Mitochondria are small cellular constituents that generate cellular energy (ATP) by oxidative phosphorylation (OXPHOS). Dysfunction of these organelles is linked to a heterogeneous group of multisystemic disorders, including diabetes, cancer, ageing-related pathologies and rare mitochondrial diseases (MDs). With respect to the latter, mutations in subunit-encoding genes and assembly factors of the first OXPHOS complex (CI) induce isolated CI deficiency and Leigh syndrome (LS). This syndrome is an early-onset, often fatal, encephalopathy with a variable clinical presentation and poor prognosis due to the lack of effective intervention strategies. Mutations in the nuclear DNA (nDNA)-encoded \(Ndufs4\) gene, encoding the NADH:Ubiquinone oxidoreductase subunit S4 (NDUFS4) of CI induce “mitochondrial complex I deficiency, nuclear type 1” (MC1DN1) and LS in pediatric patients. A variety of (tissue-specific) \(Ndufs4\) knockout mouse models were developed to study the LS pathomechanism and intervention testing. Here, we review and discuss the role of CI and \(Ndufs4\) mutations in human MD, and review how the analysis of \(Ndufs4\) knockout mouse models has generated new insights into the MC1DN1/LS pathomechanism and its therapeutic targeting.
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Abbreviations: Δψ = electrical potential across the mitochondrial inner membrane; ΔpH = proton gradient across the mitochondrial inner membrane; αHB = α-hydroxybutyrate; βHB = β-hydroxybutyrate; AA = amino acid; AAV = adeno-associated virus; Acetyl-CoA = acetyl coenzyme A; AD4 = N-acetyl cysteine amide; AF = assembly factor; AHB = α-hydroxybutyrate; AKT = Ak strain transforming; apoA-1 = apolipoprotein A1; AR = autosomal recessive; BCAA = branched-chain amino acids; BN-PAGE = blue-native polyacrylamide gel electrophoresis; CAMK2 = calcium/calmodulin dependent protein kinase 2; Cer = cerebellum; CI-CV = complex I to complex V; CGN = granule neurons; CIRBP = cold-inducible RNA-binding protein; CKM = muscle creatine kinase; CO = carbon monoxide; COHb = carboxyhaemoglobin; CoQ = coenzyme Q10; CS = citrate synthase; DA = dopamine(rgic); DAT = dopamine transporter; DHAP = dihydroxyacetone; DMG = N-dimethylglycine; DMKG = dimethyl α-ketogluatrate; DOPAC =
3,4-dihydroxyphenylacetic acid; EGR = electroretinography; ESC = embryonic stem cell; ETC = electron transport chain; F6P = fructose-6-phosphate; FA = fatty acid; FADH$_2$ = flavin adenine dinucleotide; FFA = free fatty acid; FCCP = carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; FeS = iron-sulfur; FGF21 = fibroblast growth factor 21; FMN = flavin mononucleotide; FN = fastigial nucleus; FRC = fructose; G3P = glycerol-3-phosphate; GAL = galactose; GCL = retinal ganglion cell layer; GDH = glutamate dehydrogenase; GFAP = glial fibrillary acidic protein; GLC = glucose; GPD1 = glycerol-3-phosphate dehydrogenase 1; GPe = external globus pallidus; GSH = glutathione; HIF = hypoxia-inducible factor; HIF1$\alpha$ = hypoxia-inducible factor 1-alpha; HPV = hypoxic pulmonary vasoconstriction; HVA = homovanillic acid; ICV = intracerebroventricular; IGF1R = insulin-like growth factor 1 receptor; IMS = intermembrane space; IO = inferior olive; IP = intraperitoneal; IV = intravenous; KI = knock-in; KO = knockout; LAC = lactate; LD = lipid droplet; LDH = lactate dehydrogenase; LDHA = lactate dehydrogenase A; LPPR1 = phospholipid phosphatase related 1; LS = Leigh syndrome; LV = left ventricular; LVEF = left ventricular ejection fraction; LVM = left ventricular mass; MC1DN1 = mitochondrial complex I deficiency, nuclear type 1; MD = mitochondrial disease; MEF = mouse embryonic fibroblast; MHC = myosin heavy chain; MIM = mitochondrial inner membrane; MOM = mitochondrial outer membrane; MPTP = mitochondrial permeability transition pore; MPST = mercaptopyruvate sulfurtransferase; MSN = medium spiny neurons; MT/mt = metallothionein; mtDNA = mitochondrial DNA; mTOR = mechanistic target of rapamycin; mTORC1/2 = mTOR complex 1/2; NAD$^+$ = oxidized nicotinamide adenine dinucleotide; NADH = reduced nicotinamide adenine dinucleotide; NAMPT = nicotinamide phosphoribosyltransferase; nDNA = nuclear DNA; NDUFS4 = NADH:Ubiquinone oxidoreductase subunit S4; NEFA = non-esterified fatty acid; NLS = nuclear location signal; NMD = nonsense-mediated decay; NMN = nicotinamide mononucleotide; OB = olfactory bulb; OCR = oxygen consumption rate; O$_2$HB = oxygen-bound haemoglobin; OPA1 = Optic Atrophy 1; OXPHOS = oxidative phosphorylation; P7C3 = pool 7 compound 3; PARP = Poly(ADP-ribose)polymerase; PD = postnatal day; PDH = pyruvate dehydrogenase; PDK = pyruvate dehydrogenase kinase; Pi = inorganic phosphate; PKA = protein kinase A; PKC = protein kinase C; PMF = proton-motive force; pO$_2$ = oxygen partial pressure; PPARA = peroxisome proliferator-activated receptor-alpha; PARG = peroxisome proliferator-activated receptor-gamma; PPP = pentose phosphate pathway; PYR = pyruvate; RBM3 = RNA-binding motif protein 3; RCR = respiratory control ratio; RGC = retinal ganglion cell; ROS = reactive oxygen species; S6K1 = ribosomal protein S6 kinase 1; SBAC = starburst amacrine cell; sgRNA = single guide RNA; SINE = short interspersed nuclear element; SNr = substantia nigra pars reticulata; TCA =
tricarboxylic acid; TRX = thioredoxin; VA = volatile anesthetics; VN = vestibular nuclei; WB = whole body; WB-KO = whole body knockout
1. Mitochondria and oxidative phosphorylation

Mitochondria are constituents of virtually every eukaryotic cell. These organelles generate cellular energy in the form of adenosine triphosphate (ATP) and also play a central role in reactive oxygen species (ROS) and redox metabolism, fatty acid (FA) oxidation, heme biosynthesis, apoptosis induction, heat generation and calcium (Ca\(^{2+}\)) homeostasis\(^1\)-\(^5\). Structurally, mitochondria consist of a matrix compartment surrounded by the mitochondrial inner membrane (MIM) and mitochondrial outer membrane (MOM) and an in-between intermembrane space (IMS). The MIM contains numerous folds (“cristae”) which enlarge its surface area. Several nutrient-dependent pathways can deliver substrates for mitochondrial ATP generation by the MIM-embedded oxidative phosphorylation (OXPHOS) system\(^6\)-\(^8\). These pathways include: (1) the cytosolic glycolysis-mediated conversion of glucose (GLC), galactose (GAL) or fructose (FRC) into pyruvate (PYR), which subsequently enters the mitochondrion. There, PYR is converted into acetyl coenzyme A (Acetyl-CoA) that is used as a substrate for the tricarboxylic acid (TCA) cycle to generate reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH\(_{2}\)), (2) entry of glutamine into the TCA cycle and (3) entry of FAs into β-oxidation and the TCA cycle. In addition to OXPHOS, ATP is also generated by the glycolysis pathway in the cytosol and mitochondrial TCA cycle. Under physiological conditions, OXPHOS generates the majority of ATP in most cells. However, during mitochondrial dysfunction increased glycolytic ATP production can compensate for loss of OXPHOS-mediated ATP generation. This compensation involves the conversion of glycolysis-generated PYR into lactate (LAC), which leaves the cell and acidifies the extracellular environment\(^9\),\(^10\). The OXPHOS process requires the combined action of four electron transport chain (ETC) complexes (complex I-IV; CI-IV) and an ATP-producing fifth complex (CV or F\(_o\)F\(_1\)-ATPase) by a chemiosmotic coupling mechanism\(^11\). ETC action sustains a matrix-directed proton-motive force (PMF) by transporting electrons from NADH (via CI) and FADH\(_{2}\) (via CII) to molecular oxygen (O\(_2\); via CIV). This process further requires coenzyme Q\(_{10}\) (CoQ) and cytochrome-c (cyt-c), which mediate electron transport from CI/CII to CIII and from CIII to CIV, respectively. Of note, electrons can also enter the ETC via alternative CoQ-converging pathways\(^12\),\(^13\). These are often tissue-specific and include: (1) the electron-transferring flavoprotein (ETF)-ubiquinone oxidoreductase, (2) \(s,n\)-glycerophosphate dehydrogenase and (3) dihydrolorotate dehydrogenase. At CI, CIII and CIV, protons (H\(^+\)) are transported from the mitochondrial matrix to the IMS, leading to a trans-MIM electrical potential (\(Δψ\)) and chemical proton gradient (\(ΔpH\)), which together constitute the PMF\(^6\). Matrix reentry of
H⁺ is then used at CV for ATP generation\textsuperscript{14}. In addition, the PMF is also crucial to sustain other mitochondrial functions including ion/metabolite exchange and protein import\textsuperscript{4,15,16}.

