NDUFS4 deletion triggers loss of NDUFA12 in Ndufs4−/− mice and Leigh syndrome patients: A stabilizing role for NDUFAF2

Merel J.W. Adjobo-Hermansa, Ria de Haasb, Peter H.G.M. Willemsa, Aleksandra Wójtalac, Sjeneet E. van Emst-de Vriesa, Jori A. Wagenaarsa, Mariel van den Brandb, Richard J. Rodenburgb, Jan A.M. Smeitinka, Leo G. Nijtmansb,1, Leonid A. Sazanovb, Mariusz R. Wieckowskic,⁎, Werner J.H. Koopmana,⁎⁎

a Department of Biochemistry, Radboud Institute for Molecular Life Sciences, Radboud Center for Mitochondrial Medicine, Radboudumc, Nijmegen, the Netherlands
b Department of Pediatrics, Radboud Center for Mitochondrial Medicine, Radboudumc, Nijmegen, the Netherlands
c Nencki Institute of Experimental Biology, Warsaw, Poland
d Institute of Science and Technology, Klosterneuburg, Austria

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ABSTRACT
Mutations in NDUFS4, which encodes an accessory subunit of mitochondrial oxidative phosphorylation (OXPHOS) complex I (CI), induce Leigh syndrome (LS). LS is a poorly understood pediatric disorder featuring brain-specific anomalies and early death. To study the LS pathomechanism, we here compared OXPHOS proteomes between various Ndufs4−/− mouse tissues. Ndufs4−/− animals displayed significantly lower CI subunit levels in brain/diaphragm relative to other tissues (liver/heart/kidney/skeletal muscle), whereas other OXPHOS subunit levels were not reduced. Absence of NDUFS4 induced near complete absence of the NDUFA12 accessory subunit, a 50% reduction in other CI subunit levels, and an increase in specific CI assembly factors. Among the latter, NDUFAF2 was most highly increased. Regarding NDUFS4, NDUFA12 and NDUFAF2, identical results were obtained in Ndufs4−/− mouse embryonic fibroblasts (MEFs) and NDUFS4-mutated LS patient cells. Ndufs4−/− MEFs contained active CI in situ but blue-native-PAGE highlighted that NDUFAF2 attached to an inactive CI subcomplex (CI-830) and inactive assemblies of higher MW. In NDUFA12-mutated LS patient cells, NDUFA12 absence did not reduce NDUFS4 levels but triggered NDUFAF2 association to active CI. BN-PAGE revealed no such association in LS patient fibroblasts with mutations in other CI subunit-encoding genes where NDUFAF2 was attached to CI-830 (NDUFS1, NDUFV1 mutation) or not detected (NDUFS7 mutation). Supported by enzymological and CI in silico structural analysis, we conclude that absence of NDUFS4 induces near complete absence of NDUFA12 but not vice versa, and that NDUFAF2 stabilizes active CI in Ndufs4−/− mice and LS patient cells, perhaps in concert with mitochondrial inner membrane lipids.

1. Introduction
Mitochondrial complex I (CI or NADH:ubiquinone oxidoreductase; EC 1.6.5.3) is the first and largest (~1 MDa) complex of the oxidative phosphorylation (OXPHOS) system, which further consists of four other multi-subunit complexes (CII-CV; [1]). CI transfers electrons from NADH to ubiquinone and uses the energy released by this process to convey protons (H⁺) from the mitochondrial matrix across the mitochondrial inner membrane (MIM). This process contributes to establishment of a trans-MIM proton-motive force (PMF) that is used to...

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generate ATP by chemiosmotic coupling [2]. CI consists of three functional modules [3]: the N-module (NADH binding and oxidation), the Q-module (electron transfer to ubiquinone) and the P-module (proton pumping). Structurally, CI is L-shaped and consists of 45 subunits, one of which (NDUFA8) occurs twice in the fully assembled complex [4,5]. For its catalytic activity CI requires 14 core proteins [6], half of which is encoded by the mitochondrial DNA (mtDNA: MTND1, MTND2, MTND3, MTND4, MTND4L, MTND5, MTND6) and the remainder by the nuclear DNA (nDNA: NDUFV1, NDUFV2, NDUF51, NDUF52, NDUF53, NDUF57, NDUF58). In addition to this catalytic core, CI contains 30 currently-identified nDNA-encoded accessory subunits of largely unknown function [7]. Biogenesis of CI is assisted by at least 14 nDNA-encoded assembly factors and the current evidence suggests that its assembly occurs by a mechanism in which five CI protein modules are pre-formed (N, Q/P, a, Pq, b, Pd-a and Pd-b) and subsequently combined [8]. The structural and functional architecture of CI was described at various levels of detail in diverse biological models like the yeast Y. lipolytica, the eubacterium Thermus thermophilus, bovine heart, ovine heart and porcine heart [4,9-14]. At a higher level of organization, CI forms a supramolecular assembly with CIIV and CIV [15], the structure and potential functional aspects of which were presented [13,16-19].

