Cell Metabolism
Short Article

The Respiratory Chain Supercomplex Organization Is Independent of *COX7a2l* Isoforms

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SUMMARY

The organization of individual respiratory chain complexes into supercomplexes or respirasomes has attracted great interest because of the implications for cellular energy conversion. Recently, it was reported that commonly used mouse strains harbor a short *COX7a2l* (*SCAFI*) gene isoform that supposedly precludes the formation of complex IV-containing supercomplexes. This claim potentially has serious implications for numerous mouse studies addressing important topics in metabolism, including adaptation to space flights. Using several complementary experimental approaches, we show that mice with the short *COX7a2l* isoform have normal biogenesis and steady-state levels of complex IV-containing supercomplexes and consequently have normal respiratory chain function. Furthermore, we use a mouse knockout of *Lrpprc* and show that loss of complex IV compromises respirasome formation. We conclude that the presence of the short *COX7a2l* isoform in the commonly used C57BL/6 mouse strains does not prevent their use in metabolism research.

INTRODUCTION

Mitochondria are the cellular power plants that produce the bulk part of the energy currency ATP through energy conversion by the oxidative phosphorylation (OXPHOS) system. The OXPHOS system, which is located in the inner mitochondrial membrane, is composed of two functional entities, i.e., the respiratory chain and the phosphorylation system, which includes the ATP synthase and carriers, such as the ATP/ADP carrier and the phosphate carrier. One important feature of mitochondria is that they harbor their own genome, mtDNA, which encodes 13 of the subunits of the enzyme complexes constituting the OXPHOS system, whereas the remaining ~90 subunits are encoded by nuclear genes and imported into mitochondria (Hällberg and Larsson, 2014). Historically, the mitochondrial respiratory chain has been defined as an ensemble of complexes I, II, III, and IV, some of which, i.e., complexes I, III, and IV, couple redox reactions to proton extrusion. The respiratory chain complexes are enriched in the tubular inner mitochondrial membrane invaginations called cristae (Busch et al., 2013; Gilkerson et al., 2003; Vogel et al., 2006) and were initially thought to be randomly distributed as independent entities (Hackenbrock et al., 1986). However, this view has been challenged based on a wealth of structural and functional analyses that support a model that the individual respiratory chain complexes interact to form stable supercomplexes (Genova and Lenaz, 2014; Schägger and Pfeiffer, 2000). One of the most important arguments for a higher-order organization of the respiratory chain was provided by the use of blue native polyacrylamide gel electrophoresis (BN-PAGE), which showed that respiratory chain complexes from a wide range of organisms can be extracted in supramolecular assemblies when mitochondria are solubilized using mild detergents (Acín-Pérez et al., 2008; Dudkina et al., 2010; Schägger, 2001). In mammalian mitochondria, supercomplexes consisting of complexes I, III, and IV provide the bulk part of the proton motive force and are termed respirasomes (Schägger and Pfeiffer, 2000). Although the supramolecular organization of the respiratory chain complexes has been extensively documented in different model organisms, the existence of factors mediating respirasome assembly or their stabilization have remained elusive (Schägger and Pfeiffer, 2000, 2001). Interactions between respiratory chain complexes within supercomplexes were originally described to be dependent on cardiolipin-protein interactions, in contrast to the ATP synthase dimer complexes, which depend on specific protein-protein interactions (Genova and Lenaz, 2014; Pfeiffer et al., 2003). Two proteins, Rcf1/HIG2A and Rcf2, were recently reported to mediate supercomplex assembly in yeast and in mammals (Chen et al., 2012; Strogolova et al., 2012; Vukotic et al., 2012). In addition, the COX7a2-like protein (COX7a2l, also called supercomplex assembly factor I, SCAFI), originally identified as an estrogen-responsive element (Watanabe et al., 1998), was reported to be essential for formation of complex IV-containing supercomplexes (Lapuente-Brun et al., 2013) and stabilization of the respirasome (Ikeda et al., 2013).