2. Complex I of the oxidative phosphorylation system

NADH:ubiquinone reductase (EC 1.6.5.3.) or complex I (CI) is the first and largest (~1 MDa) OXPHOS complex, which couples H⁺ transport to electron transfer from NADH to CoQ\textsuperscript{6,17-21}. It was estimated that CI action is responsible for sustaining ~40% of the total PMF\textsuperscript{22}. CI is composed of 44 different subunits (\textbf{Table 1}), fourteen of which are “core subunits” that suffice to carry out CI catalytic function\textsuperscript{22}. In humans, seven hydrophobic core subunits are encoded by the mitochondrial DNA (mtDNA): ND1, ND2, ND3, ND4, ND4L, ND5 and ND6, whereas the remaining core subunits are nuclear DNA (nDNA)-encoded: NDUFV1, NDUFV2, NDUFS1, NDUFS2, NDUFS3, NDUFS7 and NDUFS8. In addition to these core subunits, human CI also contains 31 nDNA-encoded “accessory” (or “supernumerary”) subunits. Although the function of these subunits still is largely unknown, it is expected that they play a role in CI assembly, stabilization, functional regulation, prevention of electron escape and the formation of OXPHOS supercomplexes (see below). Currently, 14 assembly factors (AFs) have been identified that mediate CI biogenesis: NDUFAF1, NDUFAF2, NDUFAF3, NDUFAF4, NDUFAF5, NDUFAF6, NDUFAF7, NDUFAF8, NUBPL, TIMMDC1, ECSIT, ACAD9, TMEM126B and FOXRED\textsuperscript{19,23}. All of these AFs are nDNA-encoded. Structurally, CI is L-shaped and consists of a hydrophobic MIM-embedded membrane arm and a matrix-protruding peripheral arm (\textbf{Fig. 1}). It appears that CI can exist in active and inactive states, which are associated with changes in CI structure\textsuperscript{21,24}. Functionally, CI consists of three modules: N, Q and P (\textbf{Table 1}), with the P module comprising of two submodules (Pp and Pd). Electrons enter the N-module via an NDUFV1-bound flavin mononucleotide (FMN), after which the Q-module transports them to CoQ via eight iron-sulfur (FeS) clusters (N1a/NDUFV2, N1b/NDUFS1, N2/NDUFS7, N3/NDUFV1, N4/NDUFS1, N5/NDUFS1, N6a/NDUFS8, N6b/NDUFS8). The P-module mediates the trans-MIM proton pumping from the matrix to the IMS\textsuperscript{19,22,25}. At a higher level of organization, CI can assemble into supercomplexes (a.k.a. “respirasomes”) consisting of CI\textsubscript{1}CIII\textsubscript{1}, CI/CIII\textsubscript{2}/CIV, CI\textsubscript{2}CIII\textsubscript{2}/CIV\textsubscript{2}, CI\textsubscript{2}CII\textsubscript{2}CIII\textsubscript{2}/CIV\textsubscript{2} or potentially even larger oligomers. It was hypothesized that formation of these supercomplexes mediates substrate channeling, prevents electrons from escaping and thereby ROS production, and/or serves protein stabilization purposes\textsuperscript{26-34}. 
3. Isolated complex I deficiency and Leigh syndrome

Mutations in nDNA- or mtDNA-encoded OXPHOS genes impair mitochondrial energy metabolism and result in multisystemic diseases, a heterogeneous group of severe, often fatal, pathologies affecting ~1:5000 live births6,35,36. However, mitochondrial dysfunction is not only observed in “rare” mitochondrial syndromes, but also in more common diseases such as Parkinson’s disease, Alzheimer’s disease, age-related frailty, cancer, epilepsy, diabetes and obesity. In this sense, studying rare mitochondrial diseases (MDs) is of great value to better understand mitochondrial dysfunction-related diseases with a higher incidence. We previously proposed to classify monogenic MDs as “primary” and “secondary” disorders37. This classification is based upon whether the disease arises from a mutation in a mitochondrial protein-encoding gene (primary disorder) or from an outside influence on mitochondria (secondary disorder). The latter include for instance viral infections, environmental toxins and off-target drug effects38,39. In this review we focus on isolated CI deficiencies due to nDNA mutations (OMIM252010). The latter often induce Leigh syndrome (LS; OMIM256000)40,41, first described in 1951 by Denis Archibald Leigh42. Currently, mutations in 24 CI subunit-encoding genes have been implicated in LS (Table 1). In addition, LS has been linked to mutations in various AFs of CI (NDUFAF1, NDUFAF2, NDUFAF3, NDUFAF4, NDUFAF5, NDUFAF6, NDUFAF8 and FOXRED1). LS is also referred to as subacute necrotizing encephalopathy and is characterized by an early-onset, often fatal, neurodegenerative disorder presenting with bilateral, symmetric lesions in the brainstem, midbrain, pons, thalamus, basal ganglia and cerebellum43-48. LS patients are born without obvious clinical abnormalities and have a normal prenatal development49. Most patients start developing symptoms within the first year of life and the majority dies before the age of 3 years. Mutations are generally transferred to the offspring in an autosomal recessive (AR) manner50, although X-linked inheritance (NDUFA1 mutation) has also been reported51. Symptoms primarily develop in high-energy demanding tissues, such as the central nervous system, skeletal muscle and heart37,49,52. LS presents with variable clinical symptoms such as progressive encephalopathy, failure to thrive, hypotonia, psychomotor retardation, breathing difficulties, recurrent vomiting, dysphagia, nystagmus, ataxia, neuropathy, hypertrophic cardiomyopathy, loss of vision, impaired hearing, seizures, lactic acidosis and eventually early death43,44,46-48. Similar to most other MDs, the prognosis of CI deficiency-linked LS is poor, as currently no effective treatment strategies exist40,54,55.

4. The NDUFS4 subunit of complex I
In the context of MD, pathogenic mutations in the nDNA-encoded \textit{NDUFS4} gene (OMIM602694) induce “mitochondrial complex I deficiency, nuclear type 1” (MC1DN1; OMIM252010) and LS. \textit{NDUFS4} is located on chromosome 5q11.2, contains five exons and encodes the accessory NADH-dehydrogenase subunit S4 (NDUFS4) of CI, also referred to as the “AQDQ” or “18-kDa” subunit\textsuperscript{43,48,56,57}. The NDUFS4 pre-protein is 175 amino acids (AA) long, with its ATG start codon located in exon one and the TGA stop codon in exon five, and contains a mitochondrial targeting sequence (MTS) of 42 AAs. The latter is removed following mitochondrial import yielding a mature protein of 133 AAs, which is highly similar between human and mouse (Fig. 2A-B-C). NDUFS4 contains two predicted phosphorylation sites (R-X\textsubscript{1-2}-S/T-X)\textsuperscript{58} for AMP-activated protein kinase A (PKA). These are located at position 36-38 (RTS/STS) in the MTS, and at position 171-173 (RVS) in the pre-protein (Fig. 2A-B). Evidence was provided that NDUFS4 exists in non-phosphorylated and phosphorylated forms and that PKA-mediated phosphorylation can occur at Ser173 of the RVS site\textsuperscript{59}. Of note, another study concluded that not NDUFS4 but another CI accessory subunit of 18-kDa (NDUFB11) was phosphorylated\textsuperscript{60}. It was proposed that NDUFS4 phosphorylation affects its mitochondrial import, MTS removal and subsequent incorporation in CI\textsuperscript{57,59,61,62}. In addition, PKA-mediated NDUFS4 phosphorylation might stimulate CI activity\textsuperscript{61}. Within ovine CI, the NDUFS4 subunit is located at the interface between the N- and Q-module (Fig. 1) and interacts with various other CI subunits within the N-module (NDUFV1, NDUFV2, NDUFV3-10, NDUFS1, NDUFA12) and Q-module (NDUFS3, NDUFS8, NDUFA6, NDUFA9). In case of bovine CI, the NDUFS4 protein also interacts with NDUFS2 but not with NDUFV2 (Table 2). Taken together, the current experimental data suggests that the NDUFS4 subunit is potentially PKA-phosphorylated and plays a key role in CI assembly and/or stabilization (likely involving N-to-Q-module attachment), thereby exerting control over CI levels and/or activity\textsuperscript{63,64}.

5. \textit{NDUFS4} mutations and human mitochondrial disease

Pathogenic mutations in \textit{NDUFS4} linked to MC1DN1/LS are inherited in an AR manner. Several of these mutations have been described (Table 3), occurring in intronic and exonic nDNA regions (Fig. 2D). The first reported mutation (c.466-470dup) induced a 5 bp duplication leading to a frameshift (K158fs) in a patient with LS-resembling symptoms. This mutation resulted in an elongation of the mature protein by 14 AA and destruction of the RVS phosphorylation consensus site\textsuperscript{56}. Hereafter, various other \textit{NDUFS4} mutations associated with LS, Leigh-like syndrome and/or MC1DN1 were presented including: (1) a homozygous c.462del mutation disrupting the
NDUFS4 reading frame\textsuperscript{44}, (2) introduction of a stop codon resulting in degradation of transcribed product and suppression of the truncated NDUFS4 subunit\textsuperscript{43,61,65} and (3) nonsense mutations leading to premature truncation of NDUFS4\textsuperscript{66}. Analysis of 14 patients with pathogenic NDUFS4 mutations demonstrated that the age of death varied between 3.6 and 27 months\textsuperscript{87}. Compatible with the latter study, analysis of a cohort of 22 patients (18 families) yielded an average age of disease onset of 4.5±4.4 months and an average age of death of 10±7.7 months\textsuperscript{48}. The latter study further reported that NDUFS4 mutations were associated with diverse clinical features including: (1) CI deficiency in muscle (N=18 patients) and fibroblasts (N=12), (2) LAC elevation in plasma (N=16) and cerebrospinal fluid (N=11), (3) lesions of the brainstem (N=14) and basal ganglia (N=9), (4) cortical atrophy (N=3), (5) hypotonia (N=22), (6) developmental arrest-regression (N=11), (7) absence of eye contact (N=10), (8) apneic episodes (N=10), (9) pyramidal signs (N=6), (10) epilepsy/seizures (N=4), (11) movement disorder (N=2), (12) microcephaly (N=1), (13) ocular abnormalities (N=11), (14) hypertrophic cardiomyopathy (N=5), (15) feeding problems/failure to thrive (N=8), and (16) ragged-red fibers and lipid accumulation in muscle biopsies (N=4).

6. Impact of NDUFS4 mutations in patient-derived cells

In patient-derived primary skin fibroblasts, NDUFS4 mutations generally result in reduced NDUFS4 mRNA levels and the absence of NDUFS4 protein, associated with reduced levels of the CI holocomplex and catalytic activity\textsuperscript{44,45,46,53,64,68,69}. In general, the expression/activity of OXPHOS complexes other than CI is not affected by NDUFS4 mutations and/or absence of the NDUFS4 protein\textsuperscript{45,56,64,70}. On native gels (\textit{i.e.} using blue-native polyacrylamide gel electrophoresis; BN-PAGE), NDUFS4-mutated patient fibroblasts exhibit complete absence of the fully assembled CI holocomplex. Instead, these cells display a catalytically-inactive CI subcomplex with a size of \textasciitilde830-kDa (“CI-830”)\textsuperscript{44,45,68,69,71}. This suggests that NDUFS4 mutations impair CI biogenesis and/or stability\textsuperscript{68}. Analysis of patient-derived cells further demonstrated that NDUFS4 mutations were associated with partial \(\Delta\psi\) depolarization, increased ROS and NAD(P)H levels, aberrations in cytosolic and mitochondrial calcium/ATP homeostasis and altered mitochondrial morphology\textsuperscript{49,69}.

7. The Ndufs4 whole-body (WB) knockout (KO) mouse model

In human patients, the majority of the currently known pathogenic NDUFS4 mutations lead to absence of (full-length) NDUFS4 protein (Table 3). These mutations are inherited in an AR
manner and therefore present in every cell. Therefore, from a genetic point of view whole-body (WB) Ndufs4 deletion is a valid strategy to create mouse models of MC1ND1/LS for pathomechanistic analysis. Therefore, below we will primarily focus on the Ndufs4 whole body KO mouse model (Ndufs4+/−-WB), which was created by deleting exon two of NDUFS4 (Fig. 2B), resulting in a frameshift that prevented formation of NDUFS4 protein\(^72\). Among Ndufs4 KO models, these mice are most extensively studied (Supplementary Table 1) and applied in intervention studies (Supplementary Table 2). However, to allow better interpretation of the Ndufs4+/−-WB model, results obtained with relevant (tissue-specific) Ndufs4 KO mouse models will also be discussed. Mouse models focusing on the impact of Ndufs4 deletion on inflammation, bone resorption and immune cell fate are presented elsewhere\(^73,74\).

