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Amelioration of Leigh syndrome induced by mouse blastocyst complementation with a mutant human mitochondrial ATP synthase 6

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Abbreviations:

MRI: magnetic resonance imaging; PERG: pattern electroretinogram; FERG: flash electroretinogram; TEM: Transmission electron microscopy; OXPHOS: oxidative phosphorylation; RGC: retinal ganglion cell; INL: inner nuclear layer; ONL: outer nuclear layer.

Abstract

Mutations in mitochondrial genes are primary causes of multiple inherited disorders, especially affecting tissues with high aerobic energy demands such as the eye, brain, heart, and skeletal muscles. Such mitochondrial diseases are untreatable, have poor prognoses, and relevant animal models are few. Here we microinjected into the mouse blastocyst an adeno-associated virus (AAV) that we redirected to mitochondria to deliver the mutant human adenosine triphosphate (ATP) synthase subunit 6 gene (m.8993T>G) responsible for Maternally Inherited Leigh Syndrome, a neurologic disease renowned for causing rapidly fatal encephalomyelopathy in childhood or Neuropathy, Ataxia and Retinitis Pigmentosa in adults. Mice bred over six generations exhibited hallmarks of the human disease with one or multiple systemic symptoms including early death, paralysis, hunching, vision loss and seizures. Necropsy revealed spongiform encephalopathy, retinal degeneration, and cardiomegaly. Intravenous injection of a mitochondrially directed AAV serotype 9 vector containing wild-type *ATP6* after disease onset, conferred prolonged survival and reduced paralysis. When administered prior to disease onset, all treated mice survived, and visual and motor function improved. These results support further development of gene therapy strategies involving intravenous delivery of mito-targeted AAV9.

Keywords: mito-targeted AAV; mutant m.8993T>G *ATP6*; Leigh Syndrome and Neuropathy, Ataxia and Retinitis Pigmentosa (NARP)

Leigh syndrome (LS) is a progressive neurodegenerative disease that affects as many as 1 in 40,000 live births^{1,2}. It is caused by mutations in nuclear or mitochondrial genes that encode structural components or assembly factors of the electron transport chain and ATP synthase complexes I-V that are responsible for eukaryotic energy production by oxidative phosphorylation (OXPHOS). Over 75 loci on more than 60 genes affecting complexes I-V have been linked to LS with about 25% of cases caused by mutations of mitochondrial DNA (mtDNA)³. LS-associated mtDNA mutations include 6 genes in complex I, COX-III in Complex IV, and the ATP6 subunit of Complex V^{1,3}. Because all mammalian cells contain numerous mitochondria, and correspondingly numerous mt-genomes, mutated and wild type mtDNA can coexist in conditions known as heteroplasmy. In general, symptoms of disease only manifest when the ratios of mutated to wild type mtDNA are increased to levels that impair OXPHOS sufficiently to restrict physiological functions.

The typical onset of LS is 3-12 months with 83% of cases presenting by age 2yr and evidence of congenital abnormalities in 22% of cases^{1,3}. Although there is more variability in adult-onset LS, the core features are similar. LS subjects typically present with closely similar brain MRI scans that identify symmetrical necrotizing lesions of the basal ganglia, thalamus, brainstem and cerebellum^{1,3}. Symptoms vary and can include psychomotor arrest or decline, hypotonia, spasticity, ataxia, dystonia, lethargy, seizures, infantile spasms, movement disorders, dysphagia, abnormal ocular movements, hearing loss and respiratory failure^{1,4,5}. Mouse models with mutations or deletions of OXPHOS-related genes that mimic the human LS condition have been described for both nuclear and mitochondrial origin genes^{1,6-11}. These mouse models have brain MRI profiles that closely mimic human LS, and symptoms that include progressive encephalopathy, growth retardation, hypothermia, ataxia, hypotonia, lethargy, failure to thrive, breathing irregularities and early death.

There is no cure for LS or indeed any of the up to 300 other mitochondrial diseases that have been described^{1,12} although there is evidence for successful palliative pharmacological treatments, and molecular genetic therapies to correct or mitigate the effects of the mutations are in the pipeline^{1,4,8,12}. Mouse models

have been developed to mimic LS associated with nuclear gene mutations in complexes I, II, and IV, the most common being the complex I NDUFS4 knockout mouse that replicates multiple traits of human LS and has been instrumental in developing therapies^{4,8,13-16}. Our group recently described a mouse model that mimics Leber hereditary optic neuropathy (LHON), a mitochondrial disease caused by mutations of the mitochondrial encoded NADH ubiquinone oxidoreductase subunit 4 (ND4) gene. Mouse germline delivery of a mutant human ND4 gene in an AAV vector targeted to the mitochondria produced mice with hallmark symptoms of LHON that were significantly reversed by subsequent intravitreal delivery of the wild type ND4 gene⁹.

Here we describe mouse germline delivery of a mutant ATP6 gene, responsible for the most common form of maternally inherited LS (MILS) and related Neuropathy, Ataxia and Retinitis Pigmentosa (NARP). Progeny of mice created by blastocyst transduction of a human mitochondrial-targeted mutant ATP6 AAV containing a T to G transition that causes substitution of arginine for leucine at amino acid 156 of the ATP6 gene, developed multiple system disorders including premature death, paralysis, vision loss, seizures, and cardiomegaly. To varying degrees, these hallmarks of human MILS were passed along the maternal lineage for six generations. Symptoms were partially mitigated by intravenous administration of a mito-targeted AAV9 containing the wild-type human ATP6 allele delivered either prior to or after disease onset.

Results

Development of transgenic ATP6 (A6) mice by blastocyst injection of mitochondrially directed AAV2 carrying mutant human m.8993T>G ATP6. The mitochondrial double membrane has proven to be a formidable barrier to manipulating the mitochondrial genome. Still, viruses¹⁷, linearized and double-stranded DNA appear to traverse them¹⁸ and express exogenous mitochondrial genes¹⁹. To generate a mutant A6 mouse, we created the mutant human m.8993T>G ATP6 gene by site-directed mutagenesis of sc-HSP-ATP6 plasmid, which ATP6 gene was synthesized and fused a FLAG epitope followed by a stop codon (without a tRNA)²⁰ and mCherry using the mitochondrial genetic code, into a self-complementary AAV serotype 2, (scAAV) plasmid (sc-HSP-mutATP6FLAG+mCherry) (Fig. 1a, described in methods).

To direct the vector to mitochondria, we appended a cytochrome oxidase subunit 8 (COX8) pre-sequence into the N terminus of one of the three AAV capsid proteins (VP2)¹⁰. Three plasmids including modified pVP2cox8, sc-HSP-ATP6mutFLAG+mCherry, and pDG with mutations at positions 444, 500 and 730 (Y444F, Y500F, Y730F) to induce robust transduction²¹ were applied to produce the mitochondrial targeted scAAV2-mutATP6FLAG AAV2 viral vector. After packaging, mito-targeted scAAV2 aliquots were microinjected into mouse blastocysts and implanted into pseudo-pregnant females (Fig.1a). Of fifty-three founder mice generated by such blastocyst injections, six females with high ocular expression of mCherry, visualized by laser scanning ophthalmoscopy, were used to generate a total of 462 additional F1-F6 A6 mice. (Fig.1b, c & Supplementary Table 1). The final A6 colony included 252 females, 203 males and 60 unsexed pups that died within 8 days of birth. The litter size mostly ranged from 8 to 12 pups. High early mortality rates were a major characteristic of all generations (Supplementary Table 2).