It has been suggested that supercomplex formation is of key importance for the stability of the individual respiratory chain complexes and that it may be initiated already at the assembly stage of complex I (Moreno-Lastres et al., 2012; Ugalde et al., 2004). Furthermore, supercomplex formation has been proposed to reduce the diffusion distance of ubiquinone and cytochrome *c*, the two mobile electron carriers of the respiratory chain, thereby increasing the electron transport efficiency and reducing reactive oxygen species production (Genova and Lenaz, 2014; Maranzana et al., 2013). Despite these hypotheses, the functional roles of the supercomplex assemblies remain unclear. Contrary to what has

been reported in yeast (Rigoulet et al., 2010), a recent report from the Enriquez laboratory (Lapuente-Brun et al., 2013) proposed that altered supercomplex assembly provides a mechanism for physiological regulation of energy metabolism in mammals by providing alternate paths for electrons derived from metabolism of specific substrates. When performing a single-nucleotide polymorphism (SNP) analysis, the authors discovered that some commonly used mouse strains, such as C57BL/6J and BALB/c, are homozygous for a six-base-pair deletion of the *COX7a2l* gene, which leads to the production of an unstable, short COX7a2l isoform that cannot support supercomplex formation (Lapuente-Brun et al., 2013). Consequently, mice with the short *COX7a2l* isoform were shown to lack complex IV-containing supercomplexes and to have aberrant respiratory chain function.

The report that COX7a2l is a novel supercomplex factor necessary for respirasome formation has attracted a lot of attention and was recently discussed in a Preview in *Cell Metabolism* (Barrientos and Ugalde, 2013). The results from the Enriquez laboratory (Lapuente-Brun et al., 2013) showing that C57BL/6 mice cannot form respirasomes have alarming consequences, as they challenge the interpretation of experimental results from a large number of mouse models generated in the C57BL/6 background. This mouse strain is widely used in metabolism research (Agostino et al., 2003; Chen et al., 2003; Diaz et al., 2005; Li et al., 1995), in studies of basic mechanisms regulating mitochondrial function (Cámara et al., 2011; Metodiev et al., 2009, 2014) and in aging research (Ross et al., 2013; Trifunovic et al., 2004). In fact, the C57BL/6 is one of the most widely used mouse strains, and it was the first one to have its genome sequenced (Waterston et al., 2002). Furthermore, the importance of the C57BL/6 mouse as a standard mammalian model organism was demonstrated by the fact that this mouse strain was recently sent to space to study the physiological and metabolic impact of long-term space flights (Sychev et al., 2014). In contrast to the report from the Enriquez laboratory (Lapuente-Brun et al., 2013), we have previously observed respirasomes when performing knockout studies in C57BL/6N mice (Milenkovic et al., 2013; Sterky et al., 2012). However, in these previous studies, we had not performed detailed analyses of supercomplex composition or assessed the *COX7a2l* genotype. To gain further insights into the role of *COX7a2l* isoforms in control of respiratory chain function and supercomplex formation, we extensively characterized the commonly used C57BL/6J and C57BL/6N mouse strains. We report here that both of these mouse strains indeed are homozygous for the short isoform of *COX7a2l*, but this circumstance does not affect their respiratory chain function or the maximal oxidative capacity. Furthermore, the steady-state levels of supercomplexes and respirasome formation are not affected in these mice. Therefore, we conclude that mice harboring the short *COX7a2l* isoform have no gross bioenergetic aberrations that prevent their use in metabolism research.