### 7.1. The phenotype of Ndufs4+/−-WB mice

Heterozygous (Hz) Ndufs4+/−-WB mice displayed no phenotype when compared to wildtype (WT) mice\(^72\). In contrast, Ndufs4+/−-WB mice appeared smaller and displayed hair loss by postnatal day (PD) 21, whereas hair grew back during the next hair-growth cycle. Detailed analysis revealed that Ndufs4+/−-WB mice display an infiltration of inflammatory monocytes and macrophages and elevated mRNA levels of inflammatory markers in their skin\(^73\). The latter might explain the sudden hair loss and be linked to the systemic inflammation reported in Ndufs4+/−-WB mice\(^73\). Until PD30, mice appeared healthy and displayed similar behavior (e.g. grooming, feeding, socializing) as WT littermates. After PD30, mice became lethargic, hypothermic, blind and eventually developed severe ataxia, marked by uncoordinated gait/balance (also see below) and hindlimb clamping. Additionally, mice developed abnormal oxygen saturation, heart rate, breathing patterns and displayed elevated cerebral and serum LAC levels (see Supplementary Table 1 for further phenotypic information). These symptoms progressively worsened and mice died at ~PD50\(^72,75-79\). Neurologically, Ndufs4+/−-WB mice developed bilateral spongiform lesions in the vestibular nuclei (VN), and neurodegeneration in various other brain regions including the olfactory bulb (OB), optic chiasm, optic tract, superior colliculus, interpeduncular nucleus, lateral lemniscus, trapezoid body, cochlear nuclei, fastigial nucleus (FN), inferior olivary complex, nodulus (X) and the uvula (IX) granular and molecular layer\(^75,77,80\). In addition, progressive microgliosis was observed from PD26 (mid-stage disease) onwards\(^75,77\). It is expected that the VN/FN lesions contribute to the breathing abnormalities observed in these mice\(^75\). Ndufs4+/−-WB mice also displayed (mild) glial lipid droplet (LD) accumulation in OB/VN, periaqueductal gray, cerebellum, dorsal motor nucleus, vagus and abducens nuclei. Accumulation of LD in glial cells is strongly associated with neuroinflammation and progression of neurodegeneration in Drosophila.
mutants and *Ndufs4*+/−-WB mice. The latter study also demonstrated that LD accumulation correlated with elevated ROS levels, and that both phenomena were mitigated by antioxidants. This suggests that oxidative stress can induce LD accumulation during mitochondrial dysfunction. Therefore, ROS-induced lipid peroxidation and ensuing neuroinflammation might be part of the pathomechanism in *Ndufs4*+/−-WB mice.

### 7.2. The behavior of *Ndufs4*+/−-WB mice

Due to their severe and progressive phenotype, early death and small size it is challenging to analyze *Ndufs4*+/−-WB knockout mice in behavioral studies. Mice suffered vision loss at PD21, as evidenced by an absent B wave in their electroretinogram and their failure to recognize a visual cliff. Until ~PD30 normal locomotor activity during both day and night cycle was observed. Gait analysis performed at PD30, PD35 and PD40 revealed severe impairment. From PD35 onwards the mice: (1) failed to maintain balance on a ledge, (2) failed in a negative geotaxis test, (3) displayed a decline in locomotor activity in the open field test, (4) exhibited deteriorating muscle strength on the wire grip hang test, and (5) were unable to remain on a rotating rod as long as WT littermates. Although we do not have information at the level of individual animals, the water intake of *Ndufs4*+/−-WB mice is ~50% of that of WT mice and further decreases around PD32-36. However, it was reported that food consumption of *Ndufs4*+/−-WB animals was within the normal range during day, night and fasting. This leaves open the question why *Ndufs4*+/−-WB animals have a smaller size. Between PD26-37 mice were housed in metabolic cages. Weight loss occurred from PD35-40 onwards, which coincides with the reduced water intake and ataxia induction. This might suggest that food intake is normal prior to this time window, but *Ndufs4*+/−-WB animals start to eat less and lose weight once the disease phenotype becomes more severe. Supporting this idea, conditional *Ndufs4*+/−-KO animals displayed reduced food intake coinciding with the appearance of symptoms.

### 7.3. Sensitivity to anesthetics of *Ndufs4*+/−-WB mice

It is well established that the use of anesthetics in MD patients requires careful consideration. In this sense, *Ndufs4*+/−-WB mice displayed resistance against ketamine sedation, but were hypersensitive to volatile anesthetics (VA), such as isoflurane and halothane. It is still incompletely understood why ketamine resistance occurs, but this phenomenon might be linked to its different mode-of-action when compared to VAs. In this sense, it was suggested that ketamine action is mediated by increased neuronal activation and cortical synchronization rather than neuronal inactivation. Moreover, ketamine may induce the anesthetized state via a different target.
than other anesthetics. Glutamatergic neuron-specific Ndufs4 knockout animals (see below) also displayed hypersensitivity to VAs, whereas animals with GABAergic neuron- or cholinergic neuron-specific Ndufs4 loss (see below) did not display this phenomenon. The increased VA sensitivity of Ndufs4+/−-WB mice may be due to VA-induced CI inhibition and the key role played by CI function in maintaining neuronal activity. These findings suggest that excitatory glutamatergic transmission is the major contributor to the VA hypersensitivity of Ndufs4+/−-WB mice. Mechanistically, it was reported that VA rapidly depleted the blood levels of β-hydroxybutyrate (βHB) in neonatal mice. In this study VA concentrations well below those required for anesthesia were applied and depletion of β-HB was mediated by citrate accumulation, malonyl-CoA production by acetyl-CoA carboxylase, and inhibition of fatty acid oxidation. Analysis of PD17 mice revealed significantly higher β-HB levels in Ndufs4+/−-WB neonates relative to their WT littermates. It was concluded that CI is not the direct target of VA mediating the acute β-HB effect in neonates, but may contribute to the increased LAC observed in VA-exposed animals.

7.4. Tissue analyses of Ndufs4+/−-WB mice

Comparison of residual CI activities in isolated mitochondria between seven tissues of WT, Ndufs4+/−-WB and Ndufs4−/−-WB mice revealed normal values for Ndufs4+/−-WB and reduced but non-zero values for Ndufs4−/−-WB animals. The latter equaled: 44% of WT (heart), 29% (muscle), 26% (brain), 25% (kidney), 19% (liver), 17% (pancreas) and 9% (lung). No significant differences between WT and Ndufs4−/−-WB tissues were detected with respect to CII-CV activities. A differential reduction in CI activity was observed in various KO brain regions: 14% of WT (OB), 25% (brainstem), 28% (cerebellum) and 62% (anterior cortex). It was hypothesized that, for the affected brain regions, the inability of mitochondrial energy production to meet cellular demands is responsible for the observed neurodegeneration and disease progression in Ndufs4−/−-WB mice. In contrast to the findings of Calvaruso et al., the original study reported no CI activity in liver of Ndufs4−/−-WB mice. As suggested in the latter paper, it is to be expected that differences in sample preparation will lead to different amounts of functional CI due to its destabilization by NDUFS4 absence. Using BN-PAGE, inactive CI-830 and active ~200-kDa CI-subassemblies were observed in heart, muscle, brain and kidney. The smaller subassembly at least contained the NDUFV1 and NDUFV2 subunit, and therefore likely represents the N-module of CI. These data suggest that Ndufs4−/−-WB tissues display a reduced but not zero CI activity due to a destabilized CI holo-complex. This is compatible with a follow-
up proteomics analysis, demonstrating that overall CI subunit levels were reduced by ~50% in brain, liver, heart, kidney, diaphragm and skeletal muscle of Ndufs4-/--WB mice\textsuperscript{64}. The latter study also demonstrated that overall CII-CV subunit levels were not greatly affected in these tissues. Analysis of the CI structure revealed that NDUFS4 and the CI accessory subunit NDUFA12 interact with each other and with CI subunits of the N- and Q-module (Table 2). Remarkably, absence of the NDUFS4 protein induced near complete absence of the NDUFA12 subunit and increased the protein level of NDUFA2 (an AF of CI) in brain, liver, heart, kidney, diaphragm and skeletal muscle of Ndufs4-/--WB animals\textsuperscript{64}. This finding was confirmed in Ndufs4-/--WB-derived mouse embryonic fibroblasts (MEFs; see below), Ndufs4-/- brainstem glutamatergic neurons\textsuperscript{94} and NDUFS4-mutated LS patient cells. Compatible with previous data\textsuperscript{91}, Ndufs4-/--WB-derived MEFs displayed in situ CI activity, but BN-PAGE analysis revealed that NDUFAF2 attached to an inactive CI subcomplex (CI-830) and inactive assemblies of higher molecular weight. Interestingly, NDUFA12 absence did not reduce NDUFS4 levels but triggered NDUFAF2 association to active CI in NDUFA12-mutated LS patient cells\textsuperscript{64}. This strongly suggests that absence of NDUFS4 induces absence of NDUFA12 but not vice versa. Association of NDUFAF2 with active CI was not observed in BN-PAGE experiments with mutations in other CI subunit-encoding genes where NDUFAF2 was attached to CI-830 (NDUF1, NDUFV1 mutation) or not detected (NDUF7 mutation). Taken together (Fig. 3A), the above evidence supports a model in which absence of NDUFS4 induces: (1) absence of NDUFA12, (2) attachment NDUFAF2 to stabilize active CI in situ, (3) a reduced stability of N- to Q-module attachment\textsuperscript{95} and, (4) a reduced, but not zero, level of active CI. In addition to NDUFAF2 binding, in situ CI stabilization might also involve MIM lipids and/or interaction with other ETC complexes\textsuperscript{29,91,96-98}. However, in multiple models (ovine, porcine, human) NDUFS4, NDUFA12 and their interacting subunits (Table 2) were not involved in ETC supercomplex interactions\textsuperscript{29-32}.

### 7.5. Skeletal muscle bioenergetics of Ndufs4-/--WB mice

In soleus muscle of Ndufs4-/--WB mice, ATP levels were slightly decreased and CI activity was reduced, whereas phosphocreatine (PCr), creatine (Cr) and inorganic phosphate (Pi) levels were normal\textsuperscript{72}. Compatible with these observations, the histology, physiology and metabolism of soleus muscle tissue appears to be minimally affected by Ndufs4 deletion. Enzymological analysis of isolated skeletal muscle mitochondria\textsuperscript{99} demonstrated a 79% reduction in maximal CI activity (V\textsubscript{max}), which was paralleled by a 45-72% increase in V\textsubscript{max} for CII, CIII, CIV and citrate synthase (CS). Using integrated in silico and experimental analysis, the same study demonstrated that the maximal rates of mitochondrial PYR oxidation and ATP production were not significantly
affected in muscle mitochondria of Ndufs4−/−-WB mice. Computer modeling further predicted that CI deficiency alters the concentration of intermediate metabolites, increases mitochondrial NADH/NAD+ ratio and stimulates the lower half of the TCA cycle, including CII\textsuperscript{99}. The computer model further predicted that CI deficiency only has a major metabolic impact when its activity decreases below 10% of normal levels, compatible with a biochemical threshold effect, and that mouse skeletal muscle mitochondria display a substantial CI overcapacity, minimizing the effect of CI dysfunction on mitochondrial metabolism. Both CI overcapacity and the biochemical threshold effects likely differ between tissues and therefore could be involved in the tissue-specific impact of Ndufs4 deletion\textsuperscript{100-102}.

7.6. Metabolome analysis of Ndufs4−/−-WB mouse brain and skeletal muscle tissue

A disturbed redox balance is often associated with accumulation of PYR, LAC and alanine. Accordingly, PYR, LAC and glycolytic intermediates were increased in whole-brain of Ndufs4−/−-WB mice at PD30\textsuperscript{103}. Likewise, the ratios of alanine, leucine and isoleucine relative to glutamic acid, as well as PYR/LAC and PYR/acyl-carnitine ratios, were increased in lesion-prone brain regions (i.e. brainstem, cerebellum and OB) of male Ndufs4−/−-WB mice at PN45-50. These alterations may be due to an increased NADH/NAD+ ratio, which controls the formation of AAs and LAC, while the increased PYR/acyl-carnitine ratio suggests NAD+ -dependent acetyl-CoA formation from PYR\textsuperscript{93}. Glutathione (GSH) was increased in brainstem, OB and lesion-resistant brain regions. Analysis of skeletal muscle revealed decreased levels of PYR, glycerol, alanine and LAC\textsuperscript{104,105}. In addition, Ndufs4−/−-WB skeletal muscle also displayed decreased levels of N, N-dimethylglycine, fatty acid acyl-carnitines (chain lengths of: C0, C3, C4, C5, C6, C8, C12, and C16) and 2-aminoadipate\textsuperscript{105}. Similar alterations were observed in Ndufs4−/−-WB brains\textsuperscript{93}, suggesting that fatty acid β-oxidation and AA catabolism is altered. Furthermore, muscle creatinine and creatine levels were decreased as well as fumarate, malate and succinate. The latter may indicate an increased use of succinate to fuel CII thereby bypassing CI. Proline, hydroproline, citrulline, glutamate and glutamine were decreased\textsuperscript{105}. Similar metabolic alterations were observed in lesion-prone brain regions of male Ndufs4−/−-WB mice\textsuperscript{93}. These metabolic alterations may indicate altered proline metabolism that has been suggested to play a central role in mTOR signaling\textsuperscript{106,107}. Branched-chain amino acids (BCAAs) were increased, while butyryl(C4)- and isovaleryl(C5)-carnitine were decreased in lesion-prone brain regions\textsuperscript{93}. BCAA accumulation may be a consequence of inhibition of the NAD+-dependent reaction catalyzed by branched-chain α-
keto acid dehydrogenase due to redox imbalance. In addition, glutamate, aspartic acid and α-hydroxyglutaric acid were also decreased in Ndufs4−/−-WB brains, which could point to a compensatory increase of α-ketoglutaric acid to the TCA cycle. These alterations are in line with disruptions of neuronal transport systems and the beneficial effects of mTOR complex 1 (mTORC1) inhibition that have been attributed to a restored glutamine/glutamate/α-ketoglutaric acid axis in presymptomatic Ndufs4−/−-WB mice. PYR and alanine levels tended to be increased in all brain regions, LAC was only increased in lesion-prone brain regions. The OB showed higher levels of other glycolysis and pentose phosphate pathway-related intermediates, which was not observed in other brain regions. Lastly, metabolic changes were observed in dihydroxyacetone phosphate and glycerol-3-phosphate (G3P). These metabolites link glycolysis, lipid metabolism and OXPHOS. G3P was most severely decreased in lesion-prone brain regions, suggesting that G3P oxidation is involved in fueling respiration in CI deficiency. Collectively, an adaptive increase in glutamate, α-hydroxyglutaric acid and G3P oxidation seems to drive energy-generation in lesion-resistant brain regions, while these mechanisms appear less effective in lesion-prone brain regions where CI activity is more severely reduced. In this context, analysis of brainstem tissue from Ndufs4−/−-WB mice suggested glycolysis impairment, as supported by increased levels of fructose-6-phosphate (F6P), G3P and dihydroxyacetone (DHAP), which are glycolysis intermediates upstream of the NADH-producing step. Suggestive of an increased brainstem NADH/NAD⁺ ratio, the latter study also demonstrated increased α-hydroxybutyrate (αHB) and LAC in the brainstem, reduced aspartate levels in the brainstem, and increased αHB plasma levels.

7.7. Analysis of Ndufs4−/−-WB mice-derived cell models

To gain further insight into the cellular consequences of Ndufs4 deletion, immortalized mouse embryonic fibroblasts (MEFs) were generated from Ndufs4−/−-WB mice. Mitochondrial fractions isolated from these MEFs lacked NDUFS4 protein, displayed virtually no CI activity and reduced CII, CIII and CIV activity. Also, SCC activity (a combined measure of CII and CIII activity) was reduced whereas CV and CS activities were normal. Similar to tissues of Ndufs4−/−-WB mice, BN-PAGE analysis of mitochondrial fractions demonstrated the presence of CI-830 and ~200-kDa CI-subassemblies, whereas the levels of fully-assembled CII–CV were not affected in Ndufs4−/−-MEFs. Using intact cells, it was observed that Ndufs4 knockout displayed a ~50% reduced O₂ consumption that was, however, sensitive to acute treatment with the CI inhibitor rotenone. The latter demonstrates that active CI (i.e. a complex in which the N-module is attached to the Q-module) is present in Ndufs4−/−-MEFs. As stated above, it is likely that differences in sample...
preparation leads to different amounts of functional CI due to its destabilization by NDUFS4 absence. This explains why no CI activity was detected in mitochondrial fractions of \textit{Ndufs4}^{-/-} MEFs, whereas rotenone effectively inhibited O$_2$ consumption in intact \textit{Ndufs4}^{-/-} MEFs$^{115}$. Complementation of the \textit{Ndufs4}^{-/-} cells with the WT gene increased the levels of catalytically active CI and O$_2$ consumption. At the intact cell level, \textit{Ndufs4}^{-/-}-MEFs displayed an increase in the combined mitochondrial autofluorescence signal of NADH and NADPH and LAC release, an increased NADH/NAD$^+$ ratio and a slight $\Delta\psi$ hyperpolarization. In contrast, \textit{Ndufs4}^{-/-}-MEFs displayed no detectable alterations in NADPH/NADP ratio, glucose consumption, protein expression of hexokinases (I and II), pyruvate dehydrogenase (PHD) phosphorylation, total ATP content, free cytosolic ATP concentration, cell growth rate and levels of hydroethidium (HEt)-oxidizing ROS$^{115}$. These findings indicate that \textit{Ndufs4} deletion destabilizes CI and suggest that \textit{Ndufs4}^{-/-}-MEFs display an increased mitochondrial NADH level and are slightly more glycolytic than WT cells. However, alterations in cell metabolism might be difficult to detect in the MEFs given their intrinsic bioenergetic properties (e.g. a relatively high glycolytic rate). Analysis of primary muscle- and skin-derived fibroblasts from \textit{Ndufs4}^{-/-}-WB mice revealed that these cells displayed no active CI on BN-PAGE, increased levels of HEt-oxidizing ROS and minor aberrations in mitochondrial morphology$^{116}$. \textit{Ndufs4}^{-/-}-fibroblasts proliferated normally and displayed no obvious apoptotic signs$^{116}$. These findings are compatible with results obtained in patient-derived primary skin fibroblasts harboring \textit{NDUFS4} mutations$^{49,64,68,69}$, and suggest a connection between CI deficiency, ROS levels and (regulation of) mitochondrial structure in the fibroblast cell model$^{2,4,117,118}$. In summary, we conclude that the cellular consequences of \textit{Ndufs4} deletion (co)depend on the type of cell, cell metabolic state, culture conditions, external substrate (e.g. glucose) concentration and immortalization status.

8. Results with other whole-body \textit{Ndufs4} KO mouse models

In general, results obtained with other whole-body knockout models (Supplementary Table 1) were highly similar (e.g. CRISPR-Cas9 \textit{Ndufs4}^{-/-} mice) but not always identical (e.g. \textit{Ndufs4}^{fky/fky}, \textit{Ndufs4}^{GT/GT} mice) to those from \textit{Ndufs4}^{-/-}-WB mice. For instance, \textit{Ndufs4}^{fky/fky} mice displayed a reduced CI activity in brain, heart, muscle, liver and kidney but presented with symptoms earlier than \textit{Ndufs4}^{-/-}-WB animals$^{95,119}$. Similar to the \textit{Ndufs4}^{-/-}-WB mouse model, NDUFS4 protein was not detectable in MEFs from \textit{Ndufs4}^{fky/fky} mice, and these cells displayed the CI-830 subassembly, reduced CI activity, reduced O$_2$ consumption, but normal ATP content and LAC production$^{119,120}$. MEFs from \textit{Ndufs4}^{fky/fky} and \textit{Ndufs4}^{-/-} (Hz) 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\textsuperscript{119}. In addition to these genetic changes, \textit{Ndufs4} deletion induced shifts in acyl-carnitine and AA levels in \textit{Ndufs4}\textsuperscript{ky/ky}, MEFs, pointing towards a reverse TCA flux\textsuperscript{95}. \textit{Ndufs4\textsuperscript{GT/GT}} mice displayed a milder phenotype than \textit{Ndufs4}\textsuperscript{−/−}-WB animals and their degree of CI deficiency was less than in \textit{Ndufs4}\textsuperscript{−/−}-WB and \textit{Ndufs4}\textsuperscript{ky/ky} mice\textsuperscript{121,122}. Relative to their WT littermates, \textit{Ndufs4\textsuperscript{GT/GT}} mice were hyperactive, which might indicate increased restlessness, a symptom that is also observed in MD patients\textsuperscript{123}. \textit{Ndufs4\textsuperscript{GT/GT}} mice displayed a normal maximal ATP production capacity and mitochondrial content in left hippocampus and, similar to \textit{Ndufs4\textsuperscript{−/−}}-WB and \textit{Ndufs4}\textsuperscript{ky/ky} mice, displayed a normal CII and CIII activity in left hippocampus and frontal cortex. \textit{Ndufs4\textsuperscript{GT/GT}} mice presented with elevated levels of acyl-carnitine C3, C4 and C12 in the frontal cortex\textsuperscript{133}, whereas no alterations were found in whole brain tissue\textsuperscript{121}. In addition, AA metabolism and TCA metabolites were also altered in brain tissue. These findings are compatible with a reverse TCA cycle flux in \textit{Ndufs4\textsuperscript{GT/GT}} mice due to altered brain bioenergetics\textsuperscript{121}. Individuals with MD often present with diabetes\textsuperscript{124}, and therefore, plasma corticosterone and glucose levels were measured in \textit{Ndufs4\textsuperscript{GT/GT}} mice. Relative to WT littermates, \textit{Ndufs4\textsuperscript{GT/GT}} mice presented with normal plasma glucose and insulin levels but lower baseline plasma cortisone levels. The latter increased upon chronic unpredictable stress\textsuperscript{121}. Given their minor CI deficiency and phenotype \textit{Ndufs4\textsuperscript{GT/GT}} mice are less suited as animal models of LS. However, these mice could be of particular value for pathomechanistic analysis of mild CI deficiencies\textsuperscript{121}.