Mutations in CI structural subunits induce isolated CI deficiency (OMIM 252010) and are primarily associated with Leigh syndrome (LS; [20,21]). This syndrome is generally characterized by neurodegeneration, variable symptoms, mitochondrial dysfunction and bilateral CNS lesions [22]. The NDUF54 gene encodes the 18-kDa NDUF54 (NADH-ubiquinone oxidoreductase Fe-S protein 4) accessory subunit of CI, which plays an important role during CI assembly and in CI stability [23-27]. At the clinical level, NDUF54 mutations primarily affect the brainstem, basal ganglia and (less frequently) the cerebral cortex and are typically associated with hypertonia, abnormal ocular movements, visual impairment, psychomotor arrest/regression and episodes of respiratory failure [28]. On average, mutations in the NDUF54 subunit induce a 50% reduction of CI activity in patient fibroblasts and cause death within 10 months after birth [28].

Various Ndufs4−/− mouse models have been generated to study the LS pathomechanism and aid the development of therapeutic strategies [29,30]. For instance, whole-body Ndufs4−/− mice present with many of the clinical features observed in LS patients and are widely used. These KO animals display an isolated CI enzymatic deficiency, appear healthy until 5 weeks after birth but die at ~7 weeks [31-33]. Interestingly, mice with a specific knockout of Ndufs4 in neurons and glia cells (NesKO mice) displayed a phenotype very similar to that of whole-body KO mice, including progressive neuronal deterioration and gliosis with early involvement of the olfactory bulb, cerebellum, and vestibular nuclei [34,35]. Specific knockout of Ndufs4 in midbrain dopaminergic (DA) neurons did not induce neurodegeneration, loss of striatal innervation or obvious Parkinson’s disease (PD) symptoms [36]. However, DA homeostasis was abnormal and Ndufs4−/− DA neurons were more vulnerable to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydrodipyrridine (MPTP), suggesting that CI deficiency might contribute to PD pathophysiology [36]. The latter study also revealed that heart-specific Ndufs4 knockout induces only a mild CI deficiency in vivo. The above results, combined with the fact that: (1) lifespan was not affected in heart-specific Ndufs4−/−/− mice [36,37] and (2) whole-body KO and NesKO mice displayed near identical clinical phenotypes [34], suggest that the clinical phenotype associated with Ndufs4 KO is primarily due to brain abnormalities [38]. In this sense, evidence was provided that lesions within the dorsal brain stem vestibular nucleus (VN) and deep cerebellar fastigial nucleus (FN) contribute to dysregulation of the central respiratory network, leading to respiratory failure and death of Ndufs4 KO mice [35]. To gain insight into the pathomechanism of Ndufs4 gene deletion, we applied for the first time quantitative mass spectrometry to determine the levels of OXPHOS subunits and assembly factors in various tissues from whole-body Ndufs4−/− mice (brain, liver, heart, kidney, diaphragm, skeletal muscle) and analyzed LS patient cells harboring nDNA-encoded CI mutations (NDUF51, NDUFS4, NDUFS7, NDUFV1, NDUF12, NDUFA2). We demonstrate that Ndufs4 gene deletion reduces CI subunit levels and propose that NDUF52 and possibly MIM lipids can stabilize active CI at the cellular level.

2. Materials and methods

2.1. Animal breeding, tissue dissection and proteomics analysis

Initial breeding pairs of heterozygous Ndufs4+−/− mice [31] were kindly donated by the Palmiter laboratory (Howard Hughes Medical Institute, University of Washington, Seattle). Mice were group-housed at the Central Animal Facility (CDL) of the Radboud University at 22 °C with a day/night rhythm of 12 h. The animals had ad libitum access to food and water and were fed on a standard animal diet (V1534-300 R/M-H; Sniff GmbH, Soest, Germany). Ndufs4 whole-body knockout (Ndufs4−/−; KO) and wild-type (WT) mice were generated by crossing homozygote males and females as described previously [31,39]. The genotype of the mice was confirmed by polymerase chain reaction. Animal experiments were approved by the Committee for Animal Experiments of the Radboud University Nijmegen Medical Center Nijmegen, The Netherlands, in accordance with Dutch laws and regulations and performed under the guidelines and regulations of the Dutch Council for Animal Care. For proteomics analysis, three WT mice (2 male, 1 female) and three KO (Ndufs4−/−) mice (2 male, 1 female) were sacrificed 6 weeks after birth by decapitation. Different tissues were isolated and extensively washed in PBS and stored in liquid nitrogen. In case of the brain, a slice (1–1.5 mm thickness) was manually dissected and contained parts of the following brain areas: cerebral cortex, hippocampal formation, thalamus, hypothalamus, internal capsule, optic tract and the amygdala (Allen mouse brain atlas; http://mouse.brain-map.org/). For comparison, other organs (from 1 KO and 1 WT animal; both males) were included in the proteomics analysis: liver, kidney, diaphragm, heart, and skeletal muscle from the hind leg (containing: musculus vastus medialis, musculus gracilis, rectus femoris, musculus biceps femoris, musculus semitendinosus, musculus semimembranosus and musculus adductor). All tissues were extensively washed in PBS and stored for further studies in liquid nitrogen. Liquid chromatography-mass spectrometry (LC-MS/MS) was carried out at the Thermo Fisher Center for Multiplexed Proteomics (Dept. of Cell Biology, Harvard Medical School, Cambridge, MA, USA). Peptide fractions were analyzed using an LC-MS3 data collection strategy on an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). A detailed description of the proteomics analysis is provided in the Supplement. Cluster analysis of the complete detected proteome (6551 proteins) demonstrated that the WT and KO animals clustered in two distinct groups (data not shown). In this study we focused on the OXPHOS system. The remainder of the proteomics data will be presented in a follow-up manuscript (in preparation).

2.2. Mouse embryonic fibroblasts

Immortalized mouse embryonic fibroblasts (MEFs) of Ndufs4 KO and WT mice were generated previously [40]. MEFs were cultured at 37 °C in a humidified atmosphere (95% air and 5% CO2) in DMEM medium (#10938, Invitrogen, Carlsbad, CA, USA), containing 25 mM d-glucose and supplemented with 1-glutamine (4 mM), sodium pyruvate (1 mM), 10% (v/v) Fetal Calf Serum (FCS, Greiner Bio-one, Frickenhuesen, Germany) and 0.1% (v/v) Gentamycin (Invitrogen).

2.3. Primary human skin fibroblasts

Human Fibroblasts were obtained following informed parental consent and according to the relevant Institutional Review Boards from skin biopsies of a healthy individual (CT5120) and various patients (P)
with complex I (CI) deficiency (OMIM 252010). Control and patient fibroblasts were previously characterized at the genetic, biochemical and cellular level (e.g. [41–44]). All patients displayed an isolated CI deficiency in muscle tissue and cultured fibroblasts and were negative with respect to mitochondrial DNA (mtDNA) alterations previously associated with CI deficiency. Patient cell lines (Supplementary Table S1) harbored mutations in various CI structural subunits: NDUFS7 (S7-P5175), NDUFV1 (V1-P5866), NDUFA12 (A12), NDUFS1 (S1-P6173) and NDUFS4 (S4-P4608, S4-P5260). Also a patient with a mutation in the CI assembly factor NDUFA2 (AF2-P8872) was included. Fibroblasts were cultured in Medium 199 (M199; #22340-020; Invitrogen) in a humidified atmosphere (95% air, 5% CO2) at 37 °C. The culture medium contained Earle’s salts, 25 mM HEPES, 5.5 mM D-glucose, 0.7 mM l-glutamine, 10% (v/v) fetal calf serum, 100 IU/ml penicillin and 100 IU/ml streptomycin.

2.4. SDS-PAGE and BN-PAGE analysis of brain slices, mouse embryonic fibroblasts (MEFs) and patient fibroblasts

Denaturating SDS-PAGE and blue-native-PAGE (BN-PAGE) followed by Western blotting were performed as described in the Supplement.

2.5. Data analysis, molecular graphics and protein homology structural modeling

Comparison of proteome data between brain slices of WT and KO animals was performed using the Benjamini-Hochberg (BH) procedure [45]. KO/WT protein ratios with a BH-P-value < 0.05 were considered to be significantly changed. Unless stated otherwise, all other data was compared using an independent Student’s t-test. Averages are presented as mean ± SEM (standard error of the mean). Cluster analysis was performed using Ward’s method and the Euclidian distance [46]. Statistical and cluster analyses were carried out using OriginPro software (OriginLab Corporation, Northampton, MA, USA). Molecular graphics were created using the PyMOL Molecular Graphics System Version 2.0 (Schrödinger-LLC, Mannheim, Germany). The homology model for NDUFA2 was generated using the Phyre2 server (Protein Homology/analogyY Recognition Engine V2.0; [47]) with default settings. The server used the cryo-EM model of *Yarrowia lipolytica* NDUFA2 (chain k in PDB 6RFQ). Ovine NDUFA2 is 32/44% identical/similar to ovine NDUFA12 and 38/55% to *Yarrowia lipolytica*, so the structural model of the assembly factor in *Yarrowia* [14] represents a better starting model than the homologous ovine NDUFA12 subunit. The generated homology model was aligned to NDUFA12 subunit and thus placed in its location within the complex (PDB 5LNK), with all the subunits missing in the 830 kDa subcomplex removed.

3. Results

3.1. Ndufs4 gene deletion reduces the protein levels of CI structural subunits and increases the levels of specific CI assembly factors in mouse tissues

To determine whether *Ndufs4* knockout affected the protein level of OXPHOS subunits and assembly factors, we performed a quantitative proteome analysis of mouse tissues (brain, heart, skeletal muscle, liver, kidney and diaphragm; Supplementary Fig. S1). Importantly, given the brain-specific phenotype in whole-body *Ndufs4* knockout (KO) mice (see Introduction) we focused our statistical data analysis on brain tissue (3 KO vs. 3 WT animals), to which the other tissues were compared (1 KO vs. 1 WT animal). In total, the relative protein expression level (KO/WT) of 86 different OXPHOS structural subunits was quantified (Fig. 1A; Supplementary Table S2). With the exception of NDUVF3 and NDUFA1, knockout of *Ndufs4* significantly reduced the levels of all CI structural proteins (Fig. 1A; marked in red characters). In contrast, *Ndufs4* knockout did not reduce the levels of other OXPHOS complex (CII-CV) subunits. With the exception of NDUFA1, of which CI contains two copies (α and β), all OXPHOS subunits occur only once in each OXPHOS complex. This allows determining the relative amount of each OXPHOS complex by calculating the average level (KO/WT) of its constituting subunits. In brain and diaphragm, *Ndufs4* knockout reduced the average CI subunit level by more than 60% relative to WT, whereas in liver, heart, kidney and skeletal muscle this reduction was significantly less (Fig. 1B; left panel). In case of CI, its average subunit level was not reduced in any of the tissues and significantly increased in heart and kidney (Fig. 1B; right panel). *Ndufs4* knockout did not generally affect the average subunit level of CIIII, CIIV and CV (Fig. 1C). Proteome analysis highlighted 34 OXPHOS assembly factors (Fig. 1D; Supplementary Table S2). Focusing on CI in the brain, *Ndufs4* gene deletion specifically increased the protein levels (KO/WT) of NDUFA1F, NDUFA2, ECSIT, ACAD9, FOXRED1 and TMEM186 whereas it did not affect the levels of NDUFA3, NDUFA4, NDUFA5, NDUFA6, NDUFA7, NUBLP, TIMMDC1, TMEM70, TMEM126B and TMEM261. Expression of assembly factors for other OXPHOS complexes was not affected in KO brain, except for the CV assembly factor ATPAF2, which also appeared increased in the other KO tissues (Fig. 1D). These results demonstrate that *Ndufs4* knockout reduces the level of CI subunits and increases the level of specific CI assembly factors.

3.2. Ndufs4 gene deletion induces combined loss of NDUFS4 and NDUFA12 protein and increases NDUFA2 protein levels in mouse tissues

Focusing on CI, we next compared the expression level (KO/WT) of its structural subunits and assembly factors in brain with that of the other tissues (Fig. 2A). In general, protein levels in the brain (x-axis) correlated well with other tissues (y-axis; thick line: y = x). Cluster analysis highlighted four distinct groups (Fig. 2A; colored ovals). As expected, *Ndufs4* gene knockout induced a complete loss of NDUFS4 protein in all KO tissues. However, this knockout also greatly reduced the levels of NDUFA12 (Fig. 2A; pink oval). No NDUFS4 peptide fragments were detected whereas NDUFA12 fragments were virtually absent (Supplementary Fig. S2), which demonstrates that the overall level of this protein is greatly reduced. Relative to other CI subunits the reduction in brain NDUFS4 and NDUFA12 level was 12-fold larger. All KO mice tissues displayed reduced levels of CI structural subunits (Fig. 2A; purple oval), a moderate increase in various CI assembly factors (Fig. 2A; orange oval), and a 2-fold higher level of the CI assembly factor NDUFA2 (Fig. 2A; green oval). SDS-PAGE analysis of KO brain tissue yielded protein levels that correlated well with our proteome analysis, and confirmed that NDUFS4 was absent, NDUFA12 levels were greatly reduced and NDUFA2 was increased (Supplementary Fig. S3). Recent data suggests that functional CI is assembled via a mechanism in which five CI protein modules are pre-formed (N, Q/Pp-a, Pp-b, Pd-a and Pd-b) and subsequently combined [8]. Within the CI structure NDUFS4 is localized close to the boundary between the N- and Q-module (Fig. 4A). Compatible with this localization, quantification of module-specific subunit expression levels (Supplementary Table S1) revealed that *Ndufs4* knockout reduces the average subunit level (KO/WT) of the N- and Q-module to a greater extent than that of the Pp- and Pd-module (Fig. 2B). Collectively, these results demonstrate that the complete absence of NDUFS4 in all tissues is associated with: (i) near complete absence of NDUFA12, (ii) increased levels of NDUFA2, and (iii) reduced stability of N- to Q-module attachment.

3.3. Ndufs4 gene knockout is associated with an NDUFA2-containing CI subassembly that incorporates into larger OXPHOS assemblies in mouse embryonic fibroblasts

To study the effects of *Ndufs4* gene knockout on CI assembly/stability, we analyzed mouse embryonic fibroblasts (MEFs) derived from WT and *Ndufs4*−/− mice [40]. Supporting our proteomics results, NDUFS4 was not detected, NDUFA12 was greatly reduced and NDUFA2 was increased in KO-MEFs (Fig. 3A–B). To visualize the effect
of Ndufs4 knockout on the levels of CI and its incorporation in OXPHOS assemblies of higher molecular weight, we performed blue native gel electrophoresis (BN-PAGE) of WT and KO MEFs (Fig. 3C). Given the reduction in N- and Q-module subunits in Ndufs4−/− tissues (see above) we detected CI using an antibody against its NDUFA9 subunit, which is essential in stabilizing the junction between these modules [48]. This suggested that WT-MEFs contain a fully assembled “free” CI and a CI + CIII2 assembly, whereas KO-MEFs contain a smaller CI subassembly of ~830-kDa in a free (“CI-830”) and CIII2-attached form (“CI-830 + CIII2”). In-gel activity (IGA) analysis demonstrated that CI was active in its free and CIII-bound form in WT-MEFs, whereas free and CIII2-attached CI-830 were inactive in KO-MEFs (Fig. 3C; “IGA”). In WT-MEFs, low levels of NDUFAF2 were detected in two distinct bands associated with CI-830 (Fig. 3C; “NDUFAF2”). In KO-MEFs, NDUFAF2 levels were much higher than in WT-MEFs and localized in three distinct bands representing CI-830, CI-830 + CIII2 and an even larger assembly potentially containing CIV (“CI-830-CIII2-CIV?”). The latter assembly was not detected in WT-MEFs. These BN-PAGE results suggest that a large and parallel reduction in the levels of NDUFS4 and NDUFA12 stimulates association of NDUFAF2 with a catalytically inactive CI-830 subcomplex and that CI-830-NDUFAF2 is incorporated into larger assemblies.

3.4. The loss of NDUFS4 greatly reduces NDUF12 protein levels in primary fibroblasts from patients with isolated CI deficiency

To determine the relevance of the findings mentioned above in human CI deficiency, we next compared the levels of NDUFS4, NDUF12, NDUFAF2 and NDUFA9, as well as CI assembly/stability/IGA patterns, in fibroblasts from LS patients. These patient cells displayed an isolated CI deficiency and carried mutations in NDUFS7, NDUFV1, NDUFA12, NDUFS1, NDUFS4 and NDUFAF2 (Supplementary Table S1).

SDS-PAGE demonstrated that NDUFS4 was undetectable in patient cells with NDUFS4 mutations, greatly reduced in cells with NDUFS7, NDUFV1, NDUFA12 or NDUFAF2 mutations, and not reduced in cells with an NDUF12 mutation (Fig. 3D). Confirming our results in Ndufs4−/− mouse tissues and MEFs, NDUF12 was not detected in cells from NDUFS4 patients (Fig. 3E). For the other mutations, NDUF12 was either not detected (NDUF12 patient cells) or reduced (NDUF57, NDUFV1, NDUFA12 patient cells). NDUFAF2 was not detected in NDUF12 patient cells, slightly reduced in NDUF12 patient cells and not reduced in NDUFS7, NDUFV1, NDUFA12 and NDUFS4 patient cells (Fig. 3F). The levels of NDUFA9 were reduced in all cases except for NDUFV1 patient cells (Fig. 3F).
Although present in control and NDUF57 patient cells (Fig. 3F), NDUFAF2 was not detected in the CI complex using BN-PAGE in these cells (Fig. 3G; "NDUFAF2"). In contrast, NDUFAF2 was attached to the CI-830 subcomplex in NDUF54, NDUF51 and NDUFV1 patients. 

NDUFA12 patient cells displayed an association of NDUFAF2 with a catalytically active CI assembly (Fig. 3G; arrowheads). IGA analysis revealed that CI activity was reduced (NDUFS1, NDUFS7, NDUFS1, NDUFAF2, NDUFA12, NDUFS4) or not detectable (NDUF54) at the position of fully assembled CI (Fig. 3G; "IGA"). In addition, residual CI activity in NDUF54 patient cells was reduced more (i.e. to 7-14% of control) than in NDUFA12 patient cells (42% of control; Supplementary Table S1).

Taken together, these results demonstrate that absence of NDUF54 due to NDUF54 mutations is associated with near complete loss of NDUFA12 and increased levels of NDUFAF2 in LS patient cells. We further observed that when NDUFA12 is absent, the CI assembly factor NDUFAF2 can replace this structural subunit in the presence of NDUF54 yielding catalytically active CI.

4. Discussion

Mutations in the NDUF54 gene, encoding an accessory subunit of mitochondrial complex I (CI), induce isolated CI deficiency and Leigh syndrome (LS) in pediatric patients. Using quantitative proteomics we here compared the level of OXPHOS subunits and assembly factors in tissues from Ndufs4−/− mice. It was observed that Ndufs4 knockout reduces CI subunit levels to a greater extent in brain and diaphragm relative to liver, heart, kidney and skeletal muscle (Fig. 1B). This result, combined with the fact that different organs display different thresholds below which an OXPHOS enzymatic deficiency induces pathology [49], suggests that the low CI subunit levels in brain, and possibly diaphragm, are responsible for the brain-specific clinical phenotype and breathing deficits in Ndufs4−/− mice [51–53].

4.1. Ndufs4 gene deletion does not reduce the level of NDUFV3, NDUFAB1 and subunits of OXPHOS complexes II, III, IV and V

In case of the CI subunit NDUFV3, a 10-kDa (NDUFV3-10) and 50-kDa (NDUFV3-50) form were detected [50–52]. The number of peptides for NDUFV3-10 and NDUFV3-50 in the brain equaled 1 and 7, respectively. Therefore caution should be taken in the quantitative interpretation of the KO/WT ratio for NDUFV3-10, which was reduced to 0.5475 but did not reach quantitative significance. In the ovine CI structure only the NDUFV3-10 form was detected (Fig. 4A), suggesting that NDUFV3-10 is a genuine CI subunit whereas NDUFV3-50 is not. Similar to NDUFV3-10, the KO/WT ratio for NDUFAB1 (equaling 0.7804) appeared reduced relative to WT but this reduction was not statistically significant. This might be due to two copies of the NDUFAB1 subunit being present in CI (Fig. 4A; α and β) and one of these being destabilized by the absence of NDUF54 to a greater or lesser extent. In addition, NDUFAB1 can interact with other proteins including LYRM2 and the mitochondrial ribosome-linked proteins MALUS1 and AltMiD51 [53]. These interactions might stabilize (and partially) protect NDUFAB1 from degradation under NDUF54-deficient conditions. Although their functional role is still controversial [54], the structures of the CI + CII2 + CIV “supercomplex” and the CI2 + CI22 + CIV2 “megacomplex” were recently presented [13,16–18]. We observed that Ndufs4−/− gene deletion greatly reduces CI subunit levels without lowering these levels for CII, CIII, CIV and CV. This argues against in situ structural stabilization of these complexes by CI in our experimental model.

4.2. Ndufs4 gene deletion increases the level of specific CI assembly factors

In mouse brain, Ndufs4 knockout was paralleled by an increase in the levels of specific CI assembly factors (NDUFAF1, NDUFAF2, ECSIT, ACAD9, FOXRED1 and TEMEM186), whereas the levels of CI, CII, CIV
and CV assembly factors were not affected. There was one exception to this pattern, the CV assembly factor ATPAF2 being increased in brain and the other KO tissues of the KO animals. This might suggest that ATPAF2 plays a role in CI assembly and/or stabilization. All of the above factors bound to CI assembly intermediates in 143B osteosarcoma cells [8]. NDUFAF1, ECSIT, ACAD9 and TMEM186 were present in a 357-kDa intermediate together with putative assembly factor COA1 (not detected in this study), assembly factor TMEM126b (not altered in KO brain) and structural subunits MTND2, MTND3, NDUFC1 (not detected in this study) and NDUFC2. Later during CI assembly, MTN6E and MTND4L bind to the 357-kDa intermediate to form the Pp-b module [8]. The Pp-b intermediate subsequently combines with two structural subunits (NDUFA10 and NDUFS5) and the Pd-a module, which contains the structural subunits NDUFB1 (not detected in this study), NDUFB5, NDUFB6, NDUFB10, NDUFB11, MTND4 and the assembly factors TMEM70 (not altered in KO brain), FOXRED1 and...
ATP5SL (not altered in KO brain). Most assembly factors (except TMEM186 and COA1), dissociate from CI at the final stage of CI assembly [8]. Among the assembly factors, the level of NDUFAF2 was most highly increased in $\text{Ndufs4}^{-/-}$ mouse tissues, $\text{Ndufs4}^{-/-}$ MEFs and $\text{Ndufs4}$-mutated patient cells. In contrast, a similar loss of NDUFA12 was not observed in a mutated Yarrowia lipolytica strain lacking NDUFS4 [14]. However, our mouse and human cell data is supported by studies in HEK293T cells [7].

4.3. $\text{Ndufs4}$ gene deletion induces near complete loss of NDUFA12 and increases NDUFAF2

In addition to an increase in NDUFAF2, loss of NDUFS4 was accompanied by near complete loss of NDUFA12 in $\text{Ndufs4}^{-/-}$ mouse tissues, $\text{Ndufs4}^{-/-}$ MEFs and $\text{Ndufs4}$-mutated patient cells. In contrast, a similar loss of NDUFA12 was not observed in a mutated Yarrowia lipolytica strain lacking NDUFS4 [14]. However, our mouse and human cell data is supported by studies in HEK293T cells [7].
highlighting differences between yeast and mammalian cells/tissues. Inspection of the CI ovine and bovine cryo-EM structures [4,5], demonstrates that NDUFS4 and NDUFA12 physically interact and straddle the peripheral arm of CI from both sides, thereby linking its N and Q module (Fig. 4A–B). These studies also highlighted interactions between NDUFS4 and NDUFV1, NDUFV2 (not in bovine), NDUFV3-10, NDUFS1, NDUFS2 (not in ovine), NDUFS3, NDUFS8, NDUFA6 and NDUFV9 (Fig. 4A; underlined subunits). All of these subunits are part of the N- or Q-module, compatible with our observation that combined loss of NDUFS4/NDUFA12 reduces the levels of N- and Q-module subunits to a greater extent than the levels of Pp- and Pd-module subunits (Fig. 2B). In patient cells, the absence of NDUFA12 was not

| SDS-PAGE | In situ | BN-PAGE |
|----------|---------|---------|
| **CONTROL CELLS** | | |
| 100% NDUFS4 | Stable | N | q | MIM |
| 100% NDUFA12 | | b | ba | a |
| 100% NDUFAF2 | | Pd | Pp |
| **NDUFA12 patient** | | Unstable | N | q | MIM |
| 215% NDUFS4 | | b | ba | a |
| No NDUFA12 | | Pd | Pp |
| 89% NDUFAF2 | | |
| **NDUFS7 patient** | | Stable | N | q | MIM |
| 34% NDUFS4 | | b | ba | a |
| 82% NDUFA12 | | Pd | Pp |
| 121% NDUFAF2 | | |
| **NDUFV1/NDUFS1 patients** | | Unstable | N | q | MIM |
| 15/33% NDUFS4 | | b | ba | a |
| 74/11% NDUFA12 | | Pd | Pp |
| 141/121% NDUFAF2 | | |
| **NDUFAF2 patient** | | Stable | N | q | MIM |
| 82% NDUFS4 | | b | ba | a |
| 8% NDUFA12 | | Pd | Pp |
| No NDUFAF2 | | |
| **Ndufs4−/− mouse / NDUFS4 patients** | | Highly unstable | N | q | MIM |
| No NDUFS4 | | b | ba | a |
| No NDUFA12 | | Pd | Pp |
| 120/130% NDUFAF2 | | |

Fig. 5. Integrated summary of results. Empirical data from SDS-PAGE, BN-PAGE and CI activity measurements was combined to deduce the in situ activity and stability of MIM-embedded CI in Ndufs4−/− knockout tissues and LS patient cells with NDUFA12, NDUFS7, NDUFV1, NDUFS2, NDUFAF2 and NDUFS4 gene mutations. The relative cellular level of NDUFS4, NDUFA12 and NDUFAF2 is indicated in the column marked “SDS-PAGE”. The presence of assembled and active CI (CI) and the CI-830 subcomplex are indicated in the column marked “BN-PAGE” (see Discussion for details).
paralleled by reduced NDUFS4 levels. This suggests that NDUFS4 stabilizes NDUFA12 but not vice versa, and that the presence of NDUFS4 is more relevant for N- to Q-module attachment than NDUFA12.

Interestingly, studies in Yarrowia lipolytica revealed that deletion of the NUMM (NDUFS6 homolog) gene, which encodes the NUMM/NDUFS6 accessory subunit of CI, blocks a late step in CI assembly [58]. In this model system, NUMM/NDUFS6 gene deletion was associated with assembly of all CI core/accessory subunits except N7BM (NDUFA12 homolog), and firm association of N7BML (NDUFAF2 homolog) with a CI assembly intermediate. It was concluded that N7BM/NDUFA12 replaces N7BML/NDUFAF2 during complex I biogenesis [58]. The recently published cryo-EM structure from complex I obtained from a mutant Yarrowia lipolytica strain lacking NDUFS6 supports these findings [14]. Previous analysis of patient cells demonstrated that the absence of NDUFS4 and NDUFS6 induced formation of an 800-kDa subcomplex to which NDUFAF2 was attached [55–57]. The latter and our current findings support a mechanism based on evidence in Neurospora crassa, which proposes that NDUFAF2 is bound to a late CI assembly intermediate and dissociates upon binding of an NDUFA12/NDUFS4/NDUFS6-containing “regulatory” module [59]. However, differences between yeast and mammals do exist, as exemplified by the fact that, in contrast to our studies in mammals (Ndufs4−/− mouse model and patient cells), deletion of NDUFS4 does not lead to loss of NDUFA12 in Yarrowia lipolytica [14].

4.4. The NDUFAF2 assembly factor can function as a stabilizing structural CI subunit

Previous results suggest that NDUFAF2 can associate with CI-830 but not with fully assembled CI [55]. In contrast, evidence in an artificial HEK293 cell model demonstrated that Ndufa12 knockout triggers association of NDUFAF2 with active CI that appeared fully assembled [7]. Analyzing NDUFA12 patient cells, we here observed that NDUFAF2 associates with a catalytically active CI assembly, but only in the absence of NDUFA12 and presence of NDUFS4. Analysis of residual CI activity in mitochondria-enriched fractions (Supplementary Table S1) revealed that this activity is reduced to a greater extent in NDUFS4 patient cells than in NDUFA12 patient cells. This suggests that NDUFAF2 supports the formation of a catalytically active CI in the absence of NDUFA12 protein, in agreement with the presented BN-PAGE data revealing a complex within the same size range as in control cells (Fig. 3G). However, the residual CI activity in NDUFA12 mitochondria was 42% of control, whereas this activity was only 7–14% in the NDUFS4 patients. This suggests that rescue by NDUFAF2 is unable to fully restore CI catalytic activity. This provides first time evidence in a disease-model that NDUFAF2 can act as a stabilizing structural subunit by replacing NDUFA12. Homology modeling suggests how binding of NDUFAF2/B17.2 L to the CI-830 subcomplex in the absence of NDUFA12/B17.2 and NDUFS4 (18-kDa) subunit might occur (Fig. 4C). To model NDUFAF2, we used the structure of Yarrowia lipolytica NDUFAF2 that recently became available [14]. The helical extension of NDUFAF2 could sterically hinder the interaction of NDUFS6 with nascent CI. However, our findings demonstrate that the CI formed is active and migrates at as similar height in NDUFA12 patient cells as in control cells (Fig. 3G). Therefore, we hypothesize that NDUFS6 is able to bind, though possibly more weakly, to a structure in which NDUFA2 has replaced NDUFA12.

Importantly, tissues from Ndufs4−/− mice [60] and fibroblasts from patients with NDUFS4 mutations (Supplementary Table S1) still display substantial CI activity measured in mitochondria-enriched fractions. Moreover, although oxygen consumption in Ndufs4−/−/MEFs is lower than in WT-MEFs it is still acutely blocked by the CI inhibitor rotenone [40]. This demonstrates that the absence of an IGA signal in Ndufs4−/− MEFs and NDUFS4-mutated patient fibroblasts during BN-PAGE analysis does not reflect the in situ condition within living cells (Fig. 5). In this sense, the fact that BN-PAGE of NDUFS1- and NDUFA12 patient cells

4.5. Summary and conclusions

Taken together, our results demonstrate that absence of NDUFS4 greatly reduces NDUFA12 levels but not vice versa. This suggests a mono-directional stabilization of NDUFA12 by NDUFS4. We further provide evidence that NDUFAF2 stabilizes functional CI in the absence of NDUFA12 and presence of NDUFS4. The fact that absence of NDUFAF2 did not induce formation of the CI-830 subcomplex but lowered the levels of assembled and active CI in NDUFAF2 patient cells, supports such a stabilizing role and suggests that NDUFAF2 is not a true CI assembly factor but acts as a chaperone instead [61]. In the presence of NDUFS4, NDUFAF2-mediated joining is quite strong since no discrete CI subassemblies were observed in NDUFA12-mutated patient cells. Integrating our findings (Fig. 5) supports a mechanism in which NDUFAF2 stabilizes CI in situ, not only in the absence of NDUFS4 and/or NDUFA12, but also in patient cells with mutations in NDUFS1 and NDUFS4. The absence of NDUFS4 was associated with activity of CI in situ but not on BN-PAGE, which revealed an inactive NDUFAF2-CI-830 subcomplex. This suggests that the impact of BN-PAGE solubilization detergents on the level of assembled and active CI depends on CI stability. In this sense, our results not only highlight the crucial role of NDUFS4 in CI stabilization but also demonstrate that NDUFAF2 is unable to maintain N- to Q-module attachment under BN-PAGE conditions. Supporting the latter hypothesis, we have demonstrated the presence of a detached (partial) N-module using BN-PAGE analysis of Ndufs4−/− mouse tissues [60]. Since CI catalytic function requires N- to Q-module attachment, we hypothesize that additional mechanisms are involved to sustain CI in situ activity in Ndufs4−/− cells. Though still poorly understood, it is well established that lipid composition affects the conformation and activity of membrane-embedded proteins [62]. Also assembly and/or stabilization of OXPHOS complexes critically depend on specific MIM lipids [63]. Therefore we propose a model in which both NDUFAF2 and MIM lipids play a role in keeping the N- and Q-module together in the dual absence of NDUFS4 and NDUFA12. In this context, it might be possible that tissue-specific differences in NDUFAF2-mediated CI stabilization and/or MIM lipid physicochemical properties underlie the significantly greater reduction in CI subunit level observed in brain and diaphragm of Ndufs4−/− mice.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

- Jan A.M. Smeitink is the founder and CEO of the SME Khondrion B.V. (Nijmegen, The Netherlands).
- Werner J.H. Koopman is a scientific advisor of the SME Khondrion B.V. (Nijmegen, The Netherlands).
- The SME Khondrion B.V. had no involvement in the data collection, analysis and interpretation, writing of the manuscript, and in the decision to submit the manuscript for publication.
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Author contributions

Merel J.W. Adjobo-Hermans: Formal analysis; Writing - review & editing, Ria de Haas: Investigation; Methodology; Writing - review & editing, Peter H.G.M. Willems: Writing - review & editing, Aleksandra Wojtala: Investigation; Validation, Sijen E. van Emst-de Vries: Investigation; Validation, Jori A. Wagenars: Investigation; Validation, M. Wu, J. Gu, R. Guo, Y. Huang, M. Yang: Structure of mammalian respiratory supercomplex I (I1I1IVI1), Cell 167 (2019) 1598-1609.

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