Transgenic A6 mice develop human LS phenotypes including premature death, weight loss, paralysis, hunching, seizures, off balance, tumors, and visual loss. Transgenic A6 mice presented a spectrum of human-like LS or NARP phenotypes that included premature death, weight loss, paralysis, hunching, seizures, motor disfunction, tumors, and ocular defects. From the entire colony of 515 transgenic mice, F0 through F6, such human-like LS/NARP features were recorded in ~70% of animals (Supplementary Table 3). Of these, 167/515 (32%) died before 15 months including 101/515 (20%) that died before 6 months, revealing highly significant rates of premature death (Fig.1d, Supplementary Fig.1a & Table 3). Six of 53 F0 mice died before 6 months of age, including four males that died before 3 months. F1 mice incurred the highest 6-month mortality rate of 56%, significantly greater than all other groups (n=54/96), followed by an F2 mortality rate of 23% that was also significantly greater than the F3-F5 groups. Of F0 to F6 mice that survived >15-months, at least 50% displayed one or more phenotypes of hunching, paralysis, motor disfunction (off-balance), visual loss or seizures (Supplementary Table 3). Hunching was observed in 11% (38 of 350) A6 mice with a mean age 19.9 months (Supplementary Fig. 1). 3% of all generation A6 mice developed severe hinder limb paralysis with a mean age 19.6 months (Fig. 1e, Supplementary Table 3 &

129 Video 1). For motor dysfunction, we focused on F0 and F1 generations (Supplementary Table 3).
130 Accelerating rotarod testing of ambulation and balance revealed latency-to-fall times for F0-F1 mice that
131 were significantly less than the corresponding times observed for similarly aged wild type mice (Fig. 1g.).
132 Of 68/101 (67%) randomly selected F0 and F1 mice aged between 6-12 months, 59% showed significant
133 motor disfunction (Supplementary Table 3). Seizures were observed in 0.8 % of total F6 mice, with mean
134 age 22.5 months (Fig. 1f, Supplementary Table 3 & Video 2). As noted in the Table 3 Legend, such seizure
135 estimates are likely low because mice were not under constant surveillance. Reduced weight gain was
136 another significant clinical feature (Supplementary Table 3). A6 mice that succumbed to premature death
137 at age <6 months had average body weights of 17 g, significantly less than the corresponding mean weights
138 of age-matched wild types (25g), (Fig.1h). Combined assessment of ocular defects by pattern
139 electroretinogram (PERG), flash electroretinogram (ERG) and histology (H&E) revealed significantly
140 defective ocular functions in 35 of 43 (80%) A6 mice selected from F0-F2 generations (Supplementary
141 Table 3), included declines in 10 of 11 by PERG, (Fig. 1i, relative to wild types), and 5 of 11 founders by
142 FERG, (Fig. 1j, compared with wild types). Together the results support the presence of pronounced but
143 variable symptoms of A6 mice that mimic those reported for human MILS and NARP. In addition, at least
144 7% of A6 mice developed tumors with a mean age 20.1 months (Supplementary Table 3 & Fig. 6).

Figure 1 Development of transgenic A6 mice by blastocyst injection of mitochondrially directed AAV2 carrying mutant human m.8993T>G ATP6. **a.** Illustration of sc-HSP-mutATP6FLAG+mCherry plasmid containing the human mitochondrial heavy strand promoter (HSP) driving expression of the mutant human m.8993 T>G *ATP6* subunit gene fused to FLAG with a stop codon followed by mitochondrial-encoded mCherry. Founder mutant ATP6 transgenic mice generated by microinjection of mito-targeted AAV2-sc-HSP-mutATP6FLAG+mCherry into blastocysts. **b.** Mutant human m.8993T>G ATP6FLAG+mCherry detected by confocal laser scanning ophthalmoscopy (CLSO) of retinas of live A6 founder mice; infrared focused on the inner retina (left panel) and in fluorescence mode (right panel) (arrows indicate fluorescent retinal cells). **c.** Founder A6 female crossed with wild type (WT) CB57BL/6 males. **d.** Cumulative proportional survival curve of A6 mice up to 6 months after birth for each generation (n=101, $p<0.0001$, log-rank test). **e.** Transgenic A6 mouse with hind limb paralysis (n=16, see Supplementary Video 1). **f.** Transgenic A6 mouse with seizures (n=4, Supplementary Video 2). **g.** Latency-to-fall time of A6 founder mice decreased with increasing speed (4 to 14 rpm) compared to wild types (n=12, $*p<0.05$, $**p<0.01$ and $***p<0.001$). **h.** Body weights of A6 mice with symptoms of LS (premature death) are significantly less than wild type mice at ≤ 6 -month-old (n=11, $***p<0.001$). **i.** Pattern electroretinograms (PERG) illustrate significantly decreased amplitude of A6 founder mice compared with wild types at age 7 months (n=10, $***p<0.001$). **j.** Flash ERG reveals significantly reduced amplitude of A6 founder mice relative to wild types (n=5, $*p<0.05$). Statistical significance analysis of survival rates was performed by log-rank test for d.

Basal ganglia lesions, retinopathy, and central nervous system neuronal degeneration of A6 mice. To determine whether A6 mice develop lesions of the basal ganglia, a condition seen in many human cases of MILS²², we imaged five A6 and one wild type brains by MRI. In one four-month-old, female F2 A6 mouse with quadriparesis and severe weight loss (Fig.2c & Supplementary Video 3), we observed an increased T2 signal at the head of the caudate/putamen relative to the wild type, was bilateral and symmetric (Fig.2d-2e). By the same MRI technique, the other 4 mice with only mild symptomatic LS disease were similar to controls (Fig.2a-2b). Because of the high mortality rate observed in A6 mice, postmortem histological examination was primarily used to characterize anatomical pathologies. Ocular histopathology of deceased A6 mice revealed some retinas with normal ganglion cell, inner nuclear and outer nuclear layers (Fig. 2f) and some with complete loss of the photoreceptor and outer nuclear layers (Fig. 2g, Supplementary Fig1c).

Loss of the retinal photoreceptor layers is characteristic of retinitis pigmentosa (RP) of human NARP and MILS. Light microscopy of A6 mouse brains revealed foci of spongiform encephalopathy where neurons were lost, also a characteristic of human MILS (Fig. 2i-2j, Supplementary Fig. 1d)²³. Transmission electron microscopy of A6 mouse brains revealed, in addition to normal deep white matter with intact axons and myelin (Fig. 2m&n), clear cystic spaces at the lesion foci where fibers and neurons were decreased about 2-fold relative to control brains (Fig.2r). Examination of the cerebral cortices revealed increased prevalence of electron dense structures in neuronal mitochondria that coexisted with normal mitochondria, reminiscent of the mitochondrial degeneration that has been reported in MILS necropsy specimens^{24,25} (Fig. 2o-2q). Abnormal cristae of neuronal mitochondria were also apparent with cross-sectional areas up to 2-fold wilder than those of adjacent mitochondria (Fig 2s), a finding reported to be caused by loss of dimerization of the ATP synthase²⁵.

Figure 2 Brain MRI imaging and histopathology of A6 mice. **a-b.** T2-weighted MRI imaging of wild type mouse brain. **c.** Images of 4-month-old female quadriplegic A6 (F2) and wild type mice used for MRI imaging. **d-e.** T2-weighted MRI imaging of the brain of A6 mouse shown in Fig. c (see also Supplementary Video 3) showing a bilateral and symmetric T2 signal increase at the head of the caudate/putamen (arrows), relative to wild type (**a-b**). **f.** Histopathology of an A6 mouse eye shows a normal-appearing retinal ganglion cell (RGC) layer, inner nuclear layer (INL) and outer nuclear layer (ONL) (scale bar: 20 μ m). **g.** Histopathology of a different A6 mouse eye shows only the ganglion cell and inner nuclear layers with loss of the outer nuclear layers (scale bar: 20 μ m). **h.** Quantification of the ONL of A6 mice reveals significant loss relative to wild type (n=6, ***p<0.001). **i.** Histopathology of the A6 mouse brain motor cortex exhibits spongiform encephalopathy with vacuolization and loss of neurons (scale bar: 20 μ m). **j.** High magnification of the boxed area in panel (i) shows remaining neurons (arrows) and spongiform degeneration where neurons are lost (scale bar: 25 μ m). **k.** Histopathology of A6 mouse brain exhibits encephalopathy with vacuolization and loss of neurons (n=10). **l.** Transmission electron microscopy of the subcortical A6 white matter shows normal myelinated fibers. **m-n.** At the foci of spongiform encephalopathy, neurons and axons are lost leaving vacuolated spaces (*) and degenerating fibers (arrow). **o.** A6 neurons showing mitochondria with electron dense aggregates adjacent to normal mitochondria of the same cell. **p.** Boxed area of panel (o), showing mitochondria with electron dense aggregates (arrows). **q.** A6 mitochondria with widening of cristae (arrow). **r.** Quantification of the foci in A6 mouse brain shows a 2-fold increase relative to normal brain (n=3, **p<0.01). **s.** Quantification reveals 2-fold wider cristae in affected areas of A6 neuronal mitochondria relative to adjacent normal cristae (***p<0.001).

Heteroplasmy of mutant human m.8993T>G ATP6 in A6 mice. Next, we characterized expression of the human mutant ATP6 gene in mouse mitochondria of A6 founder and offspring mice. PCR analysis of DNA extracted from transgenic A6 mouse brain, spinal cord or liver mitochondria using human ATP6 specific primers revealed the presence of the mutant human *ATP6* gene (Supplementary Fig.1a&b). Sanger DNA sequencing confirmed that the PCR fragments were mutant human m.8993T>G ATP6 (Supplementary Fig.1c). SDS-PAGE western blot analysis showed that A6 mouse mitochondrial proteins reacted with FLAG and/or mCherry antibodies (Supplementary Fig. 1d). Immunofluorescence imaging of mutant human ATP6 fused FLAG revealed a perinuclear pattern in A6 mouse brain and spinal cord neuronal cells indicative of mitochondrial localization (Supplementary Fig. 1e). Then, we examined the ratio of

mutant human *ATP6* DNA relative to the endogenous mouse allele from whole tissues by semi-qPCR and quantitative real time (q-rt) PCR. The data indicate low ratios of human ATP6 to MsATP6 (Supplementary Fig.2a-c). Such ratios declined significantly in brains from subsequent F3 to F6 progeny (Supplementary Fig. 3c). Laser capture microscopy of founder mouse brain and retinal layers followed by qPCR revealed that human ATP6 levels were close to 10% in some areas of the brain, and up to 40% in the retinal outer layer (Fig. 3a & Supplementary Fig. 3d). By applying similar methods of quantification, the corresponding ratios from F1 early death brains ranged from 10%-20% (Fig. 3b), while some of those from F2 and/or later generation of mice with severe LS symptoms were as high as 70% (Supplementary Fig. 3e). Although higher mutant loads have been described in MILS and NARP autopsy specimens, low mutant loads have also been associated with shortened lifespan phenotypes²⁶⁻²⁸, consistent with proposals that factors in addition to overall mutant mitochondrial DNA load play important roles in determining disease phenotypes²⁹. In addition, based on the uneven distribution of mCherry fluorescence described above, it seems probable that the mutant *ATP6* DNA loads are dependent on the anatomical location of the affected tissues, particularly neurons (Supplementary Fig. 2f upper) and may, for example be influenced by the high bioenergetic requirements and larger mitochondrial densities of neuronal tissues.

Mutant human m. 8993T>G ATP6 protein assembles into murine complex V and impairs synthesis and hydrolysis of ATP. High magnification immunostaining revealed that the transgenic mutant human ATP6-fused flag protein complex co-localized with the mitochondrial marker Porin in A6 mouse brain and spinal cord mitochondria (Fig.3c). This finding supports the expression of mutant human ATP6 in murine mitochondria. To determine whether mutant human ATP6 protein assemble into the murine complex V, we performed one and two-dimensional blue native PAGE western blots. One dimensional blot probed with an anti-FLAG antibody identified a band corresponding to the FLAG-mutant human ATP6 fusion protein that migrated with complex V (Fig 3d, upper panel). Two-dimensional native blue/SDS-PAGE western blots also probed with anti-FLAG and anti-mCherry with a different blot, identified ~60 kDa bands that represent ATP6FLAG-mCherry (MW ~58 kDa, Fig.3d-1&2). We then reacted another gel with anti-ATP5a and

confirmed its reactivity with the ~60 kDa band (MW. ~55 kDa), the band identified as ATP6FLAG-mCherry, consistent with the migration of complex V (Fig.3d-3). With increased denaturing time the small band ATP6FLAG was decreased or lost, consistent with our results from SDS-PAGE western blots which only show the ~60 kDa, ATP6FLAG-mCherry band (Supplementary Fig. 2d). A possible explanation for a fusion protein is that the stop codon between ATP6FLAG and mCherry allow read through. To confirm the identity of complex V in western blots, the anti-FLAG antibody reactive bands were excised from one dimensional blue native gel, digested with trypsin to prevent the release of membrane bound proteins²⁴ and analyzed by mass spectroscopy. The results confirmed identity of the ATP synthase and therefore the assembly of ATP6FLAG into complex V (Supplementary Fig. 4c & Table 6). To investigate the effects of mutant human ATP6 on complex V function, we implemented in gel complex V activity assays. As shown in Fig. 3e and Supplementary Fig. 4a, we found that ATPase activity was impaired in parallel with the lost dimer bands from A6 mouse brain, spinal cord and muscle ATPase. In addition, the rate of ATP synthesis and oxidative phosphorylation (OXPHOS) in A6 brain extracts was decreased significantly, by ~ 50% compared to wild type brains (Fig. 3f). Taken together, these findings are consistent with assembly of mutant human A6 subunits into the murine complex V with corresponding loss of complex V activity, OXPHOS and mitochondrial ATP generation in brain, spinal cord and muscle of A6 mice.

Fig 3. Reduction of complex V activity and ATP synthesis by mutant human m.8893T>G ATP6. a. Quantitative rtPCR after laser capture microdissection of positive mCherry cells showing <10% ratio of mutant human ATP6 to mouse endogenous ATP6 in founder A6 mouse brains. b. Quantitative rtPCR showing ratios of mutant human ATP6 to mouse endogenous ATP6 of 10-20% in F1 premature death pup brains. c. High magnification immunostaining showing mutant human ATP6 fused flag co-localized with mitochondrial marker Porin in A6 mouse brain and spinal cord mitochondria (scale bar: 50µm). d. Two-dimensional native blue/SDS-PAGE western blots probed with antibodies against Flag and mCherry indicate migration of ATP6FLAG+mCherry (~60 kDa) consistent with complex V, and co-migrating at a similar molecular size as ATP5A (~60 kDa) (n=3, independent experiments). e. Complex V in gel activity assay showing decreased intensity of complex V dimers in A6 mouse brain relative to wild type brain (n=10). f. ATP synthesis and OXPHOS in A6 mouse brains were decreased by >50% relative to wild types (n=5, * $p<0.05$).

Construction of mito-targeted AAV9 with a cox8 signal sequence. To investigate the feasibility of a gene therapy strategy to ameliorate or preempt the disease phenotypes in this A6 mouse model of human MILS/NARP, we constructed a rescue vector by replacing the mutant human *ATP6* with wild-type human *ATP6* (Fig. 4a). Unlike AAV2, AAV9 efficiently crosses the blood-brain barrier following intravenous injection therefore we used AAV9 serotype for the rescue³⁰. A cox8 signal sequencing was added to the N terminal of the VP2 sequence to target wild type human ATP6 to mitochondria. Western blots of the packaged mito-targeted AAV9 vector stained respectively with anti-COX8 and AAV antibodies confirmed a product with the predicted slower mobility relative to the native VP2 (Fig. 4b, left and right panel).

Transduction of homoplasmic mutant m.8993T>G ATP6 cybrids with mito-targeted AAV9 carrying wild type ATP6. The presence of both mutant and wild type human *ATP6* DNA in AAV9-transduced homoplasmic mutant m.8993T>G ATP6 cybrids (NARP cells) were confirmed by amplifying DNA from transduced and non-transduced cells and digesting fragments with Sma I after 5-days selection in galactose media. The relative representation of wild type and mutant ATP6 can be determined because wild-type ATP6 lacks a Sma I site. As shown in supplementary Fig. 5a, whereas DNA from non-transduced cybrids was completely digested by Sma I, the fragments from AAV9-transduced cells were incompletely digested.

The identity of the undigested band was confirmed by excision from the gel and digestion with BstN1 that cuts wild-type ATP6, but not mutant human ATP6 (Supplementary Fig. 5a, lane 4). Using Sanger DNA sequencing, we further confirmed that the PCR fragments derived from transduced cells contained both mutant and wild-type human ATP6 alleles (Supplementary Fig. 5b, upper), whereas the amplicants obtained from uninfected cells had only the T>G allele (Supplementary Fig. 5b, bottom). The ratio of wild type to mutant ATP6 in mito-targeted AAV9 transduced cybrids, determined by relative band intensities was 25-47%. Immunofluorescence microscopy of transduced cybrids after staining for FLAG (present as ATP6FLAG in the AAV9 rescue vector) revealed perinuclear FLAG expression that co-localized with MitoTracker Red (Fig.4c). As a control, immunofluorescent images of homoplasmic m.8993T>G cybrid cells transduced with AAV9 containing wild-type ATP6FLAG without the cox8 targeting signal showed ATP6FLAG expression confined to the nucleus (Supplementary Fig.5e). Transmission electron microscopy of cybrids at 4 hours after infection with mito-targeted AAV9 revealed viral particles associated with membrane-bound organelles (Fig.4d-4f, Supplementary Fig.5c). One-dimensional blue native/PAGE western blots of mitochondria from transduced cybrids confirmed reactivity of a 550 kDa holo-complex V band with anti-FLAG antibody as well as with an anti-mt-ATP6 antibody (Fig.4g). Complex V in-gel assays comparing uninfected and transduced cybrids showed monomer and dimer bands with transduced cybrid cells with stronger intensities of assembled dimers. (Fig.4h). Together, these findings confirm successful transfer with subsequent expression and activity of wild type human ATP6 by mito-targeted AAV9 in the mitochondria of homoplasmic m.8993T>G cybrid cells.

Rescue of homoplasmic mutant m.8993T>G ATP6 cybrids by mito-targeted wild type ATP6.

Transduction of homoplasmic mutant m.8993T>G ATP6 cybrids with mito-targeted wild type ATP6-AAV9 significantly increased the viability of cells cultured in glucose-free, galactose media (selection media) relative to uninfected cybrids (Fig. 4i). Luciferin-luciferase assays to measure ATP with malate and pyruvate substrates, revealed significantly increased rates of ATP synthesis of 2.82, 3.49 and 3.01 nmol/min/μg (Fig. 4j) in cybrids transduced respectively at MOIs of 500, 1,000 and 5,000, compared with

1.3 nmol/min/μg of uninfected cybrids. Accordingly, the derived rate of OXPHOS was also significantly increased, respectively to 2.44, 2.79 and 2.7 nmol/min/μg at MOIs of 500, 1,000 and 5,000, relative to 0.93 nmol/min/μg of uninfected cybrids (Fig. 4j)

Fig. 4 Analysis of m.8993T>G cybrids (NARP cells) transduced by mito-targeted AAV9 with wild-type human *ATP6*. **a.** Illustration of the sc-HSP-ATP6FLAG plasmid (upper panel) containing the human mitochondrial heavy strand promoter (HSP) driving expression of wild-type human ATP6 fused in frame to a FLAG epitope and the sc-HSP-ATP6FLAG-mCherry plasmid (bottom panel) that contains mitochondrially encoded mCherry. **b.** Western blot of mitochondria, COX8-targeted AAV9 stained with anti-COX 8 antibody (left panel). Western blot showing that VP2-COX8 is larger than the unmodified VP2 (right panel). **c.** Cybrids infected with mito-targeted AAV9 containing wild-type ATP6FLAG selected in glucose-free galactose media exhibit perinuclear FLAG expression that co-localizes with MitoTracker Red (bottom panels, scale bar: 20 μ m). **d.** Transmission electron microscopy of cybrids transduced with mito-targeted AAV9 display mito-targeted AAV viral particles compartmentalized in membrane bound vesicles. **e-f.** Higher power images reveal that the contents of the viral particles are selectively delivered to mitochondria (arrows) rather than the nucleus (Nu). **g.** One dimensional native blue/PAGE (left panel) western blot showing a band from infected cybrids (NARP/IN) detected by anti-FLAG antibody stain (middle panel) also reacted with a mt-*ATP6* antibody confirming its identity as ATP6 (right panel). **h.** In gel complex V activity assay showing increased intensities of dimer and monomer bands of complex V in infected (NARP/IN) versus uninfected cybrids (NARP). **i.** Survival of AAV9 transduced cybrid in galactose selection media was significantly and dose-dependently increased relative to uninfected cybrid cells at MOIs of 500 and 1,000 (** p <0.01, * p <0.05). **j.** Relative to uninfected cybrids, the overall rate of ATP synthesis and OXPHOS of infected cells increased significantly and dose-dependently with MOIs of 500 and 1,000 (** p <0.01). Data are representative of three independent experiments with similar results.

Expression of wild type human ATP6 in mouse brain following intravenous delivery of mito-targeted AAV9. Because AAV9 crosses the blood-brain barrier and has been reported to rescue mice with LS caused by a nuclear gene knockout⁸, we tested AAV9 transduction first in control mice by intravenously injecting 10 μ L of self-complementary mito-targeted AAV9 (1.86×10^{12} vg/mL) expressing wild-type human ATP6FLAG+mCherry into the right orbital venous sinus. Using laser-scanning ophthalmoscopy focused on the inner retina (Fig. 5a), we detected ATP6FLAG+mCherry protein in both right (Fig. 5b) and left eyes (Fig. 5c) of live injected mice but not in noninjected control mice. Relative to noninjected mice (Fig. 5e-h), confocal microscopy of the brains of injected mice further revealed fluorescence of the ATP6FLAG+mCherry protein in the brain (lower magnification, arrow shown in Fig. 5j, Fig. 5i-5l).

Punctate and perinuclear ATP6FLAG+mCherry stained by FLAG colocalized with porin, a mitochondrial marker (arrow shown in Fig. 5n of Fig. 5m-5p and at higher magnification in Fig. 5r of Fig. 5q-5u). These findings suggest that the AAV9 vector traversed the blood-brain barrier into mitochondria of central nervous system (CNS) neurons. Therefore, we tested whether delivery of our AAV9 vector was effective in preventing and/or reversing symptoms of MILS in this mouse model.

382

383 **Figure 5 Intravenous administration of mito-targeted AAV9 with wild-type human *ATP6* to wild type mice. a.**

384 Illustration of intravenous injection into the mouse orbital venous sinus (n=7). **b.** Laser scanning ophthalmoscopy of

385 a live mouse focused on the inner retina (infrared=IR) with mCherry labeled retinal cells in the right (c) and left eyes

386 (d) (arrows indicate the optic nerve head). **e-h.** Postmortem micrographs of an untreated mouse brain reveal absence

of FLAG immunostaining. **i-l.** Micrographs of injected mouse brains show FLAG tagged ATP6 in the motor cortex (arrows) (Scale bar: 50 μ m). **m-p.** and **q-u.** Higher magnification shows punctate and perinuclear ATP6FLAG that colocalizes with mitochondrial porin (arrows, Scale bar: 20 μ m and 10 μ m).

Amelioration of LS symptoms by intravenous delivery of mito-targeted AAV9 containing wild-type human *ATP6*. Transgenic *A6* mice that developed symptoms of severe paralysis or hunching and that were assigned as high risk for imminent death, were administered intravenous injections of 1.86×10^{10} vg of mito-targeted AAV9 containing wild-type human *ATP6*. Figure 6a shows examples of the locomotion of a high-responder rescued mouse that had severe hind limb paralysis prior to treatment. Symptoms appeared to stabilize at post injection >Day 3, and the mouse survived to Day 65 (Supplementary Video 4). Rescues were initiated in two groups and survival and pathology were tracked subsequent to rescue injections (D0). Group 1 included similarly aged *A6* mice with severe hind limb paralysis. Log rank tests showed a 28-day mean survival (range 5-65 days), of such mice in the treatment group (n=11), while 5 untreated *A6* mice with similar hind limb paralysis died within 4 days of D0 (Fig.6b). In group 2, that included similarly aged *A6* mice with severe hunching, using the same protocol, the mean survival of treated mice (n=14) was 16 days (range 5-47 days), compared with <3 days for the non-rescued hunching group (n=12) (Fig. 6c). The results are consistent with the rapid expression time and phenotypic complementation by the scAAV9-*hATP6* vector seen in transduced cybrids (Fig. 4) and indicate significantly improved survival of treatment groups relative to non-rescued controls.

a

b

c

Fig. 6 Amelioration of A6 mouse LS disease onset by intravenous administration of AAV9 with mito-targeted wild type *ATP6*. **a.** A6 mouse with hind limb paralysis with mito-targeted AAV9 containing wild-type human *ATP6* delivered by i.v. injection at D0. No change in paraplegia was seen during the subsequent 2 days whereas by day 3, an improvement in hind limb function was apparent (see Supplementary Video 4). **b.** Log-rank test showing significantly extended lifespans of rescued paretic A6 mice compared with untreated controls (n=16, $p<0.0001$). **c.** Log-rank test showing significantly extended lifespans of hunched A6 mice relative to untreated hunched controls (n=26, $p<0.0001$).

Suppression of LS phenotypes and enhanced Complex V activity by early rescue. Preventative treatment was initiated in non-symptomatic 3-month F2 A6 mice that were selected from the progeny of high-mortality F1 dams. Thirty-four mice from 3 different A6 mice were divided randomly into 10 rescue and 24 non-rescue controls. The treatment group received intravenous injections of 1.86×10^{10} vg mito-targeted AAV9 rescue vector while controls received PBS. All rescue animals (100%) survived through 21 months, while untreated A6 mice sustained 8 of 24 premature deaths between 5-12 months and a cumulative

432 survival rate of 67% (Fig. 7a). Log rank tests after 15+ months, indicate 56% (9 out of 16) of untreated A6
433 mice presented severe LS phenotypes including paralysis (1), hunching (4), weight loss (3) and tumors (1),
434 whereas 80% of the treatment group were non-symptomatic. The exceptions were 2 treatment mice that
435 showed significant weight loss (Fig. 7b). Three months after injection, visual function measured by pattern
436 ERG, was significantly improved in treated A6 mice over that of the untreated group (Fig.7c).
437 Histopathological analysis confirmed that the brains and spinal cords of rescued A6 mice (Fig. 7e&h) were
438 similar to wild-type mice with markedly less vacuolization and cell loss (Fig. 7d&g) compared to the severe
439 loss seen in the non-rescue group (Fig. 7f&i). In addition, non-rescued survivors lost an average of 30%
440 body weight at age 1-year (n=25), whereas the mean weights of rescued mice were no different from age-
441 matched wild-type controls. The ratio of heart to body weight of hunching non-rescued A6 mice was almost
442 twice that of wild-type or rescued A6 mice. Cardiomegaly, a component of MILS, was also apparent only
443 in the non-rescued A6 hearts (Fig. 7l). Immunofluorescence images of mutant human ATP6 fused FLAG
444 in heart sections revealed a perinuclear expression pattern consistent with presence of the mutant human
445 ATP6 protein in heart mitochondria (Supplementary Fig. 2f, bottom panel). In-gel activity measurements
446 of ATP hydrolysis to assess complex V activity revealed significantly improved ATPase activity of rescued
447 A6 mice (brain) relative to untreated A6 mice (Fig. 7m&n). The results are consistent with efficient AAV9-
448 mediated delivery of wild type ATP6 by intravenous injection, complementation of the defective mutant
449 ATP6 and effective amelioration of the MILS phenotypes by early gene delivery in this model.

Fig.7 Rescue of A6 mouse LS phenotypes by early intravenous administration of mito-targeted AAV9 with wild type *ATP6*.

a. Cumulative proportion survival curves showing 100% survival of treated A6 mice through 12 months and untreated animals with a cumulative 67% Kaplan-Meier survival rate also at 12 months (8 of 24 died before 12 months, $p=0.0462$, log-rank test). **b.** Log rank test showing untreated A6 mice had cumulative LS phenotypes of 56% (9/16) including paralysis (1), hunching (4), weight loss (3) and tumor (1) at age 15-21 months, whereas 80% of rescued mice presented with normal (no disease) phenotypes at age 21 months, while the remaining 2 mice (20%) showed weight loss as the only identified LS-related phenotype (Log-rank, $p=0.0217$). **c.** Pattern ERG analyses of treated mice showed an almost 2-fold increase in amplitude relative to untreated controls ($p<0.001$). **(d-f)** Neuropathology respectively of wild type, rescued and non-rescued A6 mouse brains showing cystic spaces and neuronal loss only in the untreated group. **(g-i)** Neuropathology respectively of wild-type, treated and untreated mouse spinal cords showing cystic spaces and neuronal loss in untreated groups (scale bar: 20 μm). **(j)** Body weights of untreated A6 mice are significantly less than those of treated A6 mice and age-matched wild-types ($*p<0.05$, $***p<0.001$). **k.** Heart to body weight ratios of treated A6 mice are similar to those of wild-type mice with significantly less cardiomegaly than untreated A6 mice (wild-type vs A6, $****p<0.0001$; treated A6 vs A6, $***p<0.001$). **l.** Representative hearts of wild type (WT), treated A6, and untreated A6 mice with cardiomegaly. **m.** In gel activity assays for complex V showing increased intensity of dimer F0F1 ATPase bands of treated A6 mouse brain prior to disease onset compared to untreated A6 mice. **n.** Quantification of the density of the monomer and dimer F0F1 ATPase bands of treated A6 mice prior to disease-onset (A6/IN) shows significantly increased dimer F0F1 ATPase bands relative to untreated A6 mice and no significant difference from wild types ($n=3$, $***p<0.001$).

Heteroplasmy of human *ATP6* alleles in rescued A6 mice. We used PCR and restriction fragment length polymorphism (RFLP) to quantify heteroplasmy of mitochondria in the A6 rescue mouse brain, spinal cord or liver mitochondrial DNA. Sequencing of PCR amplified DNA obtained from mitochondria of rescued A6 mice revealed the presence of both wild type and mutant human ATP6 alleles (Supplementary Fig. 6a&b). To determine the relative contributions of wild type and m.8993T>G mutation of ATP6, the signal intensities of the relevant DNA bands separated by agarose gels were measured using image J and the corresponding percentages calculated for each sample. As shown in Supplementary Figure 6c, the ratio of wild type to mutant ATP6 in rescued mice was close to 50%. Western blots confirmed ~10-fold increased

intensity of the ATP6FLAG+mCherry protein band in samples from rescued versus non-rescued A6 mice from brains (Supplementary Fig. 6d&e).

Discussion

We used a mito-targeted AAV2 vector microinjected into mouse blastocysts to mediate germline transmission of mitochondrial encoded subunit 6 of the human ATPase gene variant m.8993T>G. Female founder mice with high ocular expression of the transgene were backcrossed with wild-type C57BL/6 males for 6 generations to generate a total of 53 F0 and 446 progeny F1 to F6 mice, 70% of which displayed one or more hallmark symptoms of LS/NARP. Presence of the m.8993T>G variant DNA and protein in transgenic animals, localization to mitochondria and assembly into complex V with dysfunctional F1ATPase activity were confirmed by molecular, immunoblot and immunostain analyses of brain, spinal cord, ocular or liver mitochondria. Because of the prevalence of ocular defects and high vulnerability of neuronal ocular tissues to mitochondrial variants that cause LS including the m.8993T>G mutation, we focused our molecular genetic analyses primarily on these tissues in F0, F1 and F2 mice. Combined laser capture and RT-qPCR analyses revealed variable heteroplasmy between different retinal locations that approached 40% in the outer nuclear layer and 20%-25% in the ganglion cell inner nuclear layers and around 10% in founder brain. This compared with 10%-20% in F1 brain sections. Representation of the m.8993T>G variant decreased markedly in F3-F6 generations in favor of the wild type allele, and there was evidence of positive correlations between transgene expression and retinal cell loss. Sustained disease phenotypes of F1 and F2 generation mice despite the absence of significant transgene replication, may be attributed to our selection of high expression founder mice for F0 backcrossing, tissue-specific responses to mutation load, and the apparent absence of tight associations between disease phenotypes and degrees of heteroplasmy often seen in human mitochondrial disease (see below). The results are also consistent with previous studies from our group where we reported similarly variable tissue heteroplasmy after germ line delivery of a human ND4 m11778G>A gene variant to mouse mitochondria. In these studies, we reported a maximum transgene expression of 20% in the retinal RGC layer, 0.08% in the inner nuclear layer

and undetectable expression in the outer nuclear layer. Mice with the variant mitochondrial gene displayed a robust disease phenotype that closely mimicked symptoms of human LHON disease despite the relatively low transgene penetration and limited replication⁹.

LS-related symptoms observed in 70% of F0-F6 mice included premature death, paralysis, seizures, weight loss, off balance and visual loss. Inclusive of all offspring, 32% of mice died before 15 months of age with F1 mice showing the highest mortality rate of 50% at 6-months, followed by 23% mortality of F2 mice at 6 months. Of surviving mice, ~50% became hunched, paralyzed, developed visual loss and/or seizures and weight loss with varying severity and age of onset. Together the results indicate widespread and pronounced but variable disease phenotypes of A6 mice, in many respects similar to human MILS. The presence of the most severe disease phenotypes in F0 and F1 mice and loss or reduced severity and prevalence of such phenotypes in subsequent generations in parallel with decline of mutant gene loads is consistent with the expected role of gene load in disabling mitochondrial functions and promoting disease expression in this model.

The importance of gene load has also been described in patients with MILS/NARP, and whereas there is a general association between degree of heteroplasmy and phenotype severity, precise correlations of clinical phenotype with mutation load are rare with few clear thresholds corresponding to a given phenotype, and many instances where severe symptoms are associated with lower mutant gene loads and vice versa^{1, 31, 32}.

For example, Stendel et al³³, recently quantified heteroplasmy and clinical presentation of 132 subjects with known pathogenic MT-ATP6 mutations and related the genotype with neurological manifestation of the disease. They reported high degrees of heteroplasmy but wide variations in both symptomatic and non-symptomatic individuals. For symptomatic subjects, the mean heteroplasmy was 95% with a range of 20%–100%, while for unaffected individuals the mean was 73%, also with a range of 20%–100%. Similarly, while most cases of LHON are homoplasmic, severe symptoms have been documented with as little as 33% mutated mitochondrial DNA³⁴, and a m.5545C>T mutation affecting tRNA specificity that usually carries a 70-90% threshold was reported in one case study to confer a severe disease phenotype at less than 25%

in affected tissues, and between 4 and 8% in cybrids generated using patient mitochondria³⁵. The apparent clinical dominance of such low copy number mitochondrial mutations has also been reported in rodent models^{9, 36, 37, 38}, and may reflect tissue-specific and/or environmental effects including local bioenergetic demands and levels of antioxidants^{39, 40, 41, 15}. Additionally, the basal rate of metabolism, or mass-specific-metabolic rate of mice is seven-fold greater than that of humans, placing correspondingly greater demands on mitochondrial function and energy production especially in high energy demand tissues⁴². Consequently, higher rates of ROS production, major drivers of mitochondrial disease including LS, and predicted to increase with higher mass-specific-metabolic rates, are more likely to destabilize redox homeostasis in mouse models, increasing susceptibility to defective mitochondrial function. Such activities are thought to contribute to the reduced lifespan and increased vulnerability of mice to age-related tumorigenesis relative to humans, both key characteristics of clinical LS. The apparent dominant negative actions of such mitochondrial mutations can account for the persistence of severe phenotypes in our F3 to F6 progeny despite marked dilution of the m.8993T>G variant in favor of wild type ATP6. Also, in agreement with our previous study, we found no evidence for integration of AAV-encoded ATP6 DNA into the mitochondrial genome (data not shown) and we attribute transgene dilution to low or negligible replication of episomal AAV transgene DNA, as previously reported⁹.

Whereas the correlations between disease phenotype, variant m.8993T>G load and defective ATPase activity, including the markedly higher retinal cell loss associated with variant load in the outer retinal layers of F0–F2 mice are consistent with molecular pathologies reminiscent of clinical MILS/NARP, our demonstration that the disease phenotype was rescued by AAV9 delivery of a wild type ATP6 subunit gene not only supports the link between the disease phenotypes and mutant ATP6 load, but also provides a basis for treatment of LS by gene therapy. By using the same strategy described previously by our group for rescue of LHON mice⁹, we delivered a mito-targeted wild type ATP6 subunit gene to m.8993T>G transgenic mice by orbital venous sinus injections of AAV9 to achieve efficient penetration of the blood brain barrier and gene targeting to mitochondria. As also described in the previous study, rescue was robust

especially when gene delivery was implemented prior to symptom onset. Our molecular analyses of heteroplasmy in rescued mice confirmed the co-presence of variant and rescue ATP6 DNA and protein in rescued mitochondria as well as significantly improved ATPase activity as assessed by in-gel activity of ATP hydrolysis in early rescue tissue samples. Rescued phenotypic parameters of mice containing the m.8993T>G mutation treated at age 3-months included 100% survival through 15 months without severe symptoms, compared with a 12-month 33% mortality of non-rescued transgenic mice, significantly improved visual function as well as normalized body weights and resolved cardiomegaly. In addition, histopathology confirmed markedly less vacuolization of brain and spinal cords in rescued animals and reduced cell loss.

The absence of animal models that accurately reflect genotypes and corresponding disease phenotypes of mitochondrial diseases, including MILS/NARP have hindered development of effective therapies⁴³. Nuclear localization and transcription (allotropic expression) of the ATP6 m.8993T>G mutation followed by cytoplasmic translation and transport into mitochondria was reported to mimic mitochondrial disease in mice⁴⁴. Homoplastic models can be approximated by gene knockout and such models have been used to demonstrate amelioration of disease phenotypes by AAV-based complementation of an Ndufs4 gene knockout in mouse models of LS^{4, 8}. Similarly, mutant mtDNA loading of mice with a genetically manipulated heteroplasmic m.5024C>T gene mutation was markedly reduced by AAV9-mediated delivery of mito-targeted TALENs that selectively degraded mutant mtDNA and restored normal levels of the affected mitochondrial alanine tRNA gene product⁴⁵. To our knowledge, studies reported here represent the first to directly target mitochondria with a human m.8993T>G mutation, establish and characterize a disease phenotype that is consistent with clinical presentation of patients with the same mutation, and confirm gene therapy rescue of affected animals by complementation with a similarly targeted AAV9 vector expressing wild type ATP6. The results support further development of gene therapy approaches for MILS/NARP and other mitochondrial diseases perhaps in combination with newer pharmacology based on amelioration of oxidative stress caused by excessive ROS leakage from defective mitochondria and known

to play a central role in cytotoxicity and cell death during mitochondrial disease^{15, 46}. The studies support and extend our previous work on gene therapy for LHON that are now in clinical development⁴⁷.

Materials and Methods

Ethics statement. This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All mouse studies were approved by the University of Miami Institutional Animal Care and Use Committee (IACUC).

Plasmids and constructs. A human mitochondrial *ATP6* gene fused with a FLAG epitope was synthesized by Genscript, Inc. and cloned in a self-complementary adeno-associated virus (AAV) vector, in which expression was driven by the human mitochondrial heavy strand promoter (HSP). To clone the plasmid sc-HSP-mtATP6FLAG-mCherry, plasmids sc-HSP-mtATP6 and sc-HSP-ND4-mCherry¹⁶ were respectively digested by Xba I and BamH I and religated to create sc-HSP-mtATP6FLAG-mCherry, in which mCherry follows the mtATP6FLAG stop codon (see Fig. 1A). The human mutant m.8993 T>G ATP6 was generated by site directed mutagenesis (Quikchange II XL site-directed mutagenesis kit, Stratagene) with substitution of T to G at nucleotide position 8993. The primers used for site directed mutagenesis are listed in Supplementary Table 4. Plasmid AAV9COX8 was constructed by a large fragment polymerase chain reaction (PCR) and seamless cloning of the cytochrome oxidase subunit 8 mitochondrial targeting sequence (COX8) to the N terminus of VP2 of the AAV9 capsid. The forward primer was designed using the COX8 fragment following VP2 and the reverse primer was the fragment of complementary COX8 following the C terminus of VP1. The primers designed for cloning COX8 to AAV9VP2 are listed in Supplementary Table 5.

Mito-targeted AAV vector production and purification. All mito-targeted AAV vector production and purifications were performed in-house. Mito-targeted AAV2-ATP6FLAG, AAV2-ATP6FLAG-mCherry and AAV2-mutATP6FLAG-mCherry were respectively packaged using plasmids sc-HSP-mtATP6FLAG, sc-HSP-mtATP6FLAG-mCherry or sc-HSP-mutATP6FLAG-mCherry, with pVP2COX8 AAV at ratios

1:1:4. Mito-targeted AAV2/9-ATP6FLAG-mCherry was produced by combining four plasmids: AAV2/9, sc-HSP-ATP6FLAG-mCherry, VP2COX8 and pHelper (1:1:1:2) or three plasmids: AAV2/9COX8, sc-HSP-ATP6FLAG-mCherry and pHelper (1:1:2). Briefly, 293AAV cells were cultured at 37°C to 50%-70% confluency with 10% heat inactivated (HI) FBS DMEM containing 100 U/mL of penicillin and 100 µg/mL streptomycin before transfection. Calcium phosphate transfection was performed according to a standard procedure³⁶. Vectors were purified by gradient ultracentrifuge and FPLC using a GE AKTA Purifier. The viruses were concentrated, and buffer exchanged in PBS, with a Biomax 100kDa concentrator (Millipore, Billerica, MA). Viral titers were determined by SYBR-Green real-time PCR relative to a standard curve. Viral purity was validated by Coomassie blue stained SDS-PAGE, assayed for sterility, divided into aliquots, and stored at -80 °C.

Animal Development. Microinjections of mitochondrial-targeted AAV vectors into fertilized oocytes were performed by the UNC transgenic core facility using a standard microinjection protocol³⁷. Briefly, hybrid (C57BL/6J×DBA/2J) F1 mice, referred to herein as B6D2F1 mice, were obtained from the Jackson Laboratory for production of fertilized oocytes. Superovulation was stimulated by i.p. injection of B6D2F1 females with 5 IU pregnant mare serum gonadotropin (National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases) after 46–48 hr. by injection of 5 IU human chorionic gonadotropin (HCG) (Sigma-Aldrich, St Louis, MO). Following HCG injection, females were mated with B6D2F1 stud males, and oocytes were harvested the following day. Approximately 1–2 pL of the MTS-AAV-HSPmutantATP6FLAG+mCherry virus [1.8×10^{12} vector genomes (vg)/mL] was microinjected into fertilized oocytes using a continuous flow injection mode. Surviving eggs were implanted into the ampulla of pseud-pregnant Swiss Webster (Taconic) recipient females. After weaning the resulting offspring were transferred to the University of Miami and analyzed for the presence of the transgene. Viral injections resulted in the offspring listed in Supplementary Table 1.

Cell culture and AAV transduction. Homoplasmic m.8993T>G cybrids (NARP cells from Dr. M. Tariq Bhatti) were cultured with high glucose Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen,

627 Cat.11995) with 10% HI-FBS and 1% penicillin and streptomycin containing 4.5g/l glucose, 2mM
628 glutamine and 110µg/ml sodium pyruvate, and supplemented with 50µg/ml uridine. 80% confluent cybrids
629 cells were transduced by mito-targeted AAV9/ATP6FLAG-mCherry (1.8×10^{12} vg/ml) in DMEM without
630 FBS for 2-3hrs, followed by DMEM with 10%FBS for ~36 hours and selection in glucose-free media with
631 50µg/ml galactose for 5 days. For electron microscopy (EM), cybrids on gold mesh EM grids were infected
632 with mito-targeted AAV9 containing wild-type ATP6 at MOI of 5000 and examined for AAV particles 2-
633 4 hours later without counterstaining.

634 **Cell survival assay.** Homoplasmic m.8993 T>G cybrids in 96-well plates at density 2000 cells per well
635 were infected with mito-targeted AAV containing wild-type ATP6 at multiplicities of infection (MOI) 0,
636 500, 1000, 5000 and 10,000 for 5hrs in triplicate. After five days of selection in glucose free galactose
637 media, viable cell numbers were counted using a BioRad, TC20.

638 **ATP synthesis assay.** Luciferin-luciferase assays were used to quantify ATP synthesis by cybrids.
639 Homoplasmic cybrids were seeded in 6-well plates and infected with mito-targeted AAV9-mtATP6FLAG
640 at MOIs 0, 500, 1000, 5000 and 10,000 followed by selection for 5 days and culture to confluence in normal
641 growth media with 50µg/ml uridine. Rinsed pellets of 5×10^5 cells were resuspended in ATP synthesis buffer
642 The rate of ATP synthesis of excised tissues was measured by chemiluminescence using a modified
643 luciferin-luciferase assay in digitonin-permeabilized tissues with the complex I substrates malate and
644 pyruvate in real-time using a luminometer (Optocom I; MGM Instruments, Hamden, CT) in a total volume
645 of 200µL that included the following: 160µL of Buffer A (150 mM KCL, 25 mM Tris-HCL, 2 mM EDTA,
646 0.1%BSA, 10 mM potassium phosphate, 0.1 mM $MgCl_2$, pH 7.4), 2.5 µL of 1mM Malate in buffer A, 2.5
647 µL of 1mM Pyruvate in buffer A, 5µL of 0.1 mM ADP in Buffer A, 5µL of 0.15mM adenosine
648 pentaphosphate in water, 10µL cells, 10µL of buffer B (194.4µL of solution A, 1.6µL of 100 mM Luciferin
649 in water and 4µL of 1 mg/ml Luciferase in 0.5M Tris-acetate pH7.75), 5µL of digitonin (50µg/ml) with or
650 without 5µL 0.05mg/mL oligomycin in ethanol. Oxidative phosphorylation (OXPHOS) was calculated by

total ATP (without oligomycin) minus the value with oligomycin. ATP and oxidative phosphorylation indices are expressed as units per mg protein.

PCR, Sanger DNA sequencing and RT-PCR. Mitochondria were isolated from cybrids or mouse tissues using a dedicated isolation kit according to the manufacturer's instructions (Mitosciences, Cat. MS850 and MS852). Mitochondrial DNA was extracted with a DNeasy Blood & tissues kit (QIAGEN Cat.69504). Sequences of forward and reverse primers to amplify the 604bp ATP6 fragment were respectively GCT TCA TTC ATT GCC CCC AC, and AGG CGA CAG CAG TTT CTA GG. The 695bp fragment was amplified with the human ATP6 GCT TCA TTC ATT GCC CCC AC as forward primer and Flag CTT GTC GTC ATC GTC TTT GTA GTC as reverse primer. The two sets of primers were used to amplify both human wild-type and mutant ATP6 fragments from cybrids followed by sequencing to confirm the identity. To determine relative contributions of wild type and m.8993T>G mutation ATP6, the signal intensities of the relevant DNA bands separated by agarose gel were measured using Image J.

Mitochondrial isolation, BN/PAGE, immunoblotting analyses and complex V by in-gel assay. Mitochondrial pellets were solubilized in Native Page Sample buffer with 2.5% digitonin, incubated on ice for 15 mins, centrifuged at 20,000g for 30 minutes at 4°C and the supernatants were aliquoted and stored in -80°C. Protein concentration were determined by BCA protein assay, BSA was used as a standard. Proteins separated on precast native PAGE™ 4-16% Bis-Tris gels from life technologies and processed as per manufacturer's instructions (Cat. BN1002BOX). Fifty µg of mitochondrial protein were used for one dimensional native blue gel Coomassie staining and complex V in gel assays. Transfer membranes were fixed in 8% acetic acid for 15 min, blocked for 1 hr. and detected respectively with FLAG (1:1000, Origen, Cat. No. TA100011), mCherry antibody (1:500, Abcam, Cat. No. ab125096), mt-ATP6 antibody (1:1000, Cat. PA5-103625) and ATP5A antibodies (1:1000, Abcam, ab28777). For 2-D immunoblotting, 200µg of mitochondrial protein were used and strips from the first dimension were cut and denatured in 50mM DDT one hour before running the second dimensional gel. The gels were transferred to PVDF membrane and detected respectively with FLAG, mCherry, ATP5A and mt-ATP6 antibodies. For complex V in-gel

676 activity, washed gels were incubated in 50 mM Tris pH 8.6 for one hour followed by incubation with 35
677 mM Tris, 270 mM glycine, 14 Mm MgSO₄, 5 mM ATP and 0.2% Pb (NO₃)₂ at 37°C for 3 hrs. or overnight⁴⁸.
678 After washing in water, gels were scanned and photographed. Western blot membranes were respectively
679 reacted with anti-FLAG, anti-mCherry, and anti-VDAC/Porin antibodies (Abcam, Cat.ab17434) with AAV
680 transduced samples respectively stained by cox8 and AAV antibodies (anti-cox8 antibody, Cat. LS-
681 C664745; anti-AAV B1, Progen, Cat.65158, mouse monoclonal B1). ECL chemiluminescence was used to
682 visualize the bands using a FUJI400 imager.

683 **Mass spectrometry.** Mass spectrometry (MS) was performed by the Harvard University Mass
684 Spectrometry core facility. Protein bands were excised from 1 and 2-D blue native gels, digested with trypsin
685 and subject to MS. Briefly, capillary reverse phase HPLC separation of protein digests were performed on
686 a 10-cm 75-μm inner diameter PepMap C18 column (LC Packings, San Francisco, CA) in combination with
687 an in-house capillary HPLC system operated at a flow rate of 200 nl/min. In-line mass spectrometric analysis
688 of the column eluate was accomplished by a quadrupole ion trap instrument (LCQ; ThermoFinnigan, San
689 Jose, CA) equipped with a nanoelectrospray source. Fragment ion data generated by data-dependent
690 acquisition via the LCQ were searched against the NCBI nr sequence database using the SEQUEST (Thermo
691 Finnigan) and Mascot (Matrix Science, Boston) database search engines. In general, the score for SEQUEST
692 protein identification was considered significant when dCn was equal to 0.08 or greater and the cross-
693 correlation score was greater than 2.2. MASCOT probability-based MOWSE scores above the default
694 significant value were considered for protein identification in addition to validation by manual interpretation
695 of the tandem mass spectrometry data.

696 **Magnetic Resonance Imaging (MRI).** High-resolution (4.7T magnet) MRI (Oxford Instruments Oxford,
697 UK) was performed on mildly symptomatic F0 A6 mice maintained under 1.5 to 2% isoflurane anesthesia.
698 All animals were placed in a prone position with heads held firmly by a purpose-built head coil. T2-weighted
699 images of the brain were acquired.

700 **Laser microdissection and human mtATP6 copy number analysis.** Frozen blocks of mCherry-stained
701 or unstained retina, spinal cord, brain of transgenic A6 mice were cut into 8-18 μ M and placed on director
702 slides. Laser capture microdissection was performed using a Leica LMD6500. Mitochondrial DNA was
703 extracted from mCherry positive cells. Taqman RT-PCR assays were performed to quantify human and
704 mouse mitochondrial ATP6 copy numbers with mtATP6 plasmid DNA as a standard curve (Thermo
705 scientific mouse mtATP6: Cat. Mm03649417_g1; human mtATP6: Hs02596862_g1).

706 **A6 mouse phenotype and histopathology.** Phenotypes of A6 mice including weight loss, off balance,
707 ocular defects, hunching, seizures, paralysis and mortality were observed and recorded over six generations.
708 A6 mice were weighed and anesthetized using ketamine/xylazine (i.p. 80 mg/kg and 20 mg/kg body weight).
709 Tissues of A6 mice including brain, spinal cord, eyes, optic nerve, muscle, spleen, liver and lung were fixed
710 in 10% formalin. H&E staining was performed by the histology core lab at the University of Miami. Images
711 were captured using a Leica fluorescence microscope (Buffalo Grove, IL).

712 **Brain sections and TEM.** Brains were immersion fixed in 2% paraformaldehyde, 2% glutaraldehyde in 0.1
713 M PBS buffer, and post fixed in 1% osmium tetroxide, 0.1 M sodium cacodylate-HCl buffer, pH 7.4. Tissues
714 were dehydrated through ethanol to propylene oxide, infiltrated, and embedded in epoxy resin. Semithin
715 longitudinal sections (0.5 μ m to 1 μ m) of the brain were stained with toluidine blue and placed on nickel
716 grids for ultrastructural examination using a JEOL JEM 1400 TEM and GATAN camera (Orius SC200
717 CCD). Image quantifications were performed by image J.

718 **Rotarod assay.** A6 and wild-type C57BL/6 mice motor and balance were evaluated using an LE8500
719 rotarod (Panlab, Spain) as per manufacturer's instructions. Time to fall was recorded on the instrument
720 screen with a starting speed of 4 rpm that was gradually increased. Each mouse was given one trial
721 (maximum time 10 minutes). The time of latency-to-fall and speed were recorded for each trial.

722 **Pattern and flash electrophysiology.** Mice were weighed, anesthetized with ketamine/xylazine (i.p. 80
723 mg/kg and 20mg/kg body weight) placed on a heating pad (37°C) and restrained using a bit bar and a nose

holder with unobstructed vision. A pattern electroretinogram (PERG) electrode, diameter of 0.25mm, was placed on the corneal surface by means of a micromanipulator and positioned to encircle the pupil without limiting the field of view. Reference and ground electrodes were stainless steel needles inserted under the skin of the scalp and tail, respectively. Before recording, a small drop of balanced salt solution was topically applied to the cornea to prevent drying. A visual stimulus of contrast-reversing (1Hz, 2 reversal/s) horizontal bars generated by a programmable graphic card (VSG-Cambridge Research Systems, Rochester, UK) on a cathode-ray tube (CRT) display (Sony Multiscan 500) was aligned with the projection of the pupil. At a viewing distance of 15 cm, the stimulus field covered an area of 69.4 x 63.4 deg. Patterns had a fixed mean luminance of 50 cd/m². Retinal signals were amplified (10,000-fold) and band-pass filtered (1-30 Hz). Three consecutive responses to 600 contrast reversals each were recorded. The responses were superimposed to check for consistency and then averaged. To obtain a corresponding index of outer retinal function, flash electroretinogram (FERG) was also recorded in response to strobe flashes of 20 cd/m²/s superimposed on a steady background light of 12 cd/m² and presented within a Ganzfeld bowl. Under these conditions, rod activity is largely suppressed while cone activity is minimally suppressed. Averaged PERG and FERG were automatically analyzed and evaluated the major positive and negative waves by Sigma Plot (Systat software Inc., San Jose, CA).

Virus administration. All animal procedures were conducted in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and in accordance with the University of Miami Institutional Animal Care and Use Committee policies. Death was not an intentional endpoint and mice were euthanized when moribund. Mice were sedated by inhalation with 1.5% to 2% isoflurane. A local anesthetic (proparacaine HCl) was applied topically to the cornea and then a 32gauge needle attached to the Hamilton syringe. Visual and motor functions were assessed and recorded by PERG, Flash ERG and Rotarod assays. Mice samples were collected and analyzed as described above.

Statistical analysis. All data plotted with error bars are expressed as the mean $\pm$ SEM. The *P* values were generated by analyzing data with student's t-tests, one-way ANOVA or two-way ANOVA using GraphPad

749 Prism 7 software. Cumulative proportional survival rate statistical analysis was performed by Kaplan-Meier
 750 analyses and statistical analyses were performed with log-rank test.

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Author contributions H. J. Y was responsible for creating and characterizing the LS mouse model, rescue vector construction and production, data compilation, data analysis, and manuscript writing. K.A.W contributed data analysis and manuscript writing. M.T.B., W.W.H., and A.S.L., proof reading and provided reagents. J. G. initiated, designed and directed the study, performed hands on application of mouse MRI, EM, and AAV injections, and contributed importantly to writing and communicating the first version of the manuscript.

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