Supplementary Table 1: Selected *Ndufs4* knockout mouse models

NAME	DESCRIPTION	PHENOTYPE	REFS
WHOLE-BODY KNOCKOUT MODELS			
Ndufs4$^{-/-}$-WB	**Whole-body *Ndufs4* knockout mice.** These animals were generated by deleting exon two of *Ndufs4*, resulting in a frameshift that prevented formation of NDUFS4 protein.	Healthy until ~5 wks of age at which ataxic signs begin. Death at ~7 wks. Progressive encephalopathy, failure to thrive, lethargy, hypothermia, loss of motor skills, epilepsy, cardiac and breathing abnormalities. Decreased total body fat, increased brain pO_2, impaired HPV, alopecia, systemic (and neuro) inflammation, high bone mass, defect in osteoclastogenesis, decreased bone resorption, increased apoptosis in RGC, increased cleavage of caspase 8 in affected brain regions, mTORC1 hyperactivity, increased S6 and IGF1R phosphorylation and protein carbonylation, liver fat droplets and free fatty acid defects, RGC loss, abnormal ERG responses, blindness, elevated serum LAC, triglyceride and NEFA levels, oxidative stress, increased protein succination and ubiquitination. Reduced glutamine/glutamate/α-ketoglutarate axis in brain and altered anesthetics sensitivity. Lipid accumulation, degradation and gliosis in cerebellum, OB and VN. Reduced synaptophysin expression. Structural CI proteins were decreased in brain, liver, heart, kidney, diaphragm and skeletal muscle. Increased levels of alanine, leucine, isoleucine, glutathione and BCAAs and increased PYR/LAC and PYR/acyl-carnitine ratios in brainstem, cerebellum and OB. Glutamic acid, aspartic acid, α-hydroxyglutaric acid were decreased and dihydroxyacetone phosphate and G3P metabolites were changed. OB showed higher levels of glycolysis and pentose phosphate pathway-related intermediates. Decreased levels of several acyl-carnitines, *N, N*-dimethylglycine, 2-aminoadipate, proline, hydroproline, citrulline, glutamate and glutamine in skeletal muscle and brainstem, cerebellum and OB. Decreased creatinine, creatine, PYR, glycerol and LAC levels in skeletal muscle. Absence of NDUFS4 accompanied by near complete absence of NDUFA12, increase in NDUFA2, and reduced stability of N- to Q-module attachment. No CI and decreased CIII activity in submitochondrial particles. Halved CI-driven O_2 consumption in intact tissues. No assembled CI on BN-PAGE gels. Reduced NAD^+/NADH ratio and mitochondrial hyperpolarization in MEFs. Fibroblasts show increased ROS levels and aberrant mitochondrial morphology. Muscle displays normal morphology, low NADH dehydrogenase activity, subsarcolemmal mitochondrial aggregation, normal oxygen consumption, ATP production and phosphocreatine concentration.	**64, 72, 73, 75, 78-81, 92, 93, 99, 103-105, 113-116, 125, 137, 169, 173, 195-199**
Ndufs4$^{+/-}$	**Heterozygous *Ndufs4* KO mice** were generated by deleting exon two of *Ndufs4*, resulting in a frameshift that prevented formation of the NDUFS4 protein.	Partially reduced CI activity. No changes in overall body weight, liver or heart weight. No elevated ROS levels due to upregulation of GSH and TRX systems. Compromised heart recovery and augmented damage following reperfusion. Increased ROS release after reperfusion injury.	**72, 200**

