 hours was removed from the analysis as this period was used to allow the mice to aclimatize to their new environment. Data collected from the second 24-hour period was used for all subsequent analysis. Energy expenditure was calculated as follows EE = CV x VO₂ (where CV = Calorific value = 3.815 + 1.232 x RER and VO₂ = VO₂o - VoO₂i (o = outflow, i = inflow). EE values were normalized to lean mass using multiple linear regression analysis (ANCOVA) as described previously (McMurray et al., 2013).

**Intraperitoneal Glucose Tolerance Test (IPGTT)**

Mice were fasted overnight and IPGTT were performed the following morning. On the morning of the IPGTT, mice were weighed and a local anesthetic was administered to the mouse tail (EMLA cream, Eutectic mixture of Local Anesthetics Lidocaine / Prilocaine, AstraZeneca, UK). A blood sample was collected from the mouse tail at time point zero in Lithium-Heparin microvette tubes (CB30, Sarstedr, Numbrecht, Germany) to establish a baseline blood glucose level. Mice were then administered an intra-peritoneal injection of 2 g glucose / kg body weight (20% glucose in 0.9% NaCl). Blood samples were then taken 60 and 120 mins post-injection. At each time point, blood glucose levels were measured using the handheld Alphatrak (Abbott) glucose monitor with a fresh Alphatrak strip (Abbott) being used for every reading.

**Tissue collection**

Mice were humanely killed at 1-, 3-, 9- and 12 months of age and tissues were harvested for analysis. Following confirmation of death: cochlea, heart, liver, kidney, iWAT, gonadal which adipose tissue, BAT and skeletal muscle were dissected. For subsequent protein, RNA and DNA analysis tissues were placed in cryotubes (Nunc, Thermo Fisher Scientific-Heraeus) and snap frozen in liquid nitrogen. Tissue samples were stored long-term at −70°C.

**Blood Biochemistry and ELISA analysis**

Food was withdrawn and mice were fasted at 8:00 AM. 4hrs later, mice were humanely killed by administration of an over-dose of anesthetic (0.2 mL of pentobarbitone) via intra-peritoneal injection in accordance with home office procedures. Once the mouse was fully anaesthetized a glass capillary is inserted into the anterior corner of the mouse eye to puncture the membrane of the retro-orbital sinus. Blood was collected from the capillary in Lithium-Heparin microvette tubes (CB30, Sarstedr, Numbrecht, Germany) to establish a baseline blood glucose level. Mice were then administered an intraperitoneal injection of 2 g glucose / kg body weight (20% glucose in 0.9% NaCl). Blood samples were then taken 60 and 120 mins post-injection. At each time point, blood glucose levels were measured using the handheld Alphatrak (Abbott) glucose monitor with a fresh Alphatrak strip (Abbott) being used for every reading.

**Mitochondrial stress test in MEFs**

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured in MEFs using the Seahorse XF24 flux analyzer (Seahorse Bioscience). Primary MEFs were seeded at a density of 40000 cells/well on XF-24 tissue culture plate and left to adhere overnight. The following day MEFs media was replaced with XF Assay Media supplemented with L-glutamine 2 mM, sodium pyruvate 2 mM, and glucose 10 mM (pH 7.4) and were incubated for 1 hour at 37°C in a CO₂ free incubator before being placed in the XF24 analyzer. OCR and ECAR measurements were measured under basal conditions and following administration of mitochondrial inhibitors oligomycin (1 μM), antymycin (1 μM) and rotenone (1 μM) or in the presence of the mitochondrial uncoupler FCCP (1 μM) (Seahorse XF Cell Mito Stress Test Kit, Agilent). Oxygen consumption rates were normalized to the number of live cells using the LIVE/DEAD Viability/Cytotoxicity kit (ThermoFisher) according to the manufactures instructions.

**Respiratory chain complex activities**

The activities of individual respiratory chain complex activities and citrate synthase, a mitochondrial matrix marker, were determined in skeletal muscle and cardiac muscle homogenates as previously described (Kirby et al., 2007).

**Western blots analysis**

Proteins were extracted from snap frozen mouse tissues using CelLytic MT Mammalian Tissue Lysis Buffer (Sigma- Aldrich) supplemented with 1 X complete protease inhibitor cocktail (1 μL / 100 μL lysis buffer, Sigma- Aldrich) and 1 X PhosStop phosphatase inhibitor cocktail (1 μL / 100 μL lysis buffer, Sigma Aldrich). Tissues were homogenized using the Precellys-24 automated homogenizer (Bertin Technologies). Tissue homogenates were centrifuged at 13,000 rpm for 15 mins at 4°C to pellet cell debris. The supernatant
tissue lysates were isolated and protein concentrations were determined using the BCA (bicinchoninic acid) Protein Assay Reagent (BioRad). Samples were diluted to 4 μg / μL in lysis buffer and supplemented with NuPAGE LDS Sample Buffer (4X) and NuPage Reducing Agent (10X) and were denatured by heating to 70 °C for 10mins. Protein samples were separated using 4%–12% linear gradient Bis-Tris ready polyacrylamide gels with 1 X MOPS electrophoresis running buffer (Invitrogen) using the XCell Surelock Mini Cell tanks (Invitrogen) at 200 V for 50 mins. Protein samples were electrotransferred from the gels onto PVDF membrane (Hybond – P, GE Healthcare Amersham) using a XCell II Blot Module (Invitrogen). Protein membranes were blocked in 5% non-fat milk Tris Buffered Saline with Tween 20 (TBST, Merk) (non-phosphor antibodies) or 5% Bovine Serum Albumin TBST (phosphor-antibodies) at room temperature for an hour or overnight at 4 °C before being incubated with primary antibodies overnight at 4 °C. Protein membranes were washed 3-5 times in TBST for 10mins at room-temperature. Secondary antibodies were diluted in 5% non-fat milk TBST. Membranes were incubated with species-specific secondary horseradish peroxidase (HRP) conjugated antibodies for 4 hr at room-temperature. Membranes were washed 5 times in TBST for 10 mins. Immunolabelled membrane were treated with Enhanced Chemiluminescence Plus (ECL plus; Amersham, GE Healthcare) and were imaged using the ChemiDoc UV chemiluminescent imager or exposure to X-ray film.

Primary antibodies used in this study: WARS2, at a 1:500 dilution (custom, Covalab); NDUF8b, at a 1:2,000 dilution (ab110242, Abcam); SDHA, at a 1:10,000 dilution (ab14715, Abcam); UQCRC2, at a 1:3,000 dilution (ab14745, Abcam); MTCO1, at a 1,200 dilution (ab14705, Abcam); ATP5A, at a 1,500 dilution (ab14748, Abcam); eIF2a, at a 1,000 dilution (#5324, Cell signaling); phosphor-Ser51-eIF2a, at a 1:1,000 dilution (#1090-1, Epitomics); ATF4, at a 1:500 dilution (sc-22800, Santa Cruz); LONP1, at a 1,000 dilution (ab103809, Abcam); CLPP, at a 1,500 dilution (ab124822, Abcam); HSP60, at a 1,100 dilution (ab46798, Abcam); HSP70, at a 1,000 dilution (ab2799, Abcam); UCP1, at a 1,200 dilution (sc-6529, Santa Cruz); Actin, at a 1,500 dilution (MAB1501, Millipore); α-Tubulin, at a 1,500 dilution (#2144, Cell Signaling); and GAPDH, at a 1,10,000 dilution (ab8245, Abcam).

In subsequent OXPHOS Western blot experiments (Figure S5) a total OXPHOS rodent WB antibody cocktail was used at a 1:1,000 dilution (ab110413, Abcam).

**Real-Time Quantitative PCR**

Total RNA was extracted from MEFs and mouse tissues using the RNeasy Mini Plus Kit (QIAGEN) according to the manufacture’s protocol. RNA concentrations were determined using a NanoDrop spectrophotometer (Thermo Scientific). RNA samples were diluted to 200 ng/μl and reverse transcription reactions were performed using Super Script III reverse transcriptase (Invitrogen) following the manufacturer’s protocol to generate 2 μg of cDNA. mRNA gene expression analysis was performed using the TaqMan system. TaqMan Gene Expression Assay reagents and TaqMan FAM dye-labeled probes (Applied Biosystems, Invitrogen, U.S.A.) were used according to the manufacturers protocol and assays were performed using an ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems). Data was normalized to house-keeping genes specific to the tissue / cell line being used. GeNORM analysis was performed for each cell / tissue used to determine the most suitable housekeeping gene. Data were analyzed using the comparative ΔΔCT method in order to determine the difference in sample groups relative to control samples. Taqman probes used in this study: WARS2 (Exon 2-3) (Mm004208865_m1), Wars2 (Exon 4-5) (Mm004208867_m1), Wars2 (Exon 5-6) (Mm004208868_m1), Pgc1α (Mm01208835_m1), Atf4 (Mm00515324_m1), Atf5 (Mm00459515_m1), Chop (Mm001135937_g1), Ftg21 (Mm00840165_g1), Tiam1 (Mm00447485_m1), Pparα (Mm00440939_m1), Ucp1 (Mm01244861_m1), Dio2 (Mm00515664_m1), Cidea (Mm00432554_m1), Pparγ (Mm00440945_m1), Cox7a1 (Mm00438297_g1) and Cox8b (Mm00432648_g1).

**Prediction of WARS2 3D structure**

The crystal structure of human WARS2 (PDB: 5EKD, Human mitochondrial tryptophanyl-tRNA synthetase bound by indolmycin and Mn*ATP. Williams, T.L., Carter Jr., C.W.) was downloaded from the PDB database (PDB; http://www.rcsb.org/). The predicted protein structure of human Wars2 was generated using PHYRE2 Protein fold recognition server (Kelley et al., 2015). The alignment and visualization of the protein structures was performed by PyMOL by Schrödinger (https://pymol.org/2/).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical tests in GraphPad Prism are indicated in the figure legends and were selected depending on whether data was normally distributed as assessed by the D’Agostino & Pearson omnibus normality test in Prism. Equal variance was assessed by an F-test in Prism and non-parametric tests used if this test was failed. Where necessary AUC’s were calculated using Prism to allow analysis of longitudinal data. Number of animals and cellular assay replicates are indicated in the figure legends.

Western blot bands were analyzed and quantified using ImageJ (Schneider et al., 2012).

Energy Expenditure adjustment for lean mass by ANCOVA using SPSS.
Supplemental Information

A Wars2 Mutant Mouse Model Displays OXPHOS
Deficiencies and Activation of Tissue-Specific
Stress Response Pathways

Thomas Agnew, Michelle Goldsworthy, Carlos Aguilar, Anna Morgan, Michelle Simon, Helen Hilton, Chris Esapa, Yixing Wu, Heather Cater, Liz Bentley, Cheryl Scudamore, Joanna Poulton, Karl J. Morten, Kyle Thompson, Langping He, Steve D.M. Brown, Robert W. Taylor, Michael R. Bowl, and Roger D. Cox
Supplementary Figure 1. Related to Figure 1. Auditory phenotyping, SNP mapping, whole genome sequencing of mouse pedigree MPC151, non-segregation of the phenotype in PP2A mice and the crystal structure of WARS2.

A) Auditory brainstem response (ABR) phenotyping of pedigree MPC151 at 12-months of age showed 5 mice with elevated hearing thresholds (red triangles) at all the frequencies tested (8, 16 and 32 kHz) and for the click stimulus, compared to their normal-hearing littermates (n=48, black triangles).

B) Body weight phenotyping of male and female pedigree MPC-151 at 12-months of age shows that 5 mice with elevated hearing thresholds (male n=3, female n=2, red triangles) also exhibit low body weight, compared to normal-hearing littermates (male n=23, female n=26, black triangles).

C) DNA from the 7 mice exhibiting hearing loss (HL) at 9-months of age and 7 normal-hearing (H) littermates was analyzed by whole genome SNP mapping. The first column indicates the mouse identification numbers and the second column their respective phenotype. The genotype of each mouse is either homozygous for C57BL/6J (black) or C3H (white) or heterozygous (grey) for each marker. The analysis defined a ~73.3Mb critical interval on Chromosome 3 between markers rs6198234 and rs6407142 (Chr3:70361430-143619317, GRCm38).

D) Sanger sequencing of MPC-151 G1 founder and MPC-151 ‘affected’ G3 (MPC151/2.10g) at the shown locations corresponding to the three identified ENU-induced missense mutations on Chr 3: Tchh-c.3314A>T, Wars2-c.349G>T and Ppa2-c.398A>T. The MPC-151 G1 founder is heterozygous for all 3 missense mutations. The MPC-151 ‘affected’ G3 is homozygous for the three missense mutations.

E) Intercross cohort mice segregating the PPA2 Y123F mutation do not exhibit a phenotype. In subpanels (A) ABR at 6 months of age and (D-G) phenotyping over a 6 month time-course in male and female mice respectively for (B,E) body weight, (C,F) fat mass and (D,G) lean mass. In subpanel (A) PPA2Y123F/Y123F n=4, PPA2Y123F/+ n=3, PP2A+/- n=2, and in subpanels (B-G) male and female for PPA2Y123F/Y123F n=6 and n=5, for PPA2Y123F/+ n=12 and n=12 and for PP2A+/- n=7 and n=4 respectively. Time course data were analyzed with a 2-way ANOVA and Bonferroni correction for multiple testing and all comparisons were non-significant. Homozygous PPA2Y123F/Y123F are blue circles, heterozygous PPA2Y123F/+ are red squares and wildtype colony-mates are black triangles.

F) The crystal structure of Human WARS2 protein (pdb id: 5ekd) colored grey aligned to the predicted protein structure of WARS2 without exon 3 colored orange. The structural prediction of the deletion of exon 3 shows that three α-helices (a, b & c) are removed in the resulting predicted Wars2 protein structure. The two exon 3 encoded alpha helices are a and b. The catalytic domain, which comprises a Rossmann fold, holds three active sites; Class I PxxxHIGH and KMSKS active-site catalytic (ATP binding) motifs, colored magenta and green respectively, and the L-tryptophan binding site, in yellow. The catalytic motifs are not directly affected by the mutation. However, these three α-helices are part of the AARs catalytic domain which synthesizes aminoacyl adenylate and moves the amino acid to the anticodon binding domain (not shown).
Supplementary Figure 2. Related to Figure 1. \textit{Wars2}^{\text{V117L-}} compound heterozygous knockout phenotyping data.

A) Schematic diagram illustrating the generation of the KOMP \textit{Wars2}-KO allele (\textit{Wars2}^{\text{tm1(KOMP)Vlcg}}). The targeting construct integrated into the C57BL/6N ES cell genome by homologous recombination, deleting 46632bp of the \textit{Wars2} gene locus, including coding regions of both \textit{Wars2}-Exon1 and \textit{Wars2}-Exon2, leading to a frame-shift and a premature stop codon. \textit{Wars2}^{\text{tm1(KOMP)Vlcg}} ES cells were microinjected into C57BL/6N blastocysts generating mosaic C57BL/6N-\textit{Wars2}^{\text{tm1(KOMP)Vlcg}} offspring. Germine transmission (GLT) of the \textit{Wars2}^{\text{tm1(KOMP)Vlcg}} construct was determined by genotyping C57BL/6N-\textit{Wars2}^{\text{tm1(KOMP)Vlcg}} x C57BL/6N offspring for the neomycin selection cassette (data not shown). Once GLT was achieved C57BL/6N-\textit{Wars2}^{\text{tm1(KOMP)Vlcg}} mice were crossed with cre-recombinase expressing mice to remove the neomycin selection cassette. \textit{Wars2}^{\text{+/-}} indicates heterozygous mice with one wildtype allele (+) and one deleted allele (-).

B) Auditory brainstem response thresholds at 4-months of age were recorded at single frequencies: 8, 16 and 32kHz, and a click stimulus. \textit{Wars2}^{\text{+/-}}, \textit{Wars2}^{\text{V117L/+}} (\textit{Tchh}^{\text{D1105V/+}}), \textit{Wars2}^{\text{+/-}} and \textit{Wars2}^{\text{V117L-}} (\textit{Tchh}^{\text{D1105V/-}}) animal numbers 5, 1, 6 and 4 respectively. Data were analyzed using a 1-way ANOVA non-parametric Kruskal-Wallis test and Dunn’s multiple comparison test between \textit{Wars2}^{\text{+/-}} compared to \textit{Wars2}^{\text{V117L-}} (\textit{Tchh}^{\text{D1105V/-}}) and \textit{Wars2}^{\text{+/-}} compared to \textit{Wars2}^{\text{V117L-}} (\textit{Tchh}^{\text{D1105V/-}}) shown as * or $ P<0.05$ respectively. Wildtype colony-mate \textit{Wars2}^{\text{+/-}} black triangles, heterozygote point-mutation \textit{Wars2}^{\text{V117L/+}}(\textit{Tchh}^{\text{D1105V/+}}) red filled circles, heterozygous knockout \textit{Wars2}^{\text{+/-}} brown filled circle and compound heterozygote \textit{Wars2}^{\text{V117L-}} blue square filled with brown.

C) Body weight, D) Fat mass, E) Lean Mass were recorded from male mice 1- to 4-months of age. \textit{Wars2}^{\text{+/-}}, \textit{Wars2}^{\text{V117L/+}} (\textit{Tchh}^{\text{D1105V/+}}), \textit{Wars2}^{\text{+/-}} and \textit{Wars2}^{\text{V117L/-}} (\textit{Tchh}^{\text{D1105V/-}}) animal numbers 8, 13, n=9 and 6 respectively. AUCs were calculated baselined to zero and \textit{Wars2}^{\text{+/-}} compared to \textit{Wars2}^{\text{V117L-}}, \textit{Wars2}^{\text{V117L/-}} compared to \textit{Wars2}^{\text{V117L-}} (\textit{Tchh}^{\text{D1105V/-}}) and \textit{Wars2}^{\text{+/-}} compared to \textit{Wars2}^{\text{V117L-}} (\textit{Tchh}^{\text{D1105V/-}}), using a 1-way ANOVA non-parametric Kruskal-Wallis test and Dunn’s multiple comparison test giving p values of 0.0691, 0.0038 and 0.006 respectively. Significance at specific time-points was calculated with a 1-way ANOVA non-parametric Kruskal-Wallis test and Dunn’s multiple comparison test and is shown in table below each figure. Wildtype colony-mate \textit{Wars2}^{\text{+/-}} black triangles, heterozygote point-mutation \textit{Wars2}^{\text{V117L/+}}(\textit{Tchh}^{\text{D1105V/+}}) red filled circles, heterozygous knockout \textit{Wars2}^{\text{+/-}} brown filled circle and compound heterozygote \textit{Wars2}^{\text{V117L-}} blue square filled with brown.
Supplementary Figure 3. Related to Figure 1. Ultrastructural analyses reveal progressive loss of outer hair cell bundles, and histology shows reduced spiral ganglion neuron number, in \textit{Wars2}^{V117L/V117L} mutant mice.

A) Scanning Electron Micrographs of the mid-coil of the cochlear sensory epithelium from \textit{Wars2}^{+/+} and \textit{Wars2}^{V117L/V117L} mice at 1-, 6-, and 12-months of age. At 1-month of age, the number and appearance of the outer hair cell stereocilia bundles are as expected and similar across genotypes. At 6-months, there is loss of outer hair cell bundles in the \textit{Wars2}^{V117L/V117L} mutant mice, which is not observed in the \textit{Wars2}^{+/+} control mice. By 12-months of age, there is a near complete loss of outer hair cell bundles in the \textit{Wars2}^{V117L/V117L} mutant mice, which is not observed in the \textit{Wars2}^{+/+} control mice. Shown are representative images from the mid region of the cochlear spiral, at least three cochleae from independent mice were imaged per region for each genotype. Scale bar 5µm. To assess the loss of outer hair cell bundles in the apical, mid and basal turns of the cochlear coil counts were undertaken to determine the number of bundles adjacent to ten pillar cells. At 1-month of age \textit{Wars2}^{+/+} (apex n=3, Mid n=12, base n=9) and \textit{Wars2}^{V117L/V117L} (apex n=5, Mid n=9, base n=9) mice have similar numbers of OHC bundles. However, by 6-months of age \textit{Wars2}^{V117L/V117L} (apex n=7, Mid n=7, base n=7) mice have a reduced number of OHC bundles in all cochlear regions compared to \textit{Wars2}^{+/+} (apex n=6, Mid n=6, base n=4) mice. At 12-months of age \textit{Wars2}^{V117L/V117L} (apex n=5, Mid n=6, base n=7) mice show a further loss of OHC bundles in all cochlear regions compared to \textit{Wars2}^{+/+} (apex n=5, Mid n=5, base n=5) mice. While \textit{Wars2}^{V117L/V117L} mice show a progressive loss of OHC bundles throughout the cochlear spiral, no significant OHC bundle loss is observed in the \textit{Wars2}^{+/+} mice up to 12-months of age. Mean ± SD. Homozygous \textit{Wars2}^{V117L/V117L} are blue squares and \textit{Wars2}^{+/+} black triangles.

B) H&E-stained mid-modiolar cochlear sections from \textit{Wars2}^{+/+} and \textit{Wars2}^{V117L/V117L} mice at 12-months of age. Visual assessment of the sections shows the number of SGN nuclei to be similar from base-to-apex in \textit{Wars2}^{+/+} mice, but there appears fewer nuclei from base-to-apex in \textit{Wars2}^{V117L/V117L} mice. Shown are representative sections from one \textit{Wars2}^{+/+} and one \textit{Wars2}^{V117L/V117L} mouse. Scale bar 500µm. To assess this apparent reduced number of nuclei, counts were undertaken to determine the number of spiral ganglion neuron nuclei within a set 5000µm² area within the apical and basal turns. This shows that in the basal turn \textit{Wars2}^{+/+} and \textit{Wars2}^{V117L/V117L} mice have comparable numbers of nuclei. In addition, \textit{Wars2}^{+/+} mice have comparable numbers of nuclei in the apical and basal turns. However, \textit{Wars2}^{V117L/V117L} mice do not have comparable numbers of nuclei in the apical and basal turns. Three sections per genotype were used for counts, obtained from independent mice, mean ± SD. Homozygous \textit{Wars2}^{V117L/V117L} are blue squares and \textit{Wars2}^{+/+} black triangles. Significance was determined using an unpaired t test: ** P<0.01, *** P<0.001, **** P<0.0001.
Supplementary Figure 4. Related to Figure 2. *W*ars2*V*117L/V117L mice have reduced body weight due to reduced adiposity. Male and female A and D) Body weight B and E) Fat mass and C and F) Lean mass in cohort 2 mice. *W*ars2*V*117L/V117L, *W*ars2*V*117L/+; *W*ars2/+ animal numbers, male and female, were 11 and 6-7, 19 and 19 and 13 and 18, respectively. AUC calculated with zero baselines and compared using a 1-way ANOVA non-parametric Kruskal-Wallis test and Dunns multiple comparison test. For AUC comparing *W*ars2+/+, *W*ars2*V*117L/V117L, *W*ars2*V*117L/+; *W*ars2+/+ and *W*ars2*V*117L/V117L, and *W*ars2/+; *W*ars2*V*117L/+; Male body weight 0.0003, <0.0001 and >0.999; fat mass <0.0001, <0.0001 and >0.999; lean mass 0.0847, 0.0005 and 0.4131 respectively; Female body weight 0.0020, 0.0005 and >0.999; fat mass 0.0016, 0.0005 and >0.999; lean mass 0.8539, 0.2825 and >0.999 respectively. Significance at specific time-points was also calculated using a 1-way ANOVA non-parametric Kruskal-Wallis test and Dunns multiple comparison test, significance between *W*ars2*V*117L/V117L and *W*ars2+/+ shown as * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001 and significant differences between *W*ars2*V*117L/V117L and *W*ars2 *V*117L/+; shown as # P<0.05, ## P<0.01, ### P<0.001, #### P<0.0001. Homozygous *W*ars2*V*117L/V117L are blue squares, heterozygous *W*ars2*V*117L/+ are red circles and wildtype colony-mate *W*ars2/+ black triangles.
Quantification of Western blots at 12 months of age Figure 4 D to J

Respiratory chain complex activities

Brain Western blots 3-5 months of age

Brain Western blots 3-5 months of age
Supplementary Figure 5. Related to Figure 4. (A) Quantification of Western blots from Figures 4 D to J. (B and C) Reduced complex I and IV activities in cardiac muscle and increased CIII activity in skeletal muscle of \textit{Wars2}^{V117L/V117L} mice by 12 months of age. (D, E and F) Reduced WARS2 and complex I and IV deficiencies in whole brain of mice at 3-5 months of age.

\textbf{A)} Quantification of 12 month of age WARS2 and respiratory chain complex subunits in multiple tissues shown in main Figure 4 western blots. \textit{Wars2}^{V117L/V117L} and \textit{Wars2}^{+/+} animal numbers were 3 each. Statistical analysis was done by multiple t-tests using the Holm-Sidak method and without assuming consistent standard deviation. Adjusted p values are shown * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

\textbf{B and C)} Quantified respiratory chain complex activities in \textbf{B)} cardiac muscle and \textbf{C)} skeletal muscle, normalized to citrate synthase activity and expressed as a percentage of average wildtype values. \textit{Wars2}^{+/+} and \textit{Wars2}^{V117L/V117L} animal numbers 5 each. Cardiac muscle CI and CIV data was analyzed using a Mann-Whitney t-test and all other data with an unpaired two-tailed t-test.

\textbf{D)} Immunoblot analysis of WARS2 and \textbf{E)} mitochondrial respiratory chain sub-unit protein levels protein in \textit{Wars2}^{V117L/V117L} \textit{Wars2}^{V117L} (C3H/Pde) whole brain samples from male mice aged between approximately 3 and 5 months of age. \textit{Wars2}^{V117L/V117L} and \textit{Wars2}^{+/+} animal numbers were 3 each. \textbf{F)} Quantification of protein blots in \textbf{D} and \textbf{E}, data plotted as Log10 relative to widltype. For statistical analysis raw data was square root transformed and analyzed by an unpaired t-test for each probe. * $P<0.05$, ** $P<0.01$. Homozygous \textit{Wars2}^{V117L/V117L} are shown as blue squares and wildtype colony-mate \textit{Wars2}^{+/+} as black triangles.
Supplementary Figure 6. Related to Figure 7. Increased plasma FGF21 is linked with systemic changes in metabolism in \textit{Wars2}^{V117L/V117L} mice.

**A and B) Plasma FGF21.** \textbf{A)} Fasted plasma FGF21 in 4-month old overnight fasted male mice. \textit{Wars2}^{V117L/V117L} and \textit{Wars2}^{+/+} animal numbers were 9 each, mean ± SD. **B)} Fasted plasma FGF21 in 3-4-month old overnight fasted male mice C57BL/6J (B6J) and C3H/Pde (C3PDE) B6J and C3PDE animal numbers were 6 and 10 respectively, mean ± SD. Significance in \textbf{A)} and \textbf{B)} calculated using a Mann Whitney 2-tailed t-test, * <0.05 and *** <0.001. Note that the C3PDE background on which the \textit{Wars2}^{V117L} mutation is maintained shows strain specific differences in FGF21 concentration. Homozygous \textit{Wars2}^{V117L/V117L} are blue squares and wildtype colony-mate \textit{Wars2}^{+/+} black triangles. Significant differences between groups shown as * P<0.05, *** P<0.001.

**C, D, E and F) plasma clinical chemistry.** \textbf{C)} Free fatty acids (FFA). \textbf{D)} glucose. \textbf{E)} triacylglycerides (TAG) and \textbf{F)} β-hydroxybutyrate levels were analysed in plasma samples collected from \textit{Wars2}^{V117L/V117L} and \textit{Wars2}^{+/+} mice at 12-months of age. \textit{Wars2}^{V117L/V117L} and \textit{Wars2}^{+/+} male and female animal numbers were 5, 6 and 9,4 respectively, mean ± SD. Data A and B analysed by Mann-Whitney test and data C and D by unpaired two-tailed t-test. Homozygous \textit{Wars2}^{V117L/V117L} are blue squares and wildtype colony-mate \textit{Wars2}^{+/+} black triangles. Significant differences between groups shown as * P<0.05, **** P<0.0001.

**G) Intraperitoneal glucose tolerance tests (IPGTT) were performed in male \textit{Wars2}^{V117L/V117L}, \textit{Wars2}^{V117L/+} and \textit{Wars2}^{+/+} mice at 6-months of age. \textit{Wars2}^{V117L/V117L}, \textit{Wars2}^{V117L/+} and \textit{Wars2}^{+/+} animal numbers were 5,7 and 5 respectively, mean ± SD. IPGTT were performed early in the morning following over-night fasting and tail blood glucose levels were taken 0, 60 and 120 mins after an intraperitoneal glucose injection. AUC was calculated with zero baselines and compared using a one-way ANOVA with Tukey’s multiple comparison test. For AUC comparisons between \textit{Wars2}^{+/+} and \textit{Wars2}^{V117L/V117L}, \textit{Wars2}^{V117L/+} and \textit{Wars2}^{V117L/V117L}, \textit{Wars2}^{V117L/+} and \textit{Wars2}^{V117L/+}, \textit{Wars2}^{+/+} and \textit{Wars2}^{V117L/+}, 0.0143, 0.0474 and 0.6545. Significance at specific time-points was calculated with a 1-way ANOVA with Tukey’s multiple comparison test. Significance between \textit{Wars2}^{V117L/V117L} and \textit{Wars2}^{+/+} shown as * P<0.05, ** P<0.01, and significant differences between \textit{Wars2}^{V117L/V117L} and \textit{Wars2}^{V117L/+} shown as # P<0.05, ## P<0.01. Homozygous \textit{Wars2}^{V117L/V117L} are blue squares, heterozygous \textit{Wars2}^{V117L/+} are red circles and wildtype colony-mate \textit{Wars2}^{+/+} black triangles.
Supplementary Figure 7. Related to Figure 7. Activation of the ISR is progressive with age, coincident with mitochondrial respiratory chain deficiencies and independent of disrupted mitochondrial proteostasis in \(\text{Wars2}^{V117L/V117L}\) heart. Immunoblot analysis of LONP1, CLPP, mtHsp70, mtHsp60, p-eIF2α, (total)eIF2α, COXI (CIV) and NDUFB8 (CI) protein levels in \(\text{Wars2}^{V117L/V117L}\) and \(\text{Wars2}^{+/+}\) heart tissue collected from female mice at A) 1-month, B) 3-months and C) 12-months of age. \(\text{Wars2}^{V117L/V117L}\) and \(\text{Wars2}^{+/+}\) animal numbers were 3 each. Note that some 12-month blots are reproduced here from display figures for ease of comparison. The controls for each gel are indicate by numbers on the right of each panel and then shown in the loading control below each panel, note that some blots are re-probed and therefore share controls. Quantification plots are shown directly below the corresponding western blot panels. Statistical analysis was done by multiple t-test (PRISM) using the Holm-Sidak method and without assuming consistent standard deviation. Adjusted p values are shown * \(P<0.05\), ** \(P<0.01\), *** \(P<0.001\). Homozygous \(\text{Wars2}^{V117L/V117L}\) are shown as blue squares and wildtype colony-mate \(\text{Wars2}^{+/+}\) as black triangles.
Supplementary Table 1, Related to Figure 2.

*Wars2<sup>−/−</sup>* causes embryonic lethality. Number of mice born per genotype from three inter-crosses: A) *Wars2*<sup>V117L/+</sup> x *Wars2<sup>−/−</sup>*, B) *Wars2*<sup>V117L/+</sup> x *Wars2*<sup>V117L/+</sup> and C) *Wars2<sup>−/−</sup>* x *Wars2<sup>−/−</sup>*. Data were analysed for deviance from expected Hardy-Weinberg ratios using a Chi-squared test.

### A) Inter-cross of *Wars2*<sup>V117L/+</sup> x *Wars2<sup>−/−</sup>* mice

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Chi-squared test p=0.1103

### B) Inter-cross of *Wars2*<sup>V117L/+</sup> x *Wars2*<sup>V117L/+</sup> mice

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Chi-squared test p=0.8647

### C) Inter-cross of *Wars2*<sup>−/−</sup> x *Wars2*<sup>−/−</sup> mice

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Chi-squared test p=<0.0001
## Supplementary Table 2, Related to Star Methods

### Oligonucleotide primer sequences

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