9. Results with neuron-specific \textit{Ndufs4} KO mouse models

\textit{Ndufs4\textsuperscript{GT/GT}} mice

Analysis of a neuron- and glia-specific model (NesKO mice) revealed a phenotype similar to that of \textit{Ndufs4}\textsuperscript{−/−}-WB animals (\textbf{Supplementary Table 1}) including failure to thrive, hypothermia, optic atrophy, cataracts, ptosis, seizures and breathing abnormalities\textsuperscript{125}. Furthermore, these animals exhibited severe progressive ataxia from ~PD35 onwards, marked by an uncoordinated gait, reduced balance, hindlimb clasp and decreased rotarod performance\textsuperscript{125,126}. These results suggest that \textit{Ndufs4} deletion in NesKO mice induces glial activation, associated with increased oxidative stress, cytokine release and eventually necrotic, but not apoptotic, cell death due to ATP depletion. Animals with specific \textit{Ndufs4} knockout in vestibular nuclei (\textbf{AAV-VN-KO mice}) also presented with a similar phenotype as \textit{Ndufs4}\textsuperscript{−/−}-WB mice\textsuperscript{75}. The development of severe breathing abnormalities in AAV-VN-KO mice suggests that VN neurodegeneration might be responsible for
these abnormalities in $Ndufs4^{-/-}$-WB mice. Glutamatergic neuron-specific $Ndufs4$ knockout (Vglut2:$Ndufs4cKO$ mice) developed several of the symptoms observed in $Ndufs4^{-/-}$-WB mice, associated with a similar phenotype and leading to early death$^{82,113,127}$. However, Vglut2:$Ndufs4cKO$ mice did have a longer lifespan (~10 wks)$^{127}$ relative to $Ndufs4^{-/-}$-WB mice$^{72,75}$. Although GABAergic neuron-specific loss of $Ndufs4$ (Gad2:$Ndufs4cKO$ mice) resulted in failure to thrive, it was not associated with clinical symptoms compared to WT littermates$^{82}$. However, mice suffered from hypothermia as early as PD20-30, exhibited (spontaneous) seizures when being handled, and displayed an increase in seizure incidence from PD50 onwards. Gad2:$Ndufs4cKO$ mice showed a different profile compared to Vglut2:$Ndufs4cKO$ mice with increased microglial and astroglial activation in the external globus pallidus (GPe), basal ganglia and the substantia nigra pars reticulata (SNr). $Ndufs4$ deletion in cholinergic neurons (ChAT:$Ndufs4cKO$ mice) did not induce development of fatal encephalopathy and animals remained clinically healthy throughout the study$^{82}$. Knockout of $Ndufs4$ in striatal medium spiny neurons (MSN $Ndufs4^{-/-}$ mice) did not affect body weight or life span up to 6 months after birth, and animals did not develop neuroinflammation$^{128}$. Finally, animals with dopaminergic neuron-specific $Ndufs4$ deletion (DA $Ndufs4^{-/-}$ mice) appeared healthy and were indistinguishable from WT littermates$^{129-131}$. In summary (Fig. 3B), the use of neuron-specific models suggests that $Ndufs4$ deletion affects glutamatergic neurons in the VN, cerebellum (Cer) and inferior olive (IO) to induce motor alterations, respiratory (breathing) abnormalities and brainstem inflammation. In addition, it appears that malfunction of GABAergic neurons in the OB, GPe and SNr likely underlies the epileptic seizures, basal ganglia inflammation and abnormal body temperature.

**10. Results with heart- and/or skeletal muscle-specific $Ndufs4$ null mice**

Next to the brain, the heart is one of the most severely affected organs in CI-deficiencies due to its high dependency on aerobic metabolism$^{132,133}$. However, in sharp contrast to the $Ndufs4^{-/-}$-WB model, heart-specific $Ndufs4$ null mice did not present with a pathological phenotype up to 1 yr. of age$^{134}$. Instead, mice developed hypertrophic cardiomyopathy marked by decreased left ventricular ejection fraction (LVEF) and increased left ventricular mass (LVM)$^{135}$. Interestingly, hypertrophic cardiomyopathy was also observed in several patients (5 out of 22) with $NDUFS4$ mutations$^{48}$. Animals with heart- and skeletal muscle-specific $Ndufs4$ knockout (Ckm-NLS-cre; $Ndufs4^{loxP/loxP}$ 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 yr. of age\textsuperscript{129}. Taken together, analysis of the heart- and/or muscle-specific \textit{Ndufs4\textsuperscript{-/-}} models suggests that the hypertrophic cardiomyopathy in \textit{NDUFS4}-mutated patients (see \textbf{section 5}) might be a secondary consequence of the CI deficiency, which develops at a later age.

\section*{11. Intervention studies in \textit{Ndufs4} mouse models}

Intervention studies primarily have been carried out using the \textit{Ndufs4\textsuperscript{-/-}}-WB mouse model. These interventions involve: (1) reducing tissue oxygenation (carbon monoxide exposure, hypoxia, phlebotomy), (2) small molecule treatment, (3) injection of induced pluripotent stem cells (iPSCs), and (4) genetic approaches (\textit{Supplementary Table 2}).

\subsection*{11.1. Reduction of tissue oxygenation}

As mitochondria are prime O\textsubscript{2} consumers, defects in the OXPHOS system might increase tissue oxygenation. Supporting this idea, venous hyperoxia was observed in MD patients and tissue oxygenation was used as a measure of impaired OXPHOS function\textsuperscript{136}. Whole O\textsubscript{2} consumption decreased over time and appeared to correlate with disease phenotype in \textit{Ndufs4\textsuperscript{-/-}}-WB mice. More detailed examination revealed that the partial oxygen pressure (PO\textsubscript{2}) in brain tissue increased in an age-dependent manner in \textit{Ndufs4\textsuperscript{-/-}}-WB mice and correlated with disease severity\textsuperscript{137}. Based on these findings, different strategies were applied to reduce tissue oxygenation in these mice. \textbf{Carbon monoxide (CO)} exposure is an established strategy to reduce tissue oxygenation which has been linked to cytoprotective effects as well as oxidative stress induction\textsuperscript{138,139}. Though various positive effects were observed (\textit{Supplementary Table 2}), chronic CO exposure induced hyperintense lesions in the caudoputamen region of the brain in \textit{Ndufs4\textsuperscript{-/-}}-WB mice, indicative of adverse effects\textsuperscript{137}. Similarly, \textbf{hypoxic conditions} prevented/mitigated part of the disease phenotype in \textit{Ndufs4\textsuperscript{-/-}}-WB mice (prolonged life span, no loss of body weight, improved motor function, prevention of hypothermia)\textsuperscript{140}. However, beneficial effects were only induced by continuous hypoxia (11\% O\textsubscript{2} for 3 wks starting from PD30) or by hypoxia applied during a late-stage of the disease (11\% O\textsubscript{2}; \textit{Supplementary Table 2}). In contrast, temporary or milder hypoxia conditions (17\% O\textsubscript{2} for 3 wks) were ineffective\textsuperscript{141,142}. Decreasing the number of circulating red blood cells by \textbf{phlebotomy} is another approach to reduce brain oxygenation by decreasing oxygen delivery. Although phlebotomy increased the life span of \textit{Ndufs4\textsuperscript{-/-}}-WB mice (\textit{Supplementary Table 2}) it only temporarily prevented development of VN lesions\textsuperscript{137}. Taken together, the above studies provide evidence that a (partial) reduction in brain oxygenation and/or oxygen delivery ameliorates the disease phenotype of \textit{Ndufs4\textsuperscript{-/-}}-WB mice. Regarding the mechanism of this
amelioration, it was proposed that simultaneous reduction of oxygen delivery and consumption may reverse disease progression by triggering adaptive programs and at the same time limiting oxygen toxicity.

**11.2. Small molecule treatment**

Intervention studies in \(Ndufs4^{-/-}\)-WB mice have been carried out with several classes of small molecules targeting: redox metabolism, mitochondrial biogenesis, energy metabolism, the mechanistic target of rapamycin (mTOR), toll-like receptor 4 (TLR4), benzodiazepine receptors and cyclic-AMP (cAMP) homeostasis. Below we summarize the impact of these interventions, whereas additional details are provided in Supplementary Table 2.

**11.2.1. Redox active compounds**

**Idebenone** is an antioxidant that rescues vision impairment in CI-deficiency-linked Leber’s hereditary optic neuropathy (LHON)\(^\text{143}\). However, idebenone treatment did not reverse visual impairment in \(Ndufs4^{-/-}\)-WB mice. N-acetyl cysteine amide (\(\text{AD4}\)) is a blood-brain-barrier (BBB) penetrating antioxidant that reduces oxidative stress\(^\text{144,145}\). AD4 delayed disease onset and reduced the severity of the disease phenotype, marked by improved motor function at PD30\(^\text{80}\). The ROS scavenger **KH176** (Sonicromanol) is based on the vitamin E-derivative Trolox\(^\text{117}\). KH176 diminished cellular damage caused by oxidative stress and improved residual enzyme activity of different OXPHOS complexes without inducing drug toxicity in LS patient-derived fibroblasts\(^\text{146}\). Although KH176 displayed various positive effects, including improved motor function and normalization of lipid peroxidation, this molecule did not improve disease onset/severity and brain pathology, and did not restore residual CI activity\(^\text{147-148}\). Individually, **KH176 and clofibrate** (see below) displayed positive effects in \(Ndufs4^{-/-}\)-WB mice. However, combined treatment with these molecules did not prolong life span or improve motor function\(^\text{148}\). NAD\(^+\) levels were reduced and NADH/NAD\(^+\) ratios were increased in brain tissue of \(Ndufs4^{-/-}\)-WB mice, suggesting that NAD(H) redox imbalance might be involved in the pathomechanism. Compatible with this hypothesis and reduced CI-mediated NAD\(^+\) formation from NADH, GAL-induced death of LS patient primary skin fibroblast with isolated CI deficiency was rescued by a cell-permeable NAD\(^+\)-variant\(^\text{149}\). However, administration of the NAD\(^+\) precursor nicotinamide mononucleotide (\(\text{NMIN}\)) prolonged life span, but did not ameliorate the clinical phenotype of \(Ndufs4^{-/-}\)-WB mice (\(\text{150}\)). **P7C3** is an aminopropyl carbazole that was first identified by an *in vivo* screen in search of chemicals that
enhanced neuron formation in the hippocampus of adult mice. It is thought that P7C3 activates nicotinamide phosphoribosyltransferase (NAMPT; a key enzyme in the NAD\(^+\) salvage pathway) thereby increasing intracellular NAD\(^+\) levels. P7C3 moderately prolonged life span, but did not increase NAD\(^+\) levels in \(Ndufs4^{-/-}\)-WB mice brains or alter NAMPT protein levels.

11.2.2. Stimulating mitochondrial biogenesis

Stimulation of peroxisome proliferator activated receptors (PPARs) and PPARG (gamma) coactivator 1\(\alpha\) (PGC-1\(\alpha\)) enhances fatty acid \(\beta\)-oxidation and mitochondrial biogenesis. Treatment with the PPARA (alpha) stimulator clofibrate prolonged lifespan and motor function of \(Ndufs4^{-/-}\)-WB mice without inducing adverse hepatic effects. Similar to clofibrate, the PPARA stimulator fenofibrate prolonged life span and partially improved motor function in \(Ndufs4^{-/-}\)-WB mice.

11.2.3. Manipulation of energy metabolism

Dimethyl \(\alpha\)-ketoglutarate (DMKG), a cell-permeable form of \(\alpha\)-ketoglutarate, was administered to \(Ndufs4^{-/-}\)-WB mice to increase brain \(\alpha\)-ketoglutarate levels. DMKG was investigated since the beneficial effects of NMN (see above) were attributed to elevated brain levels of \(\alpha\)-ketoglutarate. Moreover, DMKG increased cellular NAD\(^+\) levels and rescued GAL-induced death of primary skin fibroblasts of LS patients with isolated CI deficiency. DMKG prolonged life span and delayed onset of hind limb clasp of \(Ndufs4^{-/-}\)-WB mice. In certain models, tetracyclines induced mitochondrial proteotoxic stress impacting on mitochondrial dynamics and function. Nevertheless, tetracyclines such as doxycycline were identified as potent small molecules to rescue against cell death in low-glucose culture conditions in MD cell models. It was found that doxycycline-mediated suppression of mitochondrial translation ameliorates the neuroinflammatory profile of \(Ndufs4^{-/-}\)-WB mice.

11.2.4. Inhibition of mTOR

Rapamycin (a.k.a. sirolimus) and the macrocyclic lactone tacrolimus (a.k.a. FK-506) display powerful immunosuppressant properties. Both rapamycin and tacrolimus can bind to FK506-binding protein 12 (FKBP12), thereby inhibiting mTOR complex 1 (mTORC1), which is composed of several proteins including mTOR itself. mTORC1 positively regulates cell growth and proliferation by promoting protein and lipid biosynthesis and inhibits catabolic processes such
as autophagy\textsuperscript{159,160}. Tacrolimus was also described to inhibit calcineurin\textsuperscript{161}. Elevated mTOR levels have not been reported in \textit{Ndufs4}\textsuperscript{-/-}-WB mice, however, its downstream kinase phosphor-rpS6 was increased in whole brain homogenates from PD>45 mice\textsuperscript{103}. In contrast, elevated phosphor-rpS6 levels were not detected in presymptomatic \textit{Ndufs4}\textsuperscript{-/-}-WB mice\textsuperscript{113}, suggesting the mTOR pathway may be involved in the disease mechanism. Rapamycin delayed disease onset, prevented the development of neurological symptoms (ataxia, uncoordinated balance and hindlimb clasping) and prolonged the life span of \textit{Ndufs4}\textsuperscript{-/-}-WB mice\textsuperscript{103,162,163}. In contrast, tacrolimus did not affect disease onset or progression, suggesting that the beneficial effects of rapamycin might not be solely due to immunosuppression nor off-target disruption of calcineurin\textsuperscript{103}. Similarly, modulation of the mTOR pathway using the poly(ADP-ribose)polymerase-1 (PARP1) inhibitor \textbf{PJ34}, delayed disease onset but did not extend the life span of \textit{Ndufs4}\textsuperscript{-/-}-WB mice\textsuperscript{164}. Evidence was provided that PARP1 inhibition modulates the mTOR pathway by decreasing the phosphorylation of AMPK, Raptor, s6 kinase, Rictor and Akt. In this context, PJ34-mediated PARP1 inhibition prevented ROS-stimulated phosphorylation of these proteins\textsuperscript{165}. Evidence was provided that rapamycin reduces neuroinflammation and glial activation through inhibition of protein kinase C (PKC)\textsuperscript{166}. Treatment with pan-PKC inhibitors (\textbf{GO6983, GF109203X}) or the PKC-\(\beta\)-specific inhibitor \textbf{ruboxistaurin} prevented hair loss, prolonged survival and delayed the onset of neurological symptoms (hindlimb clasping) in \textit{Ndufs4}\textsuperscript{-/-}-WB mice. Only ruboxistaurin prevented skin inflammation and reduced glial fibrillary acidic protein (GFAP) levels and the NF-\(\kappa\)B inflammatory response in brain. These findings suggest that inhibition of PKC-\(\beta\) and NF-\(\kappa\)B-mediated inflammation increases survival of \textit{Ndufs4}\textsuperscript{-/-}-WB mice\textsuperscript{166}. Importantly, rapamycin can display severe adverse effects\textsuperscript{167}, especially in young children, which limits its therapeutic potential in LS pediatric patients\textsuperscript{103}. In this context, encapsulated rapamycin did have similar efficacy as intraperitoneal (IP) injections, but did not result in toxicity in treated mice\textsuperscript{162} potentially increasing its suitability for patient applications. In addition to encapsulated rapamycin, it was reported that the FDA-approved immunosuppressant everolimus is better tolerated by MD patients, although not all patients were responsive to this drug\textsuperscript{168}. Collectively, mTOR inhibition may prove to be a suitable strategy to augment the severe phenotype of LS patients. However, the toxicity and off-target effects of mTOR inhibitors should be closely monitored when using these compounds in clinical trials.

\section*{11.2.5. TLR4 inhibition, benzodiazepine agonist treatment and cAMP homeostasis}
The TLR4 inhibitor **TAK-242**, a drug suppressing inflammatory mediators by binding to TLR4, rescued hair loss in *Ndufs4*<sup>−/−</sup>-WB mice, suggesting that systemic inflammation in these mice is mediated by TLR4<sup>73</sup>. Similar to rapamycin, the benzodiazepine-agonist **zolpidem**, rescued visual function, prevented starburst amacrine cell (SBAC) degeneration and innate immune and inflammatory response that occurred at time of vision loss<sup>169</sup>. **Papaverine** is an opium alkaloid antispasmodic drug that elevates cyclic AMP levels by inhibiting phosphodiesterase<sup>170</sup>. Papaverine also restored visual function and prevented SBAC degeneration and innate immune/inflammatory responses that occurred at time of vision loss<sup>169</sup>.

### 11.3. Injection of human iPSC-MSCs

Mitochondria can be uni- and bi-directionally transferred between cells via cell-cell contacts (“tunneling nanotubes”)<sup>177</sup>. Using co-cultures of mesenchymal stem cells (MSCs) and CI-deficient mouse or human fibroblasts, it was demonstrated that mitochondria were transported from MSCs to CI-deficient cells<sup>172</sup>. Although ROS levels were reduced in CI-deficient cells, mitochondrial transfer efficiency was low and (partial) restoration of CI assembly/activity was not detected. Intravitreal injection of induced pluripotent stem cell-derived MSCs (iPSC-MSCs) into retinal ganglion cells (RGCs) of 3 wks old *Ndufs4*<sup>−/−</sup>-WB mice resulted in transfer of mitochondria to neurons. The latter was associated with rescue of retinal function, prevention of RGC loss and prevention of abnormal RGC activation. RGCs that received mitochondria displayed extended longevity and normalized levels of pro-inflammatory cytokines. These results demonstrate that donation of healthy mitochondria reduces retinal degeneration, likely by improving retinal mitochondrial bioenergetics<sup>173</sup>. Although certainly promising, the therapeutic impact of iPSC-MSC delivery still faces several major challenges including tumorigenicity, immune rejection and genetic instability<sup>174</sup>.

### 11.4. Genetic approaches

#### 11.4.1. Adeno-associated virus vectors

Various adeno-associated virus (AAV) vectors were used to deliver human *NDUFS4* (hNDUFS4) in *Ndufs4*<sup>−/−</sup>-WB mice. Delivery of **AAV-PHP.B-hNDUFS4** (PD26-28) extended life span and reduced disease severity (normalization of growth rate, improvement of motor coordination, preventing development of failure to thrive, epileptic seizures and cardiac abnormalities)<sup>175,176</sup>. This vector, in contrast to other tissues, did not transduce brain in newly born mice (PD1). The
latter possibly relates to inefficient AAV transport mechanisms in neonates. Bilateral delivery of an AAV Serotype 1 construct (AAV1-Ndufs4-IRES-GFP; i.e. containing the mouse Ndufs4 gene) into the VN at PD21 extended life span and delayed disease progression\textsuperscript{75}. Intravenous (IV) injection of the AAV2/9-hNDUFS4 construct resulted in AAV2/9-hNDUFS4 being present in skeletal muscle, heart and liver. However, this vector was absent in the brain and did not ameliorate clinical symptoms\textsuperscript{177}. Intracerebroventricular (ICV) injection in newborn mice increased hNDUFS4 levels in the brain but did not improve the clinical phenotype. Combined (IV+ICV) injections in newborn mice were associated with detectable levels of hNDUFS4 in brain, muscle and heart. This resulted in near complete restoration of CI activity, extended life span, increased body weight and improved motor coordination\textsuperscript{177}. These results suggest that AAV2/9-hNDUFS4 cannot pass the blood-brain barrier (BBB), and further strengthen the hypothesis that the Ndufs4\textsuperscript{-/-}-WB phenotype is brain-specific. Recently, IV injection of AAV-EF1\textalpha-GPD1 was applied to express glycerol-3-phosphate dehydrogenase 1 (GPD1) in the brain of Ndufs4\textsuperscript{-/-}-WB mice in a neuron- and glia-specific manner\textsuperscript{114}. GPD1 is a cytosolic protein, which catalyses the reversible conversion of dihydroxyacetone phosphate (DHAP) and NADH to glycerol-3-phosphate (G3P) and NAD\textsuperscript{+}. Together with the mitochondrial protein glycerol-3-phosphate dehydrogenase, it also forms a G3P shuttle that facilitates the transfer of reducing equivalents from the cytosol to mitochondria. It was found that GDP1 expression normalized several of the metabolic aberrations in the brainstem of Ndufs4\textsuperscript{-/-}-WB mice, ameliorated neuroinflammation, partially prevented motor function decline and reduction in body temperature, and extended life span to PD84\textsuperscript{144}. These results provide evidence that stimulation of G3P biosynthesis regenerates cytosolic NAD\textsuperscript{+} to mitigate the phenotype in Ndufs4\textsuperscript{-/-}-WB mice. Taken together, although AAV vector delivery might be a promising strategy for MD patients with NDUFS4 mutations, BBB permeability differs between mice and human. In this sense, the ability of AAV vectors to penetrate the BBB needs to be determined, or vector delivery should be performed directly in the affected brain region, in order to be applicable in a clinical setting\textsuperscript{176}.

11.4.2. Overexpression of specific proteins

Metallothioneins (MTs) are small intracellular proteins that are induced in fibroblasts from MD patients with isolated CI deficiency\textsuperscript{178} and potentially protect against oxidative stress. However, Mt1 overexpression in Ndufs4\textsuperscript{-/-}-WB mice (confirmed in quadriceps muscle and brain) did not prevent protein oxidation, oxidative stress or inflammation and did not rescue the disease phenotype\textsuperscript{81}. This is compatible with other antioxidant-based therapies (see above), which did not
rescue disease onset and severity. Evidence was provided that overexpression of the MIM-fusion and cristae-shaping protein **Optic Atrophy 1 (OPA1)** normalized aberrant mitochondrial cristae morphology in the forebrain and cerebellum of *Ndufs4*<sup>−/−</sup>-WB mice. OPA1 overexpression was demonstrated in various tissues (brain, skeletal muscle, heart) and paralleled by improved rotarod performance at 5 wks and a moderate increase in life span. However, no phenotypic improvement was observed at 7 wks with respect to disease progression and death of the animals. Furthermore, the therapeutic potential of OPA1 overexpression may be limited by its antiapoptotic properties.

Small molecule mTOR inhibition ameliorated the disease phenotype of *Ndufs4*<sup>−/−</sup>-WB mice (see above). In this context, it was investigated whether the serine/threonine kinase **S6K1**, which is downstream of mTORC1 and involved in ribosomal protein synthesis and autophagy induction, plays a role in disease progression. Whole body and liver-specific *S6k1* knockout prolonged the life span of *Ndufs4*<sup>−/−</sup>-WB mice and delayed onset of hindlimb clasping. In contrast, fat- or brain-specific *S6k1* showed no beneficial effects, suggesting that the mitigating effects of rapamycin-induced mTOR inhibition are partially mediated by active S6K1, although whole body or liver-specific S6K1 knockout was not as efficient as a high dose (8 mg/kg) of rapamycin. In yeast and other models, the single-subunit NADH dehydrogenase **Ndi1** functions as a non-proton pumping alternative enzyme that replaces CI and oxidizes intramitochondrial NADH. This suggests that Ndi1 expression might compensate for reduced CI-mediated NADH oxidation in *Ndufs4*<sup>−/−</sup>-WB mice. Introduction of Ndi1 in neuron- and glia-specific *Ndufs4* knockout mice (NesKO mice) did not prevent motor function and breathing abnormalities but prolonged life span to a median of >1 year. Importantly, in contrast to NesKO mice, Ndi1-expressing mice did not develop seizures, suggesting that seizures are a major cause of death in NesKO mice. These findings demonstrate that normalization of NADH oxidation partially prevents disease progression in NesKO mice, but does not fully restore neuronal function.

### 12. Summary and conclusions

Development of *Ndufs4*<sup>−/−</sup> mouse models for MC1DN1/LS delivered novel pathomechanistic insights and allowed in vivo evaluation of potential intervention strategies. Mechanistically, *Ndufs4* deletion induces the combined absence of two CI subunits (NDUFS4 and NDUFA12), paralleled by increased levels of the CI assembly factor NDUFAF2. As a consequence, the CI holocomplex becomes destabilized and, though still (partially) active in situ, dissociates upon isolation and during analysis by BN-PAGE (Fig. 3A). Comprehensive analysis of the *Ndufs4*<sup>−/−</sup>-WB model demonstrated that these mice develop a fatal encephalopathy, which closely resembles the phenotype of human MC1DN1/LS patients with *NDUFS4* mutations (Fig. 3B). *Ndufs4*<sup>−/−</sup>-WB
mice primarily presented with neurological symptoms that were similar to those of brain/neuron-specific \textit{Ndufs4} knockout animals (Fig. 3B). This strongly suggests that whole-body \textit{Ndufs4} deletion and joint absence of NDUFS4/NDUFA12 induces brain-specific pathology. The latter might be due to tissue-specific differences in CI expression levels, CI-dependent energy demand and/or biochemical threshold effects. Of note, the \textit{Ndufs4} mouse model(s) fail(s) to recapitulate the whole spectrum of manifestations encountered in different cases of LS\textsuperscript{184,185}. Moreover, it was reported that spontaneous mutations in mouse (sub)strains due to excessive breeding programs can affect animal metabolism and study outcomes. This difference in genetic background might explain why homozygous \textit{Ndufs4}\textsuperscript{+/−} KI mice were embryonically lethal, whereas \textit{Ndufs4}\textsuperscript{−/−}-WB mice were healthy until ~5 wks of age. Therefore, mouse (sub)strain selection should be scrutinized to minimize genotypic and phenotypic differences, potentially leading to misinterpretation of study outcomes\textsuperscript{186,187}. In this context, it is likely that the genetic background also impacts on the detailed disease outcome and phenotype in human MC1DN1/LS patients. Most of the interventions discussed in this review partially rescue the phenotype in \textit{Ndufs4}\textsuperscript{−/−} mice, which is promising. Although various clinical trials are starting or ongoing, there is no evidence yet supporting the effectiveness of these interventions in human MD and MC1DN1/LS patients\textsuperscript{188-190}.

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Figure legends

Figure 1 Structure of CI and location of NDUFS4 and other subunits. (A) Side view of the cryo-EM structure of CI in *Ovis aries* heart at 3.90 Å resolution (PDB accession number: 5LNK; www.rcsb.org), highlighting the position of the NDUFS4 protein (red) relative to the NDUFAB1, NDUFV3-10, NDUFS6 and NDUFA12 subunit\(^2\). The two copies (α and β) of the NDUFAB1/SDAP accessory subunit are indicated. Yellow spheres mark iron-sulfur clusters. Transmembrane helices are depicted in the MIM-embedded part. (B) Same as panel A but now highlighting the position of the NDUFS4 protein relative to the NDUVF1, NDUFS1, NDUFA9, NDUFS7 and NDUF12 subunit. (C) Same as panel B, but now depicting a view from the top and back of CI. The molecular graphics in this figure were created using the PyMOL Molecular Graphics System Version 2.0 (Schrödinger-LLC, Mannheim, Germany).

Figure 2 Sequence of the human and mouse NDUFS4 and pathogenic NDUFS4 mutations. (A) Human (*Homo sapiens*) NDUFS4 pre-protein sequence (O43181 from UniProt: www.uniprot.org). The mitochondrial targeting sequence (MTS) is highlighted in bold red. The two PKA consensus phosphorylation sites in the MTS and NDUFS4 protein are highlighted. Also, the AQDQ sequence (highlighted in pink) is indicated. (B) Same as panel A but now for the mouse (*Mus musculus*) NDUFS4 protein sequence (Q9CXZ1). In the whole-body Ndufs4 knockout animal (*Ndufs4*^-/-WB), the last part of the MTS and the first 17 AAs of NDUFS4 (highlighted in blue) were deleted\(^2\). (C) Alignment of the pre-protein sequences in panel A and B. The MTS is highlighted in bold and identical AAs are depicted in green. The mature human and mouse NDUFS4 proteins differ by only 4 AAs (highlighted in grey), rendering them 97% identical. (D) Schematic structure of *NDUFS4* (NM_002495.2) consisting of five exons (not drawn to scale). The currently known mutations are highlighted (Table 2).

Figure 3 Consequences of Ndufs4 knockout. (A) Ndufs4 knockout induces absence of the NDUFS4 subunit of CI, near complete absence of the NDUFA12 subunit and increased levels of the CI-attached NDUFAF2 CI assembly factor. This results in an unstable CI holocomplex that is present at lower levels *in situ* and therefore displays a lower activity in Ndufs4^-/- mice. Upon isolation, the unstable CI complex loses its N-module, resulting in an inactive ~800-kDa subcomplex on blue-native PAGE (BN-PAGE) gels. This figure was adapted from Ref. 64. (B) Genetic dissection of clinical signs in Ndufs4^-/-WB mice. Vglut2-expressing glutamatergic neurons mediate most of the phenotype of Ndufs4^-/-WB mice, such as motor and respiratory
alterations, while GABAergic neurons are involved in basal ganglia inflammation, development of epilepsy, and hypothermia. Conditional alteration in either population leads to reduced lifespan and decreased body weight. **Abbreviations:** Cer, cerebellum; GPe, external globus pallidus; IO, inferior olive; OB, olfactory bulb; SNr, substantia nigra pars reticulata; VN, vestibular nuclei. This figure was adapted from Ref. 82.
Figure 1
Figure 2

A

Human NDUFS4 (O43181)

MAAVMSVVLQTRLWRRAVAAALSVSRVPTRSRTSTWRKQDDQDQDTQLTVDELKDIT
TLTGVPEEHKTRVRIFVPARNMQSGHVNNTKWKMFEDTRERWENPLMGWASTADPSLN
MVLTFSKEDAVSFAEKNWSYDIEERKVPKP(KS)KYGANFWSNKRT

B

Mouse NDUFS4 (Q9CZ1)

MAAVSISVSLQAMLRRGAMATAAVSVCRVPSRLSTSTWKLDNQRTQDTQLTVDELIT
TLTGVPEEHKTRVRIFVPARNMQSGHVNNTKWKMFEDTRERWENPLMGWASTADPSLN
MVLTFSAKEDAI(A)FAEKNWSYDVEEKKVPKPS(KS)KYGANFWSNKRT

C

Mitochondrial targeting sequence (MTS)

HUMAN: MAAVMSVVLQTRLWRRAVAAALSVSRVPTRSRTSTWRKQDDQDQDTQLTVDELKDIT
MOUSE: MAAVSIYVSLQAMLRRGAMATAAVSVCRVPSRLSTSTWKLDNQRTQDTQLTVDELKDIT

HUMAN: ITTLTGVPEEHKTRVRIFVPARNMQSGHVNNTKWKMFEDTRERWENPLMGWASTADP
MOUSE: ITTLTGVPEEHKTRVRIFVPARNMQSGHVNNTKWKMFEDTRERWENPLMGWASTADP

HUMAN: LSNMVLTFSKEDAVSFAEKNWSYDIEERKVPKP(KS)KYGANFWSNKRTVSTK
MOUSE: LSNMVLTFSKEDAI(A)FAEKNWSYDVEEKKVPKPS(KS)KYGANFWSNKRTVSTK

D

Exon 1 (126bp)  Exon 2 (79bp)  Exon 3 (173bp)  Exon 4 (74bp)  Exon 5 (223bp)

Exons and mutations:
- C.443G>A
- C.99-1G>A
- C.178-2A>G
- C.221del
- C.316C>T
- C.350+5G>A
- C.462del
- C.466-470dup
- C.470-471dup
Figure 3

A

WT animal: 

Proteome

KO animal: 

KO animal: 

Stable and active

Unstable and less active

Inactive

Ndusf4

NDUFA12

Cl level

NDUFAF2

B

Leigh Syndrome model

Decreased lifespan • Reduced body weight
Feeding problems • Growth retardation
Brainstem & basal ganglia inflammation
Abnormal body temperature
Respiratory abnormalities
Epileptic seizures
Motor alteration

GABAergic neurons

Epileptic seizures
Basal ganglia inflammation
Abnormal body temperature

Glutamatergic neurons

Motor alterations
Respiratory abnormalities
Brainstem inflammation

Decreased lifespan • Reduced body weight • Feeding problems
Growth retardation
| Number | LS-linked | Name \(^a\) | Module \(^c\) | Alternative names | Remarks |
|--------|-----------|-------------|-------------|-------------------|---------|
| 1      | +         | NDUFV1      | N           | 51-kDa, Nqo1, NuоГ | Contains FMN and N3 |
| 2      | +         | NDUFV2      | N           | 24-kDa, Nqo2, NuоЕ | Contains N1а |
| 3      | −         | NDUFV3  \(^d\) | N           | 10-kDa            |         |
| 4      | +         | NDUFS1      | N           | 75-kDa, Nqo3, NuоГ | Contains N1б, N4, N5 |
| 5      | +         | NDUFS6      | N           | 13-kDa            | Contains Zn\(^2+\) binding site |
| 6      | +         | NDUFA12     | N           | 817.2             |         |
| 7      | +         | NDUFS4      | N/Q?        | AОDQ, 18-kDa      |         |
| 8      | +         | NDUFA2      | N/Q?        | B8                | Contains thioredoxin fold |
| 9      | +         | NDUFS2      | Q           | 49-kDa, Nqo4, NuоД |         |
| 10     | +         | NDUFS3      | Q           | 30-kDa, Nqo5, NuоД |         |
| 11     | +         | NDUFS7      | Q           | PSST, Nqo6, NuоБ | Contains N2 |
| 12     | +         | NDUFS8      | Q           | TYKY, Nqo9, NuоЪ | Contains N6а and N6б |
| 13     | −         | NDUFA5      | Q           | B13               |         |
| 14     | −         | NDUFA6      | Q           | B14               | LYR |
| 15     | −         | NDUFA7      | Q           | B14.5а           |         |
| 16     | +         | NDUFA9      | Q           | 39-kDa            | Short-chain dehydrogenase/reductase fold with bound NAD(P)H |
| 17     | −         | NDUFAB1     | Q & Pd      | SDAP-a, SDAP-b    | Acyl carrier protein; 2 copies present; contains phosphopantetheine cofactor |
| 18     | -         | NDUFS5      | Pp          | 35-kDa            | Quadruple CX\(_{C}\) domain; double CHCH domain |
| 19     | +         | NDUFA1      | Pp          | MWFE              | STMD |
| 20     | -         | NDUFA3      | Pp          | B9                | STMD |
| 21     | -         | NDUFA8      | Pp          | PGBK              | Quadruple CX\(_{C}\) domain; double CHCH domain |
| 22     | +         | NDUFA10     | Pp          | 42-kDa            | Nucleotidase family |
| 23     | +         | NDUFA11     | Pp          | B14.7             |         |
| 24     | +         | NDUFA13     | Pp          | B16.6             | Identical to GRIM19, STMD |
| 25     | -         | NDUFC1      | Pp          | KFYI              | STMD |
| 26     | +         | NDUFC2      | Pp          | B14.5b            |         |
| 27     | +/-       | MT-ND1      | Pp          | Nqо8, NuоЩ, ND1   | 8 TMHs |
| 28     | +         | MT-ND2      | Pp          | Nqо14, NuоН, ND2  | 11 TMHs |
| 29     | +         | MT-ND3      | Pp          | Nqо7, NuоС, ND3   | 3 TMHs |
| 30     | -         | MT-ND4L     | Pp          | Nqо11, NuоК, ND4L | 3 TMHs |
| 31     | +         | MT-ND6      | Pp          | Nqо10, NuоЪ, ND6  | 5 TMHs |
| 32     | -         | NDUFB1      | Pd          | MNLL              | STMD |
| 33     | -         | NDUFB2      | Pd          | AGGG              | STMD |
| 34     | -         | NDUFB3      | Pd          | B12               | STMD |
| 35     | -         | NDUFB4      | Pd          | B15               | STMD |
| 36     | -         | NDUFB5      | Pd          | SGDH              | STMD |
| 37     | -         | NDUFB6      | Pd          | B17               | STMD |
| 38     | -         | NDUFB7      | Pd          | B18               | Double CX\(_{C}\) domain; double CHCH domain |
| 39     | +         | NDUFB8      | Pd          | ASH1              | STMD |
| 40     | -         | NDUFB9      | Pd          | B22               | LYR |
| 41     | -         | NDUFB10     | Pd          | PDSW              |         |
| 42     | -         | NDUFB11     | Pd          | ESSS              | STMD |
| 43     | +         | MT-ND4      | Pd          | Nqо7, NuоЪM, ND4  | 14 TMHs |
| 44     | +         | MT-ND5      | Pd          | Nqо12, NuоЪL, ND5 | 16 TMHs |

CHCH, coiled-coil-helix-coiled-coil-helix; FMN, Flavin mononucleotide; LYR, member of mitochondrial LYR (LYRM) protein family; N, iron-sulfur cluster; O, for ovine CI; STMD, small single transmembrane domain; TMH, transmembrane helices. Adapted from Refs. 19,20,64,192.

- Linked to Leigh syndrome (LS; OMIM256000; compiled using a literature search and information from: www.omim.org; +/- marks a potential link).
- Human protein name according to HGNC (www.genenames.org). Core subunits in bold. Subunits encoded by the mtDNA in italic.
- Functional modules: N (NADH binding and oxidation), Q (electron transfer to ubiquinone), P module (consisting of Pp and Pd submodules, proton pumping).
- Represents the NDUFV3-10 subunit (10-kDa).
Table 2 Interactions of NDUFS4 and NDUFA12 with other CI subunits

| Subunit              | Modulea | Interacting withb |
|----------------------|---------|-------------------|
|                      |         | NDUFS4 | Bovine | NDUFA12 | Bovine |
| NDUFS1/51-kDa        | N       | X      | X      | --      | --     |
| NDUFS2/24-kDa        | N       | X      | --     | --      | --     |
| NDUFS3/10/10-kDa     | N       | X      | X      | X       | X      |
| NDUFS5/75-kDa        | N       | X      | X      | X       | X      |
| NDUFS6/13-kDa        | N       | --     | --     | X       | X      |
| NDUFA12/B17.2        | N       | X      | X      | NA      | NA     |
| NDUFS4/18-kDa        | N/Q?    | NA     | NA     | X       | X      |
| NDUFS2/49-kDa        | Q       | --     | X      | --      | --     |
| NDUFS3/30-kDa        | Q       | X      | X      | --      | --     |
| NDUFS7/PSST          | Q       | --     | --     | X       | X      |
| NDUFS8/TYKY          | Q       | X      | X      | X       | X      |
| NDUFA6/B14           | Q       | X      | X      | --      | --     |
| NDUFA7/B14.5a        | Q       | --     | --     | X       | X      |
| NDUFA9/39-kDa        | Q       | X      | X      | --      | --     |
| MT-ND1/ND1           | Pp      | --     | --     | X       | X      |
| NDUFA1/MWFE          | Pp      | --     | --     | X       | X      |

For each subunit the human (according to HGNC: www.genenames.org) and bovine names (kDa) are given. Core subunit are highlighted in **bold**, mtDNA-encoded subunits are highlighted in *italic*.

*Functional modules: N (NADH binding and oxidation), Q (electron transfer to ubiquinone), P module (consisting of Pp and Pd submodules, proton pumping).

*Interactions are marked by X and obtained from the ovine CI structure191 and bovine CI structure192.
| ClinVar - accession | Variant | Variant type | Effect at protein level | Disease phenotype | Reference |
|---------------------|---------|--------------|-------------------------|-------------------|-----------|
| VCV000006890        | c.44G>A | Single nucleotide (1 bp) | p.Trp15Ter (W15*). MTS mutation. NDUFS4 protein absent. | LS; MC1DN1        | 66        |
| VCV000496165.3      | c.99-1G>A | Single nucleotide (1 bp) | Abnormal splicing. | LS                | 193       |
| VCV000488559        | c.178-2A>G | Single nucleotide (1 bp) | Abnormal splicing. | LS                | ClinVar only |
| VCV000006888        | c.291del | Deletion (1 bp) | p.Lys96_Trp97InsTer (W96*). No full length NDUFS4 protein. | MC1DN1            | 61        |
| VCV000006889        | c.316C>T | Single nucleotide (1 bp) | p.Arg106Ter (R106*). NDUFS4 protein absent. | MC1DN1            | 61        |
| VCV000930177        | c.350+5G>A | Single nucleotide (1 bp) | Abnormal splicing. Analysis of muscle and fibroblast cDNA from the patient showed reduced expression of NDUFS4 to 13% and 18% of control levels, respectively, and the presence of abnormal transcript species indicative of splicing abnormalities was detected in muscle. | MC1DN1            | 194       |
| VCV000040257        | c.462del | Deletion (1 bp) | p.Lys154fs (K154fs). Replacement of the last 22 AAs with 34 novel AAs. RVS phospho-site destroyed. 60% reduction in NDUFS4 transcript levels due to nonsense-mediated mRNA decay. No full length NDUFS4 protein was detected in isolated mitochondria. | LS; MC1DN1        | 44        |
| VCV000006887.2      | c.466-470dup | Duplication (5 bp) | p.Lys158fs (K158fs). Mature NDUFS4 protein is 14 AA longer. RVS phospho-site destroyed. NDUFS4 protein absent. | LS; MC1DN1        | 56        |
| VCV000488560        | c.470-471del | Deletion (2 bp) | p.Lys156_Ser157InsTer | LS                | ClinVar only |

The data in this table was compiled using ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/; only mutations marked as “Pathogenic”) and OMIM (www.omim.org).

Abbreviations: LS, Leigh syndrome; MC1DN1, Mitochondrial complex I deficiency, nuclear type 1; MTS, mitochondrial targeting sequence.