Ndufs4$^{+/-}$-KI	**Heterozygous *Ndufs4* knock-in (KI) mice.** A knock-in point mutant *Ndufs4* targeting vector was designed that induced truncation of *Ndufs4* in the last exon prior to a 10-15 AA sequence. The vector was targeted to embryonic stem cells (ESCs), heterozygous ESCs were injected in blastocysts and introduced in mice to generate *Ndufs4*$^{+/-}$-KI mice.	Homozygous *Ndufs4*$^{-/-}$-KI knock-in mice were embryonically lethal (probably due to genetic background). Heterozygous *Ndufs4*$^{+/-}$-KI knock-in mice were viable. BN-PAGE for heart, skeletal muscle and brain mitochondria revealed no differences in location or intensities of CI-CV between *Ndufs4*$^{+/-}$-KI and *Ndufs4*$^{+/+}$-KI mice. Heart mitochondria displayed absence of the NDUFS4 subunit and ~20% of the mutated 14.4-kDa protein compared to WT mice. The levels of the mutated protein are lower than expected (~50%), which may be explained by NMD due to the premature stop codon present in the mutant *Ndufs4* mRNA. RCR was decreased in heart, skeletal muscle and brain mitochondria, indicating a possible defect in CI-mediated oxygen consumption. Using succinate as a substrate (CII-mediated oxygen consumption) did not result in altered RCR, indicating that there is a defect solely in CI. In addition, mitochondria isolated from these tissues also showed ~25-30% reduced CI activity. LDH levels between *Ndufs4*$^{+/-}$-KI and *Ndufs4*$^{+/+}$ mice remained similar, which may be explained by the fact that *Ndufs4*$^{+/-}$-KI mice have one WT *Ndufs4* copy.	**201**
Ndufs4$^{fky/fky}$	**Whole-body *Ndufs4* knockout mice** were obtained by insertion of the B2 SINE (short interspersed nuclear element) into *Ndufs4*.	The phenotype of *Ndufs4*$^{fky/fky}$ mice is similar to that of the *Ndufs4*$^{-/-}$-WB model, although the *Ndufs4*$^{fky/fky}$ model presents with symptoms earlier. Overall, *Ndufs4*$^{fky/fky}$ mice present with a phenotype from ~PD16 and display temporary hair loss. The animals display failure to thrive and, from 5 wks onwards, become ataxic, including tilting of the head, walking in circles and forward curling in combination with twisting of the body when resuspended by the tail. From PD40 onwards, symptoms progressively and rapidly worsen, leading to death. Similar to *Ndufs4*$^{-/-}$-WB animals, CI activity was reduced in brain, heart, muscle, liver and kidney. *Ndufs4*$^{fky/fky}$ models displayed normal activity of other OXPHOS complexes, indicative of an isolated CI-deficiency. Analysis of brain- and heart-derived mitochondria revealed a decreased ATP generation capacity. BN-PAGE demonstrated the presence of an ~830-kDa CI-subassembly and a "crippled" CI_1CIII_2 supercomplex containing 38 of the 44 CI subunits. The ~830-kDa CI-subassembly lacked the NDUFS4 subunit and N-module subunits (NDUFV1, NDUFV2, NDUFS1, NDUFS6, NDUFA12). NDUFS4 protein was not detectable in MEFs from *Ndufs4*$^{fky/fky}$ mice, and these cells displayed a ~830-kDa CI-subassembly, reduced CI activity, reduced O_2 consumption, but normal ATP content and LAC production. MEFs from *Ndufs4*$^{fky/fky}$ and *Ndufs4*$^{+/fky}$ (heterozygous) mice exhibited downregulation of genes involved in cellular function, transcriptional regulation, neural differentiation/signaling pathways, and synaptic transmission. This suggests that these cells have variable gene expression patterns in early differentiation highlighting the effect of CI dysfunction on the cell's differentiation potential. In addition to these genetic changes, *Ndufs4* deletion induced shifts in acyl-carnitine and AA levels, pointing towards a reverse TCA flux.	**95, 119, 120**
Ndufs4$^{GT/GT}$	**A milder version of the *Ndufs4*$^{-/-}$-WB mouse model.** These mice were generated by gene trap insertion in the first intron of	*Ndufs4*$^{GT/GT}$ mice displayed failure to thrive, increased sucrose consumption, impaired neurogenesis and reduced inflammation in hippocampus compared to WT littermates. Relative to their WT littermates, *Ndufs4*$^{GT/GT}$ mice were hyperactive, as demonstrated by increased activity in various behavioral tests	**121, 122**

	Ndufs4.	(rotarod, swimming, tail suspension, open field). This hyperactivity could point to increased restlessness, a symptom also observed MD patients. *Ndufs4*$^{GT/GT}$ mice had normal plasma glucose and insulin levels compared to WT littermates. Plasma cortisone levels were lower upon baseline conditions compared to WT littermates; these levels increased upon exposure to chronic unpredictable stress. Given their relatively modest CI deficiency and ensuing mild phenotype, *Ndufs4*$^{GT/GT}$ mice are compatible with pathomechanistic analysis of mild CI deficiencies and behavioral testing. Gene trap insertion induced ~50% reduction in *Ndufs4*-derived mRNA and NDUFS4 protein levels in brain tissue, associated with a ~25% reduction in CI activity. In this sense, the degree of CI deficiency was less in *Ndufs4*$^{GT/GT}$ than in *Ndufs4*$^{-/-}$-WB and *Ndufs4*$^{fky/fky}$ mice. *Ndufs4*$^{GT/GT}$ mice displayed a normal maximal ATP production capacity and mitochondrial content in left hippocampi and, similar to *Ndufs4*$^{-/-}$-WB and *Ndufs4*$^{fky/fky}$ mice, displayed a normal activity of CII and CIII in left hippocampi and frontal cortex. Targeted metabolomics revealed that *Ndufs4*$^{GT/GT}$ mice had elevated levels of acyl-carnitine C3, C4 and C12 in the frontal cortex, whereas no alterations were found in whole brain tissue. In addition, AA metabolism and TCA metabolites were also altered in brain tissue. These findings could indicate a reverse flux in the TCA cycle in *Ndufs4*$^{GT/GT}$ mice caused by altered brain bioenergetics.	
CRISPR-Cas9 *Ndufs4*$^{-/-}$	**CRISPR-Cas9-induced *Ndufs4* knockout mice.** These animals were generated by designing a sgRNA to target exon 2 of *Ndufs4*. Next, capped polyadenylated Cas9 mRNA and sgRNA was synthesized by *in vitro* transcription and co-injected in mouse embryos at the pronuclear stage. Embryos were cultured to the blastocyst stage and transplanted into pseudo pregnant female mice.	Three wks. after birth, CRISPR-Cas9 *Ndufs4*$^{-/-}$ mice lost hair that grew back in the next hair-growth cycle. Mice developed failure to thrive and lost motor function as measured by the forced swimming and open field test. Disease progressively worsened and mice eventually died at ~6 wks of age. In addition, early embryonic development was also affected as indicated by reduced development of zygotes and spontaneous ovulation arrest in female mice. NDUFS4 protein was not detected in liver.	**202**
BRAIN/NEURON-SPECIFIC KNOCKOUT MODELS			
NesKO	**Neuron- and glia-specific *Ndufs4* knockout mice.** These mice were generated by crossing the conditional *Ndufs4* mice with Nestin-Cre mice. *Pcp2-Cre* mice and *Ubc-CreERt2* mice were used to inactivate *Ndufs4* in Purkinje cells or in the adult by administration of tamoxifen, respectively. This induced specific	Similar to the *Ndufs4*$^{-/-}$-WB model, NesKO mice display failure to thrive, hypothermia, optic atrophy, cataracts, ptosis, seizures and breathing abnormalities. Furthermore, these animals exhibited severe progressive ataxia from ~PD35 onwards, marked by an uncoordinated gait, reduced balance, hindlimb clasping and decreased rotarod performance. Microscopy analysis of brain tissue demonstrated spongiform degenerations in VN, IO, FN, caudal cerebellar vermis and OB, increased vascularity in VN and cerebellum, increased astroglial and microglial activation, indicating neuroinflammation, and neurodegeneration in the cerebellum and VN. At the molecular level, NesKO mice showed low to zero residual CI enzyme activity in SMPs isolated from	**125, 126**

	loss of *Ndufs4* in neurons and astroglia.	brain, reduced OCR, increased protein oxidation, abnormal mitochondria with swollen cristae, normal muscle morphology, increased cerebellar LAC levels and increased caspase 3, but not caspase 9 cleavage.	
AAV-VN–KO	**VN-specific *Ndufs4* knockout mice.** These animals were generated by bilateral injections of adult *Ndufs4*$^{lox/lox}$ mice with adeno-associated virus (type 1) expressing CreGFP (AAV1-Cre-GFP) in the mediolateral VN.	These animals developed failure to thrive, loss of motor function (measured by rotarod performance), hindlimb clasping, breathing abnormalities and eventually died at ~7 wks. Mortality was not as marked as in *Ndufs4*$^{-/-}$-WB mice. The development of a disease phenotype suggests that the VN may be involved in the pathology of LS. Furthermore, neurodegeneration in the VN has been suggested to be responsible for the breathing abnormalities observed in *Ndufs4*$^{-/-}$-WB mice. AAV-VN-KO mice developed microglial activation ~1 month after surgery, marked by increased Iba-1 staining.	75
Vglut2:Ndufs4cKO	**Glutamatergic neuron-specific *Ndufs4* knockout mice.** These mice were generated by crossing mice with one *Ndufs4* allele deleted and expressing a codon-improved Cre recombinase (iCre) under the *Slc17a6* promoter (*Slc17a6*^{Cre}, *Ndufs4*$^{\Delta/+}$) to mice with two floxed *Ndufs4* alleles (*Ndufs4*$^{lox/lox}$).	Vglut2:Ndufs4cKO mice developed progressive motor function loss, respiratory abnormalities, body tremor, impaired balance and motor coordination, ataxia, hindlimb clasping, failure to thrive and at later disease stages hypothermia and hypotonia, eventually resulting in early death. However, mice did have a longer lifespan (~10 weeks) when compared to *Ndufs4*$^{-/-}$-WB mice (~5 weeks). Additionally, Vglut2:Ndufs4cKO mice are hypersensitive to VAs, whereas Gad2:Ndufs4cKO and ChAT:Ndufs4cKO mice did not display such hypersensitivity (see below). Eventually, Vglut2:Ndufs4cKO mice died of severe motor and respiratory deficits. Neurologically, loss of *Ndufs4* in glutamatergic neurons resulted in increased glial activation in VN, IO and FN, accompanied by increased caspase 8 activation, a marker for neurodegeneration, resembling a similar neuroinflammatory profile as *Ndufs4*$^{-/-}$-WB mice. Further characterization of Vglut2:Ndufs4cKO mice revealed that these animals displayed a reduced OCR in mitochondria isolated from VN. Proteomics analysis revealed reduced abundance of most CI subunits and increased NDUFAF2 levels. N-module subunits were most dramatically decreased, highlighting the importance of NDUFS4 in N-to-Q module attachment and stabilization (see above). Specific subunits of the Q- and P-module also displayed reduced protein levels. Acetylome analysis revealed decreased NDUFS1, NDUFAB1, NDUFA5, NDUFA9 and NDUFB3 acetylation in mitochondria from glutamatergic neurons in the VN, whereas PDH and CV subunits displayed increased acetylation. This was accompanied by reduced PDH activity in mitochondria isolated from VN and increased LAC levels in VN, suggesting that glutamatergic neurons in Vglut2:Ndufs4cKO mice are more glycolytic than of WT littermates. In this sense, Vglut2:Ndufs4cKO mice developed a similar phenotype as the *Ndufs4*$^{-/-}$-WB model. This suggests that malfunction of glutamatergic neurons is important in LS neuropathogenesis.	82, 86, 87, 94, 113, 127
Gad2:Ndufs4cKO	**GABAergic neuron-specific *Ndufs4* knockout mice.** These animals were generated by by	GABAergic neuron-specific loss of *Ndufs4* resulted in failure to thrive, but did not lead to the development of other visible clinical symptoms compared to WT littermates. However, at later disease stages mice suffered from hypothermia and	82

	crossing mice with one floxed *Ndufs4* allele and expressing Cre recombinase under the control of the *Gad2* promoter (*Gad2*$^{Cre/+}$, *Ndufs4*$^{lox/+}$) to mice with two floxed *Ndufs4* alleles (*Ndufs4*$^{lox/lox}$).	spontaneous seizures when being handled Gad2:Ndufs4cKO mice showed a different profile compared to Vglut2:Ndufs4cKO mice with increased microglial and astroglial activation in the external globus pallidus (GPe) in the basal ganglia and the substantia nigra pars reticulata (SNr). Death of Gad2:Ndufs4cKO mice was associated with seizures.	
ChAT:Ndufs4cKO	**Cholinergic neuron-specific *Ndufs4* knockout mice.** These mice were generated by crossing mice with one floxed *Ndufs4* allele and expressing Cre recombinase under the control of the *ChAT* promoter (*Chat*$^{Cre/+}$, *Ndufs4*$^{lox/+}$) to mice with two floxed *Ndufs4* alleles (*Ndufs4*$^{lox/lox}$).	ChAT:Ndufs4cKO mice did not develop fatal encephalopathy and remained clinically healthy throughout the study.	**82**
MSN *Ndufs4*$^{-/-}$	**Striatal medium spiny neuron (MSN)-specific *Ndufs4* knockout mice.** This model was generated by crossing mice expressing cre recombinase under control of the *Gpr88* promoter and heterozygous *Ndufs4* mice (*Gpr88*$^{cre/+}$, *Ndufs4*$^{\Delta/-}$) to mice with two floxed *Ndufs4* alleles (*Ndufs4*$^{lox/lox}$). *Gpr88* is selective of striatal MSNs and does not target other neuronal populations in the striatum.	No differences in body weight or life span were observed between MSN KO mice and WT littermates up to 6 months. After 3 months locomotor activity (home cage activity), motor coordination and learning (rotarod performance) progressively worsened. In contrast, associative learning remained unaffected. MSN KO mice did not develop neuroinflammation. Striata of MSN KO mice showed selective decrease of NDUFS4 protein levels. Striatal mitochondria displayed decreased CI-dependent OCR upon supplementation of the CI substrates PYR and malate and reduced maximal respiration upon addition of the uncoupler FCCP. In contrast, CII-dependent OCR upon supplementation of succinate remained unaltered compared to WT littermates.	**128**
DA *Ndufs4*$^{-/-}$	**Dopaminergic (DA) neuron-specific *Ndufs4* knockout mice.** These animals were generated by crossing *Ndufs4*$^{loxp/+}$ mice with mice carrying *DAT*-cre. Next, double heterozygous offspring were crossed to homozygous DA *Ndufs4*$^{-/-}$ mice. Alternatively, *Ndufs4*$^{loxP/loxP}$ mice were crossed with *Slc6a3*$^{iCre/+}$ mice in which *Cre* recombinase is expressed under regulatory elements for the dopamine transporter (DAT) in the *Sl6a3* resulting in *Ndufs4*$^{loxP/loxP}$:: *Slca6a3*$^{iCre/+}$ mice (DA *Ndufs4*$^{-/-}$ mice).	DA *Ndufs4*$^{-/-}$ mice appeared healthy and were indistinguishable from WT littermates. Mice showed normal motor function (Catwalk system) locomotor function (locomotor boxes; open field test) and balance and coordination (rotarod performance) up until 2 years. In contrast, cognitive function was impaired, marked by progressive loss of short- and long-term (novel object recognition test) and spatial memory (Morris water maze). Mice also developed age-dependent anxiety-like behavior (elevated-plus maze, social interaction test). Purified DAT synaptosomes showed reduced CI activity. DA neuron morphology appeared normal, DA neuron nerve terminals were not lost and DA populations were only slightly decreased after 2 years, indicating that there was no DA neuron degeneration in the substantia nigra. Closer examination revealed that DA release was decreased in striatum and amygdala, whereas serotonin levels were not affected. Furthermore the two DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in striatum were increased accompanied by increased DOPAC:DA and HVA:DA ratios. In addition, *Ndufs4* deletion in DA neurons also enhanced phospho-α-synuclein accumulation.	**129-131**

HEART- AND SKELETAL MUSCLE-SPECIFIC KNOCKOUT MODELS			
Heart-specific *Ndufs4*-null mice	**Heart-specific *Ndufs4* knockout mice.** These mice were generated by crossing mice with loxP-flanked *Ndufs4* alleles to mice that express the *Cre* recombinase driven under the α-MHC promoter or CKM-NLS-cre.	In sharp contrast to the *Ndufs4*$^{-/-}$-WB model, selective loss of *Ndufs4* in the heart did not result in a pathological phenotype up to 1 yr. of age. Instead, mice developed hypertrophic cardiomyopathy marked by decreased LVEF and increased LVM. NDUFS4 protein was not detectable in cardiac tissue of cardiac-specific *Ndufs4*$^{-/-}$ mice and associated with a ~50% reduction in myocardial CI activity. Mitochondria isolated from cardiac tissue displayed reduced CI-driven O_2 consumption, NADH accumulation (leading to a decrease in NAD^+/NADH ratio) and protein hyperacetylation, resulting in a higher susceptibility to cell death. In contrast, ATP production, ROS levels, CS activity and mitochondrial number appeared normal in cardiac tissue. Since no change in redox state was observed in cardiac tissue, it appears that induction of cardiomyopathy is primarily due to reductions in CI activity and not oxidative stress. However, in contrast to brain tissue, cardiac tissue displays a reserve capacity for CI-dependent ATP synthesis and seems to tolerate reduced CI function in unstressed mice.	**134,135,203**
Ckm-NLS-cre; Ndufs4$^{loxP/loxP}$ mice	**Heart- and skeletal muscle-specific *Ndufs4* knockout mice.** These animals were generated by crossing *Ndufs4*$^{loxp/+}$ mice with mice carrying CKM-NSL-cre. Next, double heterozygous offspring were crossed to homozygous *Ckm-NLS-cre; Ndufs4*$^{loxP/loxP}$ mice.	These mice developed an increased heart-to-body weight ratio. Although this may indicate cardiomyopathy, animals did not show signs of heart failure and remained clinically healthy up to at least 1 year of age. NDUFS4 protein was not detected in heart muscle of *Ckm-NLS-cre; Ndufs*$^{loxP/loxP}$ mice. Analysis of detergent-permeabilized mitochondrial extracts from KO hearts revealed almost complete absence of CI activity accompanied by reduced coupled CI/CIII activity. ATP production rates were slightly reduced in intact mitochondria using glutamate/malate (TCA cycle), glutamate/succinate (TCA cycle), or palmitoyl-L-carnitine/malate (β-oxidation) as substrates. In addition, CS and glutamate dehydrogenase activities per heart muscle mass were also increased, suggesting that a compensatory increase in mitochondrial mass maintains the ATP production to almost normal levels. BN-PAGE revealed ~830 kDa inactive and ~200 kDa active CI subassemblies, in accordance with other *Ndufs4* mouse models (see above), and impaired CI-containing supercomplex formation, suggesting impaired CI assembly and/or stability.	**129**

Supplementary Table 2: Selected intervention strategies in *Ndufs4* knockout mouse models

NAME	INTERVENTION	EFFECT OF INTERVENTION	REFS
WHOLE-BODY KNOCKOUT MODELS			
Ndufs4$^{-/-}$-WB	CO exposure	*Ndufs4*$^{-/-}$-WB mice were exposed to 21% CO (600 pm) in O_2 starting at PD55 for 2-3 wks. Exposure to chronic, continuous 600 pm CO in air COHb and arterial O_2Hb. After 3 wks of CO exposure haematocrit was increased, indicating that this treatment regimen efficiently caused renal hypoxia and erythropoietin production. Mice regained body weight and survived to adulthood. In addition, CO exposure also reversed lesions in the VN and reduces brain pO_2. However, mice developed hyperintense lesions in the caudoputamen region of the brain upon chronic 600 pm CO exposure, indicating adverse effects.	**137**
Ndufs4$^{-/-}$-WB	Hypoxia	Hypoxia-exposure (8.5% O_2 for 6 h) of *Ndufs4*$^{-/-}$-WB mice, induced stabilization of HIFs and activated erythropoietin-linked transcription and translation in plasma. This indicates that *Ndufs4*$^{-/-}$-WB mice can activate a hypoxic transcriptional response despite their disease phenotype. When *Ndufs4*$^{-/-}$-WB mice were exposed to hypoxia for a longer period of time (11% O_2 for 3 wks starting from PD30), they survived to adulthood, did not develop the severe phenotype, but some mice started to develop mild hindlimb clasping at ~PD120. Hypoxia treatment improved body weight gain, core body temperature and prevented loss of motor function. Furthermore, decreased NAD^+/NADH ratios in the lung and increased serum LAC levels were normalized. Hypoxia prevented development of neurological lesions and neuroinflammation in the cerebellum, OB and brainstem. However, hypoxia-exposed *Ndufs4*$^{-/-}$-WB mice still developed LV dysfunction and displayed reduced CI activity in brain tissue.	**137, 140, 141, 198**
Ndufs4$^{-/-}$-WB	Phlebotomy	Phlebotomy was carried out by bleeding mice ~200-400 microliters by tail veins 5 to 6 times with 2 to 3 d in between to allow blood volume to recover. This maneuver increased the life span of *Ndufs4*$^{-/-}$-WB mice towards ~130 d and prevented development of lesions in the VN. Lesions did, however, become apparent closer to death.	**137**
Ndufs4$^{-/-}$-WB	AD4	AD4 (150 mg/kg/day, IP, PD21-28) delayed disease onset and reduced severity of disease phenotype, marked by improved motor function by PD30.	**80**

Ndufs4$^{-/-}$-WB	Clofibrate	Treatment with the PPAR stimulator clofibrate (100 mg/kg/day, IP, from PD14 onwards) prolonged lifespan and motor function of *Ndufs4*$^{-/-}$-WB mice without inducing adverse hepatic effects. Respiratory capacity of CI-CIV was not improved upon clofibrate administration. In contrast, cholesterol metabolism was improved marked by higher cholesterol levels in bile.	**148**
Ndufs4$^{-/-}$-WB	DMKG	DMKG (300 mg/kg/day, IP, PD21 onwards), a cell-permeable form of α-ketoglutarate, was administered to increase brain α-ketoglutarate levels. DMKG prolonged life span and delayed onset of hindlimb clasping in *Ndufs4*$^{-/-}$-WB mice. In addition, DMKG also suppressed hypoxia signaling by lowering HIF1α and LDHA levels without affecting the NAD^+ pool in brain tissue, suggesting that DMKG suppresses hypoxic signaling without affecting the NAD^+ salvage pathway.	**150**
Ndufs4$^{-/-}$-WB	Doxycycline	Doxycycline (chow diet, 5,000 or 8,000 p.p.m.) was supplemented to *Ndufs4*$^{-/-}$-WB mice, and prolonged their life span and improved the growth rate and motor function (rotarod performance). Microglial activation in VN and OB was decreased, marked by decreased Iba-1 staining. In addition, doxycycline suppressed markers associated with microglial and astrocyte activation (LEG3, CD180, GFAP and S100A4), complement components (C1Qa and C1QB) and interferon response (IFIT1 and IFIT3). Anti-inflammatory response proteins (CLINT1, OTUD4 and APOA4) were upregulated. Metabolomics revealed increased metabolic signatures associated with immune, inflammatory and oxidative stress. The latter was also associated with glutamine and glutamate levels. However, CI protein levels were not restored.	**156**
Ndufs4$^{-/-}$-WB	Fenofibrate	Administration of fenofibrate (100 mg/kg/day, IP, from PD20 onwards) prolonged life span and partially improved motor function. Fenofibrate increased total unsupported cholesterol and apoA-1 supported cholesterol efflux. Accordingly, cholesterol plasma levels were elevated, whereas bile levels remained normal. Fatty-acid-driven oxidation was not enhanced in muscle fibers, indicating that fenofibrate failed to increase mitochondrial mass or stimulate fatty-acid-β-oxidation in muscle. Fenofibrate induced only mild hepatotoxicity.	**156**
Ndufs4$^{-/-}$-WB	GF109203X	Mice were treated with the pan-PKC inhibitor GF109203X (8 mg/kg/day, IP, from PD10 onwards). This prolonged survival, delayed onset of hindlimb clasping and prevented hair loss.	**166**
Ndufs4$^{-/-}$-WB	GO6983	Mice were treated with the pan-PKC inhibitor GO6983 (4 mg/kg/day, IP, from PD10 onwards). This prolonged survival, delayed onset of hindlimb clasping and prevented hair loss.	**166**
Ndufs4$^{-/-}$-WB	Idebenone	Idebenone treatment (200 mg/kg, oral, PD21-35) did not reverse visual impairment in *Ndufs4*$^{-/-}$-WB mice. Specifically, idebenone did not rescue visual function, protect against SBAC degeneration, supress innate immunity, inflammation or reduce microglial activation.	**169**
Ndufs4$^{-/-}$-WB	KH176 (Sonlicromanol)	KH176 (10 mg/kg/day, twice daily, IP, from PD14 onwards) improved the motor function of *Ndufs4*$^{-/-}$ mice, without negatively affecting body weight, indicating	**147, 148**

		that the treatment regimen was well tolerated. Furthermore, KH176 was able to normalize lipid peroxidation, a measure for oxidative stress, maintained microstructural coherence, marked by higher fractional anisotropy and reduced ganglion cell degeneration. However, KH176 did not improve disease onset or severity, brain pathologies or restore residual OXPHOS complex activities.	
Ndufs4$^{-/-}$-WB	KH176 and clofibrate	No prolonged life span or improved motor function. Increased liver weight and reduced hepatic lipid content.	**148**
Ndufs4$^{-/-}$-WB	NMN	The NAD^+ precursor NMN was administered to *Ndufs4*$^{-/-}$-WB mice (500 mg/kg, IP, twice weekly). NMN did prolong the life span, but did not ameliorate the clinical phenotype of these mice. Closer examination of disease biomarkers revealed that NMN did not normalize NADH levels, LAC levels or protein acetylation in brain tissue. However, these markers were improved in heart and skeletal muscle tissue. The therapeutic effects of NMN were mediated by elevated α-ketoglutarate levels and suppression of hypoxic signaling.	**150**
Ndufs4$^{-/-}$-WB	P7C3	P7C3 likely activates NAMPT, an important enzyme of the NAD^+ salvage pathway. P7C3 (50 mg/kg, daily, IP) moderately prolonged life span, but did not increase NAD^+ levels in mouse brain or alter NAMPT protein level.	**150**
Ndufs4$^{-/-}$-WB	Papaverine	Papaverine (20 mg/kg, IP, PD21-35) restored visual function, prevented SBAC degeneration and innate immune and inflammatory response that occurred at time of vision loss.	**169**
Ndufs4$^{-/-}$-WB	PJ34	PJ34 administration (20 mg/kg body, IP, from PD30 onwards) resulted in delayed disease onset up to PD43, marked by reduced severity of ataxia, improved rotarod performance and reduced oxidative stress in brain tissue in *Ndufs4*$^{-/-}$ mice. On a molecular level, PJ34 reduced PARP content in several tissues, showed a tissue-specific increase of mRNA levels of mtDNA and nDNA-encoded ETC subunits, increased mitochondrial membrane potential, mitochondrial area and number, and improved mitochondrial morphology. However, the disease progressed again in ageing mice and PJ34 did not extend life span.	**164**
Ndufs4$^{-/-}$-WB	Rapamycin	Rapamycin (both 8 and 20 mg/kg/day, IP, from PD10 onwards) (partially) ameliorates the phenotype of *Ndufs4*$^{-/-}$-WB mice. Rapamycin was able to delay the disease onset and prolong the life span of *Ndufs4*$^{-/-}$-WB mice. Mice did not develop neurological symptoms, such as ataxia, uncoordinated balance and hindlimb clasping. Rapamycin was able to prevent the development of neurological lesions and preserved visual function, likely by reducing inflammatory markers in retinas of *Ndufs4*$^{-/-}$-WB mice to similar levels as in WT retinas. Rapamycin attenuated metabolic defects in *Ndufs4*$^{-/-}$-WB mice, including alterations on GABA, dopamine, FFA levels and glycolytic intermediates. Transcriptional levels of mtDNA and nDNA-encoded ETC subunits and number of mitochondria remained unaltered, whereas mitochondrial area and cristae area in the cerebellum were reduced). Rapamycin increased the relative abundance of several CI subunits in brains of 30 d old *Ndufs4*$^{-/-}$-WB mice and reversed increased CIV levels. Furthermore, rapamycin decreased mTORC1 and mTORC2 core subunits (mTOR, mLST8) in KO brain. The decrease of mTORC2 was accompanied by reduced phosphorylation of AKT and PKC. Rapamycin restored	**76, 103, 162, 163, 169**

		LPPR1 (a neuronal growth promotor) and MPST (a neuroprotective mitochondrial enzyme), repressed PKC-β and induced PKA and CAMK2 phosphorylation in KO brain and restored CIRBP and RBM3 (neuroprotective cold-inducible RNA binding proteins) in both genotypes. In addition, rapamycin also decreased phosphorylation of proteins from pro-inflammatory signaling pathways, reduced brain cytokine levels and downregulated the inflammation-activating kinase IKK-α, IκB and NF-κB.	
Ndufs4$^{-/-}$-WB	Ruboxistaurin	Mice were treated with the PKC-β-specific inhibitor ruboxistaurin (10 mg/kg/day, IP, from PD10 onwards). Prolonged survival and delayed onset of hindlimb clasping. Prevention of hair loss, skin inflammation and reduced GFAP levels and NF-κB inflammatory response in brain.	**166**
Ndufs4$^{-/-}$-WB	Tacrolimus (FK-506)	Tacrolimus (0.8 mg/kg/day) did not rescue disease onset or progression.	**103**
Ndufs4$^{-/-}$-WB	TAK-242	The TLR4 inhibitor TAK-242 (10 mg/kg/day, IP, from PD22 onwards) rescued hair loss in *Ndufs4*$^{-/-}$-WB mice.	**73**
Ndufs4$^{-/-}$-WB	Zolpidem	Zolpidem (20 mg/kg, IP, PD21-35), a benzodiazepine-agonist, rescued visual function, prevented SBAC degeneration and innate immune and inflammatory responses that occurred at time of vision loss	**78**
Ndufs4$^{-/-}$-WB	Intravitreal injected human iPSC-MSCs	Rescue of retinal function decline, prevention of RGC loss and abnormal RGC activation, transfer of mitochondria to neurons in GCL, donated mitochondria extended longevity of RGC and normalized levels of pro-inflammatory cytokines.	**173**
Ndufs4$^{-/-}$-WB	AAV-PHP.B-hNDUFS4	Prolonged life span, improved growth rate, normalized motor coordination, rescue of failure to thrive, hypothermia, epilepsy and cardiac abnormalities, reduced retinal degradation, restored NDUFS4 expression in neurological regions (OB, striatum, hippocampus, midbrain, cerebellum, spinal cord and VN), liver, heart, muscle and retina, partial rescue of astrogliosis, microgliosis, lipid accumulation, formation of lesions, neurodegeneration, and CI activity and assembly, lowered serum LAC levels.	**175, 176**
Ndufs4$^{-/-}$-WB	AAV1-Ndufs4-IRES-GFP	Prolonged life span, delayed clinical disease progression, reduced gliosis and lesion in VN, normalized hypercapnic ventilatory response, normalized breathing regularities and development of lesions in striatum.	**75**
Ndufs4$^{-/-}$-WB	AAV2/9-*hNDUFS4*	IV injections restored CI assembly in liver and CI activity in all tissues in adult mice. No improvements in body weight, life span or motor function. ICV injections increased body weight, improved motor function and restored CI activity in new-born mice. Double IV+ICV injections increased body weight, improved motor function, prolonged life span and restored CI activity in muscle, heart and brain.	**177**
Ndufs4$^{-/-}$-WB	AAV-EF1α-GPD1	IV injections allowed neuron- and glia-specific GPD1 overexpression. Brain GPD1 levels were increased. Body weight loss was not rescued. Life span was extended from PD58 to PD84. Reduced the increased levels of F6P, G3P, DHAP αHB and LAC in brainstem. Increased aspartate levels in brainstem. Reduced increased αHB levels in plasma. Ameliorated neuroinflammation and partially prevented the decline in motor function and reduction in body temperature.	**114**
Ndufs4$^{-/-}$-WB	*Mt1* overexpression by crossing	No prolonged life span, failure to thrive, no improved ataxia or motor functions.	**81**

	TgMt1 mice with *Ndufs4*$^{-/-}$-WB mice.	Reduced CI activity in brain and quadriceps. MT1 overexpression in astrocytes and neurons of hippocampus and VN, increased astrocyte activation, slightly reduced microglial activation in hippocampus and VN, no improvements in protein oxidation, ROS levels or inflammation markers.	
Ndufs4$^{-/-}$*::Opa1*^{tg}	OPA1 overexpression by crossing of Opa1tg mice with *Ndufs4*$^{-/-}$-WB mice.	Prolonged life span, reduced loss of motor functions, disease progression and death at ~7 weeks. Rescued CI activity and CI-driven mitochondrial respiration. BN-PAGE showed absence of fully assembled CI. Normalized mitochondrial cristae morphology in brain.	**179**
Ndufs4$^{-/-}$-WB	*Ndufs4*$^{-/-}$-WB mice were crossed with tissue-specific *Rp6k1*$^{-/-}$ mice.	Whole body and liver-specific S6K1 knockout prolonged life span and decreased onset of hindlimb clasping of *Ndufs4*$^{-/-}$ mice, brain- or fat tissue-specific S6K1 knockout showed no beneficial effects.	**181**
BRAIN/NEURON-SPECIFIC KNOCKOUT MODELS			
NesKO + NDI1	Neuron- and glia-specific *Ndufs4* knockout mice with yeast NDI1 expression.	Prolonged life span to a median of >1 yr. Normal growth rate, no seizures, development of bilateral lesions in the brainstem and cerebellum, lesions were present in OB, increased OCR in CGNs, no reactive gliosis in OB, cerebellum or brainstem. Loss of motor function and breathing abnormalities were not prevented. Carbon flux from glucose into the TCA cycle was altered, marked by increased labeling of citrate, aspartate, malate, succinate and glutamate and decreased labeling of PYR and alanine.	**126**

Supplementary references

195. **Carspecken CW**, Chanprasert S, Kalume F, Sendensky MM, Morgan PG. Anesthetics have different effects on the electrocorticographic spectra of wild-type and mitochondrial mutant mice. *Anesthesiology.* 2018;129:744-755.

196. **Shil SK**, Kagawa Y, Umaru BA, et al. *Ndufs4* ablation decreases synaptophysin expression in hippocampus. *Sci Rep.* 2021;11:10969.

197. **Gospe SM 3rd**, Travis AM, Kolesnikov AV, et al. Photoreceptors in a mouse model of Leigh syndrome are capable of normal light-evoked signaling. *J Biol Chem.* 2019;294:12432-12443.

198. **Schleifer G**, Marutani E, Ferrari M, et al. Impaired hypoxic pulmonary vasoconstriction in a mouse model of Leigh syndrome. *Am J Physiol Lung Cell Mol Physiol.* 2019;316:L391-L399.

199. **Song L**, Cortopassi G. Mitochondrial complex I defects increase ubiquitin in substantia nigra. *Brain Res.* 2015;1594:82-91.

200. **Kuksal N**, Gardiner D, Qi D, Mailloux RJ. Partial loss of complex I due to *NDUFS4* deficiency augments myocardial reperfusion damage by increasing mitochondrial superoxide/hydrogen peroxide production. *Biochem Biophys Res Commun.* 2018;498:214-220.

201. **Ingraham CA**, Burwell LS, Skalska J, et al. NDUFS4: creation of a mouse model mimicking a Complex I disorder. *Mitochondrion.* 2009;9:204-10.

202. **Wang M**, Huang YP, Wu H, et al. Mitochondrial complex I deficiency leads to the retardation of early embryonic development in *Ndufs4* knockout mice. *PeerJ.* 2017;5:e3339.

203. **Zhang H**, Gong G, Wang P, et al. Heart specific knockout of *Ndufs4* ameliorates ischemia reperfusion injury. *J Mol Cell Cardiol.* 2018;123:38-45.