RESULTS AND DISCUSSION

CD1 and C57BL/6 Mice Contain Different *COX7a2l* Alleles but Exhibit Very Similar Mitochondrial Bioenergetic Properties

We analyzed the allelic variation of *COX7a2l* in genomic DNA extracted from CD1 and C57BL/6J mice and, in agreement with the previous report from the Enriquez laboratory (Lapuente-Brun et al., 2013), we found that C57BL/6J mice indeed are homozygous for a short isoform of the *COX7a2l* allele (Figure 1A). In addition, we analyzed the C57BL/6N mouse strain and found that it also contains the short *COX7a2l* allele in a homozygous state (Figure 1A). As the different *COX7a2l* alleles have been previously reported to modulate respiration driven by complexes I and II, we proceeded to investigate the mitochondrial bioenergetic properties in these different mouse strains. To this end, we assessed the oxygen consumption rate in isolated heart and liver mitochondria (Figures 1B and 1C). Freshly isolated heart and liver mitochondria were incubated with respiratory substrates whose metabolism results in delivery of electrons at the level of complexes I or II. In contrast to the previous report (Lapuente-Brun et al., 2013), the analyzed mouse strains exhibited similar oxygen consumption rates with complex I or complex II substrates under phosphorylating, nonphosphorylating, and uncoupled conditions (Figures 1B and 1C). Furthermore, as the respiration in intact mitochondria is known to depend on substrate transport and matrix dehydrogenase activities, we also measured respiration in permeabilized mitochondria incubated with saturating concentrations of substrates and cytochrome *c*. When succinate was added at saturating amounts, the respiration capacity (dependent on complexes II, III, and IV) was similar to the capacity found in intact mitochondria incubated with succinate (Figure 1D). However, the respiration stimulated by adding NADH (dependent on complexes I, III, and IV) or NADH and succinate (dependent on complexes I, II, III, and IV) was clearly increased, but there was no difference between mitochondria from CD1 and C57BL/6J mice (Figure 1D). Furthermore, western blot analyses of mitochondria isolated from the different strains showed indistinguishable steady-state levels of OXPHOS proteins (Figure 1E), which is in good agreement with the very similar respiration patterns (Figures 1B–1D).

Respirasomes Are Present in Mice Containing the Short *COX7a2l* Isoform

The finding that the respiratory chain capacity assessed in the presence of different substrate combinations was very similar in mouse strains harboring the short or long *COX7a2l* alleles (Figures 1B–1D) was in stark contrast to the results from the Enriquez laboratory (Lapuente-Brun et al., 2013). This surprising discrepancy prompted us to proceed to investigate the supramolecular organization of the respiratory chain by using standard BN-PAGE techniques. To this end, we solubilized mitochondria in mild detergent conditions (a ratio of digitonin to mitochondrial protein of ~6 g/g), as digitonin/protein ratios between 4 and 8 g/g are the most commonly used (Dudek et al., 2013; Maranzana et al., 2013; Moreno-Lastres et al., 2012; Schägger and Pfeiffer, 2000; Sterky et al., 2012; Wittig et al., 2006). After extraction with detergent, the respiratory chain supercomplexes were separated by BN-PAGE and analyzed by western blotting (Figure 2A) or in-gel enzyme activity assays (Figure 2B). As expected under these mild solubilization conditions, we observed ATP synthase dimers (Figure 2A, right panel), thus showing that our results are in agreement with previous reports (Habersetzer et al., 2013). Remarkably, we found no alteration in the supramolecular organization of complexes I, III, and IV in CD1, C57BL/6J, and C57BL/6N mice when using western blot analyses (Figure 2A) or in-gel

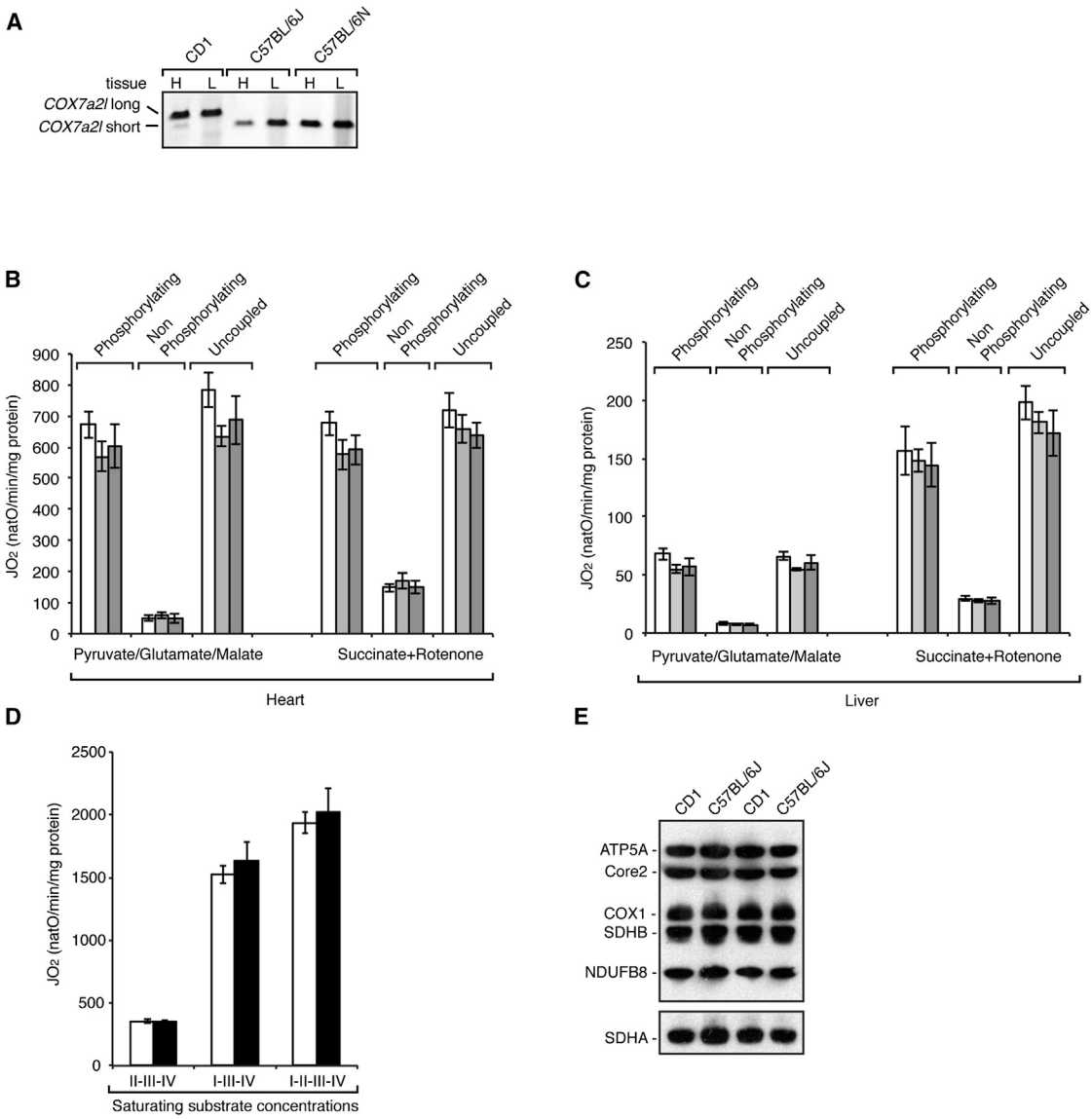

Figure 1. Different *Cox7a2l* Isoforms Have No Differential Effects on Respiratory Chain Activity

(A) PCR analysis of *Cox7a2l* alleles in heart (H) and liver (L) tissue from CD1, C57BL/6J, and C57BL/6N mouse strains.

(B) Oxygen consumption of heart mitochondria from CD1 (white bars), C57BL/6J (light gray bars), and C57BL/6N (dark gray bars) mouse strains at 10 weeks of age. n = 4; error bars indicate mean ± SEM.

(C) Oxygen consumption of liver mitochondria from CD1 (white bars), C57BL/6J (light gray bars), and C57BL/6N (dark gray bars) mouse strains at 10 weeks of age. n = 4; error bars indicate mean ± SEM.

(D) The maximal oxygen consumption rate assessed in permeabilized heart mitochondria from CD1 (white bars) and C57BL/6J (black bars) mice in the presence of saturating concentrations of cytochrome *c*, NADH (I-III-IV), succinate (II-III-IV), or both NADH and succinate (I-II-III-IV). n = 7; error bars indicate mean ± SEM.

(E) Steady-state levels of OXPHOS subunits in CD1 and C57BL/6J isolated heart mitochondria as determined by western blot analyses.

enzyme activity assays (Figure 2B), showing that the short and long *COX7a2l* isoforms do not influence supercomplex formation. To further validate our conclusions, we performed a set of western blots with additional antibodies directed against subunits of complex III and complex IV (Figures 2C and 2D). Consistent with many previous reports (Moreno-Lastres et al., 2012; Schägger, 2001; Schägger and Pfeiffer, 2000), we mainly found complex III and complex IV as free complexes or incorporated into respirasomes (Figures 2A–2C). To assess comigration of complexes more stringently and to exclude misalignment of gels, we used a two-color fluorescent labeling system allowing double immunodetection on the same western blot membrane (Figure 2D). When the migration of the labeled complex IV (Figure 2D, red) and the labeled complex III (Figure 2D, green) was compared, we found incorporation of both complexes into supercomplexes in CD1 as well as in C57BL/6J mice. The group of Ugalde (Moreno-Lastres et al., 2012) has quantitatively assessed that a minor amount of complex III (~10%) and trace

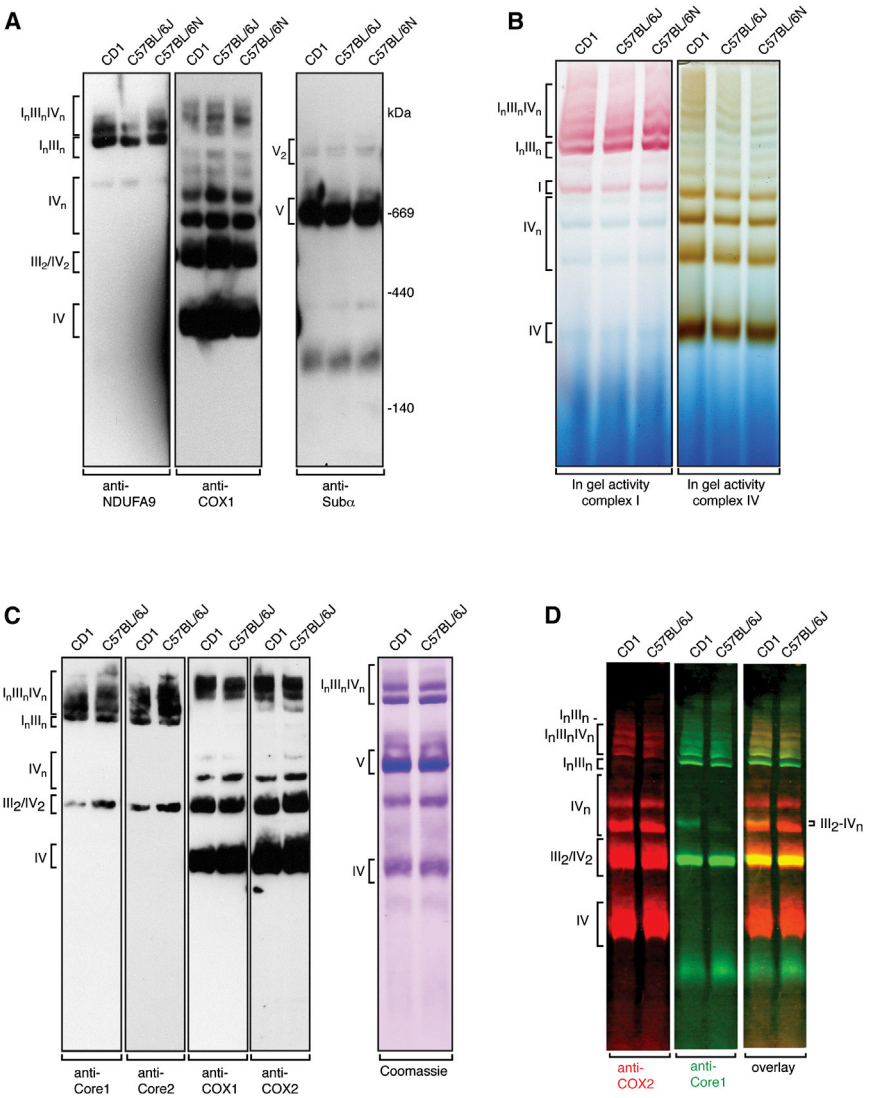

Figure 2. The Short *Cox7a2l* Isoform Does Not Affect the Supramolecular Organization of the Respiratory Chain

(A) Supramolecular organization of the OXPHOS system in different wild-type mouse strains. Heart mitochondria were extracted with a ratio of 6 g/g of digitonin to mitochondrial protein and analyzed by BN-PAGE. Immunodetection of subunits of complex I (NDUFA9), complex IV (COX1), and ATP synthase (subunit α) was performed after transfer of proteins from the BN-PAGE gel to a PVDF membrane. The positions of respiratory chain complexes and supercomplexes are indicated to the left. A representative image from five independent experiments is shown.

(B) In-gel enzyme activities of complexes I and IV in different wild-type mouse strains. The BN-PAGE conditions are as in (A). The data are representative of three independent experiments.

(C) Supramolecular organization of the respiratory chain in different wild-type mouse strains as determined by immune staining with antibodies against complex III (anti-Core1 and anti-Core2) and complex IV (anti-COX1 and anti-COX2). Coomassie Brilliant Blue staining of the gel was performed as a loading control. The BN-PAGE conditions are identical to the ones used in (A). The data are representative of three independent experiments.

(D) Supramolecular organization of the respiratory chain in different wild-type mouse strains. Western blot analyses were performed with double fluorescent detection of complex IV (anti-COX2, red color) and complex III (anti-Core2, green color). The BN-PAGE conditions are identical to the ones used in (A). The data are representative of four independent experiments.

amounts of complex IV (<3%) are found in a III_2-IV_n complex. Consistent with their results, we observed such a III_2-IV_n complex in CD1 and C57BL/6J mice (Figure 2D). The proportion of complex III_2 in the III_2-IV_n complex was reduced in the C57BL/6J strain in comparison with the CD1 strain (Figure 2D). This minor difference in complex III_2-IV_n abundance is unlikely to have any physiological impact, because CD1 and C57BL/6J mice show no difference in respiratory chain capacity or in levels of supercomplexes. Our results thus suggest that complex IV and complex III are only partly comigrating and, interestingly, that the amount of complex IV in this complex seems to be independent of complex III (Figure 2D). Furthermore, identical respirasome organization was observed using the BALB/c mouse strain also reported to be homozygous for the *Cox7a2l* short allele (Figures S1A and S1B). We thus conclude that the respirasome organization, as judged by the migration of complexes I, III, and IV, is indistinguishable in CD1, C57BL/6J, C57BL/6N, and BALB/c mouse strains when analyzed with BN-PAGE (Figures 2A–2D, S1A, and S1B).

Complex IV Is Essential for Respirasome Formation

To rule out the possibility of a fortuitous comigration between putative complex IV multimers and other types of supercomplexes and to prove that complex IV physically interacts with complexes I–III, we utilized the conditional *Lrpprc* mouse knockout (Ruzzenente et al., 2012). These knockout mice have a severe complex IV deficiency but near-normal steady-state levels of the other respiratory chain complexes (Mourier et al., 2014; Ruzzenente et al., 2012). Heart mitochondria isolated from control and conditional *Lrpprc* knockout C57BL/6N mice were analyzed by BN-PAGE, and a drastic reduction of complex IV levels was found (Figures 3A and 3B). The low residual levels of complex IV were present in a free form (IV) in the knockout mice. As a consequence of the complex IV deficiency, we observed that most of the high-molecular-weight supercomplexes (I_n-III_n-IV_n) disappeared, and only supercomplexes containing complexes I and III (I_nIII_n) remained (Figures 3A and 3B). Moreover, we found accumulation of a complex III dimer (III_2) in conditional *Lrpprc* knockout mice (Figure 3B), consistent with the observation that loss of complex IV prevents incorporation of complex III into supercomplexes (Figures 3A and 3B). In addition, it should be mentioned that the analysis of mitochondria from the conditional *Lrpprc* knockout mouse confirmed the

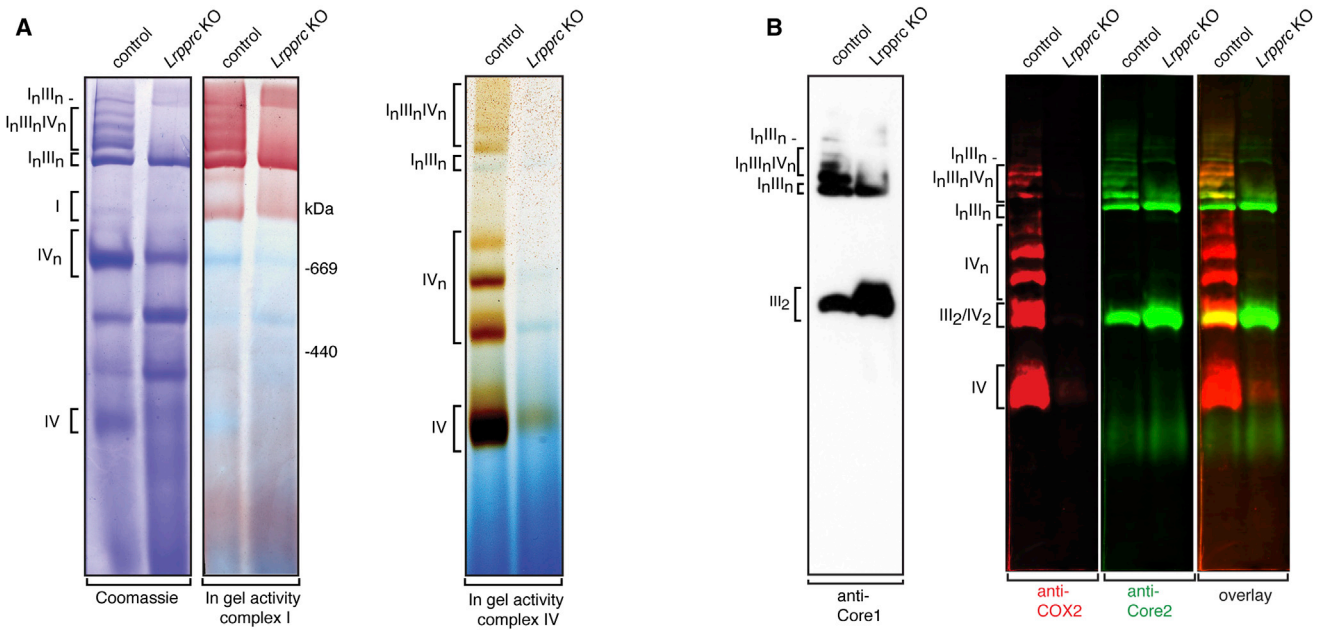

Figure 3. Complex IV-Containing Respirasomes Are Present in Mouse Strains Harboring the Short *Cox7a2l* Isoform

(A) