Genome editing in mitochondria corrects a pathogenic mtDNA mutation in vivo

Payam A. Gammage1,4, Carlos Viscomi1, Marie-Lune Simard2, Ana S. H. Costa3, Edoardo Gaude3, Christopher A. Powell1, Lindsey Van Haute1, Beverly J. McCann1, Pedro Rebelo-Guiomar1,4, Raffaele Cerutti1, Lei Zhang2, Edward J. Rebar5, Massimo Zeviani6, Christian Frezza7, James B. Stewart6 and Michal Minczuk1,*

Mutations of the mitochondrial genome (mtDNA) underlie a substantial portion of mitochondrial disease burden. These disorders are currently incurable and effectively untreatable, with heterogeneous penetrance, presentation and prognosis. To address the lack of effective treatment for these disorders, we exploited a recently developed mouse model that recapitulates common molecular features of heteroplasmic mtDNA disease in cardiac tissue: the m.5024C>T tRNAAla mouse. Through application of a programmable nuclease therapy approach, using systemically administered, mitochondrially targeted zinc-finger nucleases (mtZFN) delivered by adeno-associated virus, we induced specific elimination of mutant mtDNA across the heart, coupled to a reversion of molecular and biochemical phenotypes. These findings constitute proof of principle that mtDNA heteroplasmacy correction using programmable nucleases could provide a therapeutic route for heteroplasmic mitochondrial diseases of diverse genetic origin.

Mitochondrial diseases are a broad group of hereditary, multisystem disorders, a substantial portion of which are transmitted through mutations of mitochondrial DNA (mtDNA) with a minimum prevalence of 1 in 5,000 adults1. Human mtDNA is a small, double-stranded, multicopy genome present at ~100–10,000 copies per cell2. In the disease state, mutated mtDNA often co-exists with wild-type mtDNA in heteroplasmacy, and disease severity in conditions caused by heteroplasmic mtDNA mutations correlates with mutation load3. A threshold effect, where >60% mutant mtDNA load must be exceeded before symptoms manifest, is a definitive feature of heteroplasmic mtDNA diseases, and attempts to shift the heteroplasmacy ratio below this threshold have driven much research towards treatment of these incurable and essentially untreatable disorders. One such approach relies on directed nucleic acid editing of mtDNA using, among other programmable genome engineering tools, mitochondrially targeted zinc-finger nucleases (mtZFNs)4–6. Because mammalian mitochondria lack efficient DNA double-strand break repair pathways7, the selective introduction of double-strand breaks into mutant mtDNA leads to rapid degradation of these molecules by components of the mtDNA replisome8. As mtDNA copy number is maintained at a cell-type-specific steady-state level, selective elimination of mutant mtDNA stimulates replication of the remaining mtDNA pool, eliciting shifts in the heteroplasmic ratio.

In previous work, we have described methods for the delivery of zinc-finger proteins (ZFPs) to mitochondria in cultured cells9,10 and the assembly and function of efficient mtZFN architectures that are capable of producing large heteroplasmic shifts that result in phenotype rescue of patient-derived cell cultures11–13. Using the first available mouse model of heteroplasmic mitochondrial disease, bearing the point mutation m.5024C>T in mitochondrial tRNAAla (mt-tRNAAla), which faithfully recapitulates key molecular features of mitochondrial disorders in cardiac tissue1, we now demonstrate efficient manipulation of mtDNA heteroplasmy with concomitant rescue of molecular and biochemical phenotypes across the heart following the delivery of mtZFNs by systemically administered adeno-associated virus (AAV).

In the context of second-generation tail–tail mtZFN architectures that were shown to be efficacious in previous work14, we set out to generate pairs of ZFPs with single-nucleotide-binding specificity for m.5024C>T. As this site in the mouse mtDNA is challenging for ZFPs, a selection of targeting strategies with varying numbers of zinc-finger motifs, spacer region lengths and additional linkers were used. The assembly of candidate ZFPs yielded a library (Supplementary Fig. 1a and Supplementary Table 1) consisting of 24 unique ZFPs targeting the m.5024C>T site, referred to as mutant-specific monomer (MTM), and a single partner ZFP targeting an adjacent sequence on the opposite strand, referred to as wild-type-specific monomer 1 (WTM1). These constructs were subjected to several rounds of screening in mouse embryonic fibroblasts (MEFs) bearing ~65% m.5024C>T heteroplasmy to assess specific heteroplasmacy-shifting activity (Supplementary Fig. 1b). These screens identified consistent, specific activity of pairing MTM25/WTM1 (Fig. 1a and Supplementary Fig. 1c), which produced a shift of ~20%, from 65% to 45% m.5024C>T heteroplasmy in the MEF cell line as determined by pyrosequencing (Fig. 1b). We also confirmed exclusive mitochondrial localization of MTM25 and WTM1 in MEF cells (Supplementary Fig. 2) and then selected this pair for in vivo experiments.

MTM25 and WTM1 mtZFN monomers were encoded in separate viral genomes and encapsidated within the cardiotropic, engineered AAV9.45 serotype17 (Fig. 1c). Following tail-vein administration of 5 × 1012 viral genomes per monomer per mouse, robust expression of MTM25 and WTM1 in total mouse heart tissue was detected by western blotting (Fig. 1d). Despite equal quantities of injected viral genomes, lower expression levels of WTM1
were consistently detected, possibly due to lower stability of the translated protein. Next, various doses of mtZFN-AAV9.45 were administered to mt-tRNAAla animals harboring m.5024C>T heteroplasmacy ranging from 44% to 81% (Supplementary Table 2). As only minimal variance in heteroplasmacy is observed between tissues of the m.5024C>T mouse14, mtDNA heteroplasmacy is assessed by pyrosequencing data, expressed as the change (Δ) between the ear punch genotype (E) and in vivo mtDNA heteroplasmy modification. γH2AX, Illustration of the mtZFN strategy. WTM1 binds upstream of m.5024C>T heteroplasmy from MEFs transfected with controls or MTM25 or WTM1 at differing concentrations, facilitated by tetracycline-sensitive HHR13. The change in m.5024C>T heteroplasmy is plotted. utZFN is a mtZFN that does not have a target site in mouse mtDNA13. Concentration '-' and '+' refer to zero and maximum dose, respectively. Gray bars indicate the mean. AAV '-' and '+' refer to zero and maximum dose, respectively. Statistical analysis was performed using the two-tailed Student’s t-test; ***<P<0.00001; vehicle/mtZFN high expression: P=0.000002; vehicle/mtZFN high expression: P=0.000083. Gray bars indicate the mean. c, Scheme of in vivo experiments. MTM25 and WTM1 are encoded in separate AAV genomes, encapsidated in AAV9.45 then simultaneously administered by tail-vein (TV) injection. Animals are euthanized at 65 days post-injection. d, Western blot of total heart protein from animals injected with 5 × 10^{12} viral genomes of MTM25 and/or WTM1. Both proteins include the HA tag and are differentiated by molecular weight. This blot was performed twice with similar results. The raw data are available for this panel (Supplementary Fig. 9). CBB, Coomassie Brilliant Blue. e, Pyrosequencing of m.5024C>T heteroplasmy from ear (E) and heart (H) total DNA. The change in m.5024C>T is plotted. n=20 (vehicle), n=3 (WTM1 only) and n=4 (all other conditions) animals (Supplementary Table 2). The error bars indicate the s.e.m. Statistical analysis was performed using the two-tailed Student’s t-test. Vehicle/mtZFN low expression: P<0.00001; vehicle/mtZFN high expression: P<0.00001. Gray bars indicate the mean. AAV '-' and '+' refer to zero and maximum dose, respectively. f, Assessment of mtDNA copy number by quantitative PCR. n=8 (vehicle) and n=4 (all other conditions) animals (Supplementary Table 2). The center line is the mean and the error bars indicate the s.e.m. Statistical analysis was performed using the two-tailed Student’s t-test; P=0.007931. Central black line indicates the mean. ** indicates P<0.01, *** indicates P<0.001. were consistently detected, possibly due to lower stability of the translated protein. 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Fig. 2 | Reduction of m.5024C>T mtDNA heteroplasmy results in phenotype rescue. a, Illustration of mt-tRNA<sup>Ala</sup> bearing the m.5024C>T mutation. Given the nature and position of this mutation, transcribed tRNA molecules containing the mutation mispair are unlikely to fold correctly or be aminoacylated, resulting in reduced steady-state levels of mt-tRNA<sup>Ala</sup> at high levels of m.5024C>T heteroplasmy. b, Quantification of high-resolution northern blot data from total heart RNA extracts. mt-tRNA<sup>Ala</sup> and mt-tRNA<sup>Cys</sup> abundance was normalized to 5S rRNA. c, Principal component analysis plot of metabolomic data for intermediate-dose AAV-treated mice and age-matched, initial heteroplasmy-matched controls acquired by liquid chromatography coupled to mass spectrometry (Supplementary Table 2). d, Total metabolite levels of pyruvate from samples measured in panel c. n = 3 (vehicle) and n = 4 (AAV) animals. e, Total metabolite levels of lactate from samples measured in panel c. n = 3 (vehicle) and n = 4 (AAV) animals. f, Total metabolite levels of aspartate from samples measured in panel c. n = 3 (vehicle) and n = 4 (AAV) animals. Black and red bars indicate the mean. * indicates P < 0.05, *** indicates P < 0.001. 

This lattermost result is consistent with our previous observations<sup>13</sup>, underscoring the importance of fine-tuning mtZFN levels in mitochondria for efficient mtDNA heteroplasmy modification. AAV9.45 transduction could not be detected in non-cardiac tissues, and no shifts in heteroplasmy were detected in the liver at 65 days post-injection, irrespective of viral dose (Supplementary Fig. 3). As AAV transduction of post-mitotic tissues, particularly in short-lived mammals, is essentially permanent, a time dependence of heteroplasmy shifting is expected. Accordingly, measurements of mtDNA heteroplasmy over time in cardiac tissue demonstrate significant increases in heteroplasmy-shifting activity in the latest post-treatment time points (Supplementary Fig. 4). Despite the presence of two regions with significant homology to the mtDNA target site in the nuclear genome, no evidence for off-target effects exerted by mtZFNs could be detected, confirming previous data that mtZFN-induced DNA double-strand breaks do not result in non-homologous end-joining activity<sup>13</sup> (Supplementary Fig. 5c).

Having defined conditions within which a robust shift of m.5024C>T heteroplasmy is achieved in vivo, we next addressed disease-relevant phenotype correction in the model. A common feature of mt-tRNA mutations in mitochondrial diseases, recapitulated in the irRNA<sup>Ala</sup> mouse model<sup>15</sup>, is the instability of mt-tRNA molecules in proportion to mutant load<sup>18</sup> (Fig. 2a). To assess the effects of mtZFN treatment on the stability of mt-tRNA<sup>Ala</sup> in the hearts of animals across the dosage range, we used high-resolution northern blotting, which revealed a significant increase in mt-tRNA<sup>Ala</sup> steady-state levels (Fig. 2b and Supplementary Fig. 6) proportional to the heteroplasmy shifts detected in these mice (average m.5024C>T heteroplasmy: control, 71% pre-treatment and 73% post-treatment; low AAV dose, 73% pre-treatment and 71% post-treatment; medium AAV dose, 73% pre-treatment and 37% post-treatment; high AAV dose, 71% pre-treatment and 40% post-treatment; Supplementary Table 2).
post-treatment) (Fig. 1e and Supplementary Table 2) and consistent with previously reported data. Depletions of mtDNA copy number associated with the administration of high viral doses (Fig. 1f) did not seem to affect the recovery of mt-tRNAAla steady-state levels following heteroplasmy shift (Fig. 2b). This agrees with previously published data that even severe mtDNA depletion does not manifest in proportional changes of mitochondrial RNA steady-state levels.

To assess the physiological effects of mt-tRNAAla molecular phenotype rescue, we compared steady-state metabolite levels in cardiac tissue from mice with high m.5024C>T mutant heteroplasmy treated with the intermediate viral titer (5 × 10^12 viral genomes) with heteroplasmy-matched and age-matched controls (Supplementary Table 2). This analysis revealed an altered metabolic signature in mtZFN-treated mice compared with controls (Fig. 2c and Supplementary Fig. 7), demonstrating significantly increased pyruvate levels (Fig. 2d) and significantly decreased lactate levels (Fig. 2e), suggestive of a diminished reliance on glycolysis, coupled to increased aspartate levels (Fig. 2f), suggestive of improved mitochondrial respiration, in the treated mice. These indicators of improved mitochondrial metabolism are not observed in mice treated with the highest AAV dose (Supplementary Fig. 8), which also exhibit substantial copy number depletions (Fig. 1f). Owing to phenotypic heterogeneity of mice bearing high levels of mtDNA heteroplasmy, changes in gross cardiac function following heteroplastic shifts could not be assessed. Taken together, these data indicate that partial m.5024C>T heteroplasmy shifts (Fig. 1e) result in the recovery of mt-tRNAAla steady-state levels and the rescue of mitochondrial function (Fig. 2b–f).

Our previous reports on the use of mtZFN technology have demonstrated that these programmable nucleases can target multiple genetic lesions, producing phenotypically relevant shifts of mtDNA heteroplasmy in cellular models of mitochondrial dysfunction. Here, we have further demonstrated the flexibility and future potential of mtZFN technology by targeting another heteroplastic mutation in mouse mtDNA, m.5024C>T, manipulating the heteroplasmy of this variant both in vitro and in vivo (Fig. 1), which results in the molecular and physiological rescue of disease phenotypes in heart tissue (Fig. 2).

Despite the time elapsed since mtDNA mutations were first associated with human disease in the late 1980s and, effective treatments for heteroplastic mitochondrial disease have not been forthcoming. Preventing the transmission of mtDNA mutations through mitochondrial replacement therapy or mitochondrial donation has gained traction, although given the nature of the mtDNA bottleneck, issues surrounding carryover of mutant mtDNA, heterogeneous mitochondrial disease presentation and the subsequent lack of family history of mitochondrial disease in the majority of new cases, these approaches can only be of limited use. More recently, several intriguing molecular pathways to treatment of mitochondrial disease have been defined and explored by numerous groups; however, hopes for clinically relevant therapy for heteroplastic mitochondrial disease, thus far, remain unfulfilled. The data that we describe in this Letter, and those from Bacman et al., constitute proof of principle that somatic mitochondrial genome editing using programmable nucleases, in combination with the ever-increasing collection of engineered, tissue-specific AAV serotypes, may offer a potentially universal route to treatment for heteroplastic mitochondrial disease. Given the magnitude of in vivo heteroplasmy modification demonstrated using these tools, total amelioration of clinical symptoms and/or halting of disease progression could be expected. As such, this development has the potential to transform the prospects of many patients with mitochondrial disease, and further work enabling the translation of these tools into effective medicines is vital.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41591-018-0165-9.

Received: 15 February 2018; Accepted: 26 July 2018; Published online: 24 September 2018

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**Acknowledgements**

This work was supported by the Medical Research Council (MC_U105697135 and MC_UU_00015/4 to M.M., MC_UU_12022/6 to C.F. and MC_UU_00015/5 to M.Z.), ERC Advanced Grant (FP7-322424 to M.Z.), NRJ-Institut de France (to M.Z.) and the Max Planck Society (to J.B.S.). P.R.-G. was supported by ‘Fundação para a Ciência e a Tecnologia’ (PD/BD105570/2014). We acknowledge the important contribution to model development made by N.-G. Larsson, which was essential to this work. We are grateful to the personnel at Phenomics Animal Care Facility, Cambridge, UK, for their technical support in managing our mouse colonies. We are grateful to M. Rice, Phenomics Animal Care Facility, for technical assistance with viral administration. We thank K. Dirksen (MPI, Cologone, Germany) for isolation and immortalization of the MEFs. All FACS experiments were performed at the NIHR BRC Cell Phenotyping Hub, Cambridge, UK, by C. Bowman, E. Perez, J. Markovic Djuric and A. Petrunkina-Harrison.

**Author contributions**

P.A.G. designed the research, performed biochemical, in vitro and in vivo experiments, analyzed the data and wrote the paper. C.V. performed the in vivo experiments. M.-L.S. contributed to model characterization. A.S.H.C. and E.G. performed the mass spectrometry-based metabolic experiments and analyzed the data. C.A.P. and L.V.H. performed biochemical experiments and analyzed the data. B.J.M. performed biochemical and immunofluorescence experiments. P.R.-G. and R.C. performed the histological experiments. L.Z. designed and assembled the ZFP library. E.J.R. oversaw the ZFP library preparation. M.Z. oversaw the in vivo experiments. C.F. oversaw the mass spectrometry-based metabolic experiments. J.B.S. provided cell and mouse models and contributed to model characterization. M.M. oversaw the project and co-wrote the paper, with all authors’ involvement.

**Competing interests**

E.J.R. and L.Z. are full-time employees of Sangamo Therapeutics.

**Additional information**

**Supplementary information** is available for this paper at https://doi.org/10.1038/s41591-018-0165-9.

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**Correspondence and requests for materials** should be addressed to P.A.G. or M.M.

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Methods

Constructs, plasmids and viral vectors. All mtZFN architectures were used as reported for second-generation mtZFN, with the exception of the ZFP domains incorporated to achieve cloning of the mtZFN ZFP domains upstream of FokI (+) in the pCMCherry-3k19 vector (Addgene 104499), incorporating the 3′ hammerhead ribozyme (HHR) using 5′ EcoRI and 3′ BamHI restriction sites. This product was then PCR amplified to include a 5′ Apal site and remove the 3′ stop codon while also incorporating a T2A sequence and 3′ XhoI site. This fragment was then cloned into pCMCherry (Addgene 62803) using Apal/XhoI sites. The WT1 ZFP was separately cloned upstream of FokI (+) in the pCMCherry-3k19 vector (Addgene 104499), incorporating the 3′ hammerhead ribozyme (HHR) using 5′ EcoRI and 3′ BamHI restriction sites. The resulting product was PCR amplified to include 5′ XhoI and 3′ Apal sites allowing cloning downstream of the MTM(Z) variants. MTM25(+) and WT1(–) monomers were cloned into separate pCMCherry and pTracer vectors as described previously. Vector construction of mtZFNs intended for AAV production was achieved by PCR amplification of MTM25(+)_HHR and WT1(–)_HHR transgenes, incorporating 5′ Eagl and 3′ BgII sites. These products were then cloned into tAAV2-CMV between 5′ Eagl and 3′ BamHI sites. The FLAG epitope tag of WT1(–) was replaced with a hemagglutinin (HA) tag at the same position in the WT1(–) open reading frame by PCR. The resulting plasmids were used to generate recombinant AAV2/9.45-CMV in the UNC Gene Therapy Center, Vector Core Facility (Chapel Hill, NC, USA). The 3K19 HHR sequence was incorporated into the mtZFN-AAV9.45 viral particles at the UNC Gene Therapy Center, Vector Core Facility (Chapel Hill, NC, USA). The 3K19 HHR sequence was incorporated into the mtZFN-AAV9.45 constructs to allow ubiquitous expression of the transgene from CMV while limiting the expression level, allowing the administration of the high viral titers required to ensure effective co-transduction of cells in the targeted tissue without inducing large mtDNA copy number depletions.

Maintenance, transfection and FACS of cell cultures. Wild-type and m.5024C>T MEF cell lines were cultured in DMEM containing 2 mM l-glutamine, 110 mg/ml sodium pyruvate (Life Technologies) and 10% FCS (PAA Laboratories). Cells were transfected by electroporation using the Nucleofector II apparatus (Lonza) and a MEF1 kit and T20 program. FACS was performed as described previously. Control of mtZFN expression was achieved through titration of tetracycline into culture media, controlling the rate of HHR autocatalysis as described previously.

Use of animal models. All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 (PLP20/75386) and EU Directive 2010/63/EU. The C57BL/6J-tRNAAla mice used in this study were housed at the C57BL/6J mouse nuclear genome. PCR amplicons were subjected to Nextera sample processing and the resulting libraries were assessed by 2×150-cycle paired-end sequencing using a MiSeq instrument (Illumina). Quality trimming and 3′-end adapter clipping of sequenced reads were performed simultaneously with Trim Galore! (–paired) and aligned to GRCm38 using bowtie2. Only reads that contained the entire region of chromosome 5 (60,042,834–60,042,934) or greater than 2,590,008 reads were selected for counting with SAMtools (flagstat) and insertion/deletion count based on CIGAR string (I/D). All individual samples yielded >10,000 reads per nucleotide.

Amplicon resequencing of nuclear DNA off-target sites. Two regions of the NCBI reference sequence for C57BL/6J mouse DNA demonstrated significant homology (>75% sequence identity) with the mtZFN target site in mtDNA. Amplicons containing these sites were obtained by PCR using primers listed below: Chromosome 2 forward 5′ GGTTCTGATATCTTGGATGTTG 3′ Chromosome 2 reverse 5′ GACATGACACGATGCTGTTTG 3′ Chromosome 5 forward 5′ GCTATCTGAGCAGAGGATGCT 3′ Chromosome 5 reverse 5′ CATCAGGAGTTGAGGACAC 3′ All primers were designed using NCBI reference sequence GRCm38.p6 and NC_005089.1 for the C57BL/6J mouse nuclear and mitochondrial genomes, respectively.

RNA extraction and northern blotting. Total RNA was extracted from 25 mg mouse heart tissue using TRIzol (Ambion) by homogenization using a gentleMACS dissociator (Miltenyi). Northern blotting was performed as described previously. Briefly, 5 µg total RNA was resolved on a 10% (w/v) polyacrylamide gel containing 8 M urea. Gels were dry blotted onto a positively charged nylon membrane (Hybond-N*), with the resulting membrane crosslinked by exposure to 254-nm UV light, 120 mJ per cm2. For rRNA probes, crosslinked membranes were hybridized with radioactively labeled RNA probes T7 transcribed from PCR fragments corresponding to appropriate regions of mouse mtDNA. 5S rRNA was probed with a complementary 5′-end-labeled DNA oligo. Membranes were exposed to a storage phosphor screen and scanned using a Typhoon phosphor

DNA extraction and quantitation. DNA was extracted from both cultured cells and whole tissues using a Qiagen DNEasy Blood and Tissue kit, according to the manufacturer's instructions. Once acquired, DNA concentrations were assessed by spectrophotometry.

Pyrosequencing and quantitative PCR. Assessment of m.5024C>T mtDNA heteroplasmy was carried out by pyrosequencing. PCRs for pyrosequencing were prepared using KOD DNA polymerase (Takara) for 40 cycles using 100 ng template DNA with the following primers: m.4962–4,986 forward 5′ ATACAGTCGCGGAGCCCTTCAAG 3′ m.5060–m.5,383 reverse 5′ [Bln] GAGGGTTCGCAATATCTGTTGATT 3′ m.5,003–m.5,022 sequencing primer 5′ AGTTTAACTCTCTGATAAGG 3′ mtDNA copy number of mouse heart samples was determined by quantitative PCR using PowerUp SYBR Green Master Mix according to the manufacturer's protocol (Applied Biosystems). Samples were analyzed using a 7900HT Fast Real-Time PCR System (Thermo Fisher). The following primers were used: MT-COI forward 5′ TCTAGAAGCGAGGATACATTACT 3′ MT-COI reverse 5′ CGGGATCCAAGAAAATGTTGTTT 3′ RNaseP forward 5′ GCCTAATCCTGGACTCGTACT 3′ RNaseP reverse 5′ CTGACACCAACACAGAGTCTGAAA 3′ All primers for pyrosequencing and quantitative PCR were designed using NCBI reference sequences GRCm38.p6 and NC_005089.1 for the C57BL/6J mouse nuclear and mitochondrial genomes, respectively.

Immunodetection of proteins. The localization of proteins by immunofluorescence was included as described previously. For mouse heart tissue, 50 mg was homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8, 1% Triton X-100, 0.5% (v/v) deoxycholate and 0.1% (v/v) SDS) using a gentleMACS dissociator (Miltenyi). The resulting homogenate was centrifuged at 10,000g at 4°C for 10 min and the supernatant was then removed and centrifuged at 10,000g at 4°C for 10 min. The concentration of both cellular and tissue protein extracts was determined by bicinchoninic acid (BCA) assay (Pierce).

Amplicon resequencing of the mtDNA target site. The region m.4962–5,383, which was also used for the pyrosequencing analysis, was amplified by PCR using ubiquitinylated primers. PCR amplicons were subjected to Nextera sample processing and the resulting libraries were assessed by 2×150-cycle paired-end sequencing using a MiSeq instrument (Illumina). Quality trimming and 3′-end adapter clipping of sequenced reads were performed simultaneously with Trim Galore! (–paired) and aligned to GRCm38 using bowtie2. Only reads that contained the entire region of chromosome 5 (60,042,834–60,042,934) or greater than 2,590,008 reads were selected for counting with SAMtools (flagstat) and insertion/deletion count based on CIGAR string (I/D). All individual samples yielded >10,000 reads per nucleotide.

RNA extraction and northern blotting. Total RNA was extracted from 25 mg mouse heart tissue using TRIzol (Ambion) by homogenization using a gentleMACS dissociator (Miltenyi). Northern blotting was performed as described previously. Briefly, 5 μg total RNA was resolved on a 10% (v/v) polyacrylamide gel containing 8 M urea. Gels were dry blotted onto a positively charged nylon membrane (Hybond-N*), with the resulting membrane crosslinked by exposure to 254-nm UV light, 120 mJ per cm2. For rRNA probes, crosslinked membranes were hybridized with radioactively labeled RNA probes T7 transcribed from PCR fragments corresponding to appropriate regions of mouse mtDNA. 5S rRNA was probed with a complementary 5′-end-labeled DNA oligo. Membranes were exposed to a storage phosphor screen and scanned using a Typhoon phosphor...
imaging system (GE Healthcare). The signals were quantified using Fiji software. The following primers/oligonucleotides were used:

- **MT-TA** forward
  5′ TAATACGACTCACTATAGGGAGACTAAGGACTGTAAGACTTCATC 3′
  5′ GAGGTCTTAGCTTAATTAAG 3′
- **MT-TC** forward
  5′ TAATACGACTCACTATAGGGAGACGATTTAGAGATTTTCTC 3′
  5′ GGCTCCTAGTGATATTCATG 3′
- **MT-TL1** forward
  5′ TAATACGACTCACTATAGGGAGAGGATTTGAAC 3′
  5′ ATTAGGGTGGCAGAGCCAGG 3′
- 5S rRNA oligo:
  5′ AAGCCTACAGCACCCGGTATTCCCAGGCGGTCTCCCATCCAAGTA CTAACCA 3′

All primers for northern blotting were designed using NCBI reference sequences GRCm38.p6 and NC_005089.1 for the C57BL/6J mouse nuclear and mitochondrial genomes, respectively.

**Sample preparation and liquid chromatography coupled to mass spectrometry analysis.** Snap-frozen tissue specimens were cut and weighed into Precellys tubes prefilled with ceramic beads (Stretton Scientific). An exact volume of extraction solution (30% acetonitrile, 50% methanol and 20% water) was added to obtain 40 mg specimen per ml extraction solution. Tissue samples were lysed using a Precellys 24 homogenizer (Stretton Scientific). The suspension was mixed and incubated for 15 min at 4 °C in a Thermomixer (Eppendorf), followed by centrifugation (16,000 g for 15 min at 4 °C). The supernatant was collected and transferred into autosampler glass vials, which were stored at −80 °C until further analysis. Samples were randomized to avoid bias due to machine drift and processed blindly. Liquid chromatography coupled to mass spectrometry analysis was performed using a QExactive Orbitrap mass spectrometer coupled to a Dionex U3000 UHPLC system (Thermo Fisher Scientific). The liquid chromatography system was fitted with a Sequant ZIC-pHILIC column (150 × 2.1 mm) and a guard column (20 × 2.1 mm) from Merck Millipore and temperature maintained at 40 °C. The mobile phase was composed of 20 mM ammonium carbonate and 0.1% ammonium hydroxide in water (solvent A), and acetonitrile (solvent B). The flow rate was set at 200 µl min⁻¹, with the gradient as described previously. The mass spectrometer was operated in the full MS and polarity-switching mode. The acquired spectra were analyzed using XCalibur Qual Browser and XCalibur Quan Browser software (Thermo Fisher Scientific).

**Statistical analysis.** One-tailed and two-tailed Student’s t-tests were used to compare independent means. Statistical analysis was performed using Prism 5 software.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
All next-generation sequencing data generated in the present study are available from the BioProject database using accession PRJNA479953. All other datasets and materials are available from the corresponding authors upon reasonable request.

**References**
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Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

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The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

The statistical test(s) used AND whether they are one- or two-sided

Only common tests should be described solely by name; describe more complex techniques in the Methods section.

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A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted. Give P values as exact values whenever suitable.

For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Clearly defined error bars

State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

None Used

Data analysis

XCalibur Qual Browser, XCalibur Quan Browser, Image J, Trim Galore!, Bowtie2, SAMtools

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

NGS data generated in the present study are available from the BioProject database using accession number PRJNA479953.

Raw data is available for Fig. 1D (Fig. S9).

There are no restrictions on data availability.
Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
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- Ecological, evolutionary & environmental sciences

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
- A naive, preliminary power calculation was performed at the outset, using speculative effect sizes we hoped to observe. The effects observed were much larger than anticipated, so the number of animals used in the study was scaled back, in accordance with the three R’s principle (replacement, reduction, refinement), enshrined in UK animal research laws.

Data exclusions
- No samples or data were excluded.

Replication
- All experiments were subject to both biological and technical replicates to ensure reproducibility. The data described in the manuscript was collected from a number of independent experiments taking place over a ~3 year period.

Randomization
- Randomization was not necessary for this study. Male animals were assembled into cohorts based on similar age and heteroplasmy. Homogeneity of the control and treatment cohorts (at the pre-treatment point) was the aim.

Blinding
- Animals were given unique identifying numbers and the administration of substances was blinded. Samples for RNA, qPCR, amplicon resequencing and LC-MS analyses were blinded.

Reporting for specific materials, systems and methods

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| n/a                             | Involved in the study |
|                                 | ChIP-seq |
|                                 | Flow cytometry |
|                                 | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials
- Model cell lines and animals are available to academic and industrial scientists through the Max Planck Institute for Biology of Ageing. Requests for m.5024C>T specific mZFNs should be directed to the corresponding authors.

Antibodies

Antibodies used
- rabbit anti-TOM20 (Santa Cruz Biotechnology, sc-11415, 1:200), Alexa Fluor 647 anti-rabbit (Abcam, ab150079, 1:1000), mouse anti-FLAG (Sigma, F1804, 1:1000), Alexa Fluor 594 anti-mouse (Life Technologies, R37121, 1:1000), rat anti-HA (Roche, 11867431001, 1:200), Alexa Fluor 488 anti-rat (Life Technologies, A11006)

Validation
- All antibodies have been verified using a variety of species-specific (human, mouse) cellular models. Verification of specific detection has been determined through a mixture of microscopy, subcellular fractionation and protein expression/purification studies.
## Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s) | Max Planck Institute for Biology of Ageing, Cologne. |
|---------------------|-----------------------------------------------------|
| Authentication      | The m.5024C>T cells are unique, and were extensively characterized prior to publication (Kauppila et al., 2016, Cell Rep.) |
| Mycoplasma contamination | All cells were tested, and found to be negative, for mycoplasma. |
| Commonly misidentified lines (See ICLAC register) | None. |

## Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | Mouse, tRNA-Ala CS7/B16j, 2-8 months of age. |
|--------------------|-------------------------------------------|
| Wild animals       | N/A                                       |
| Field-collected samples | N/A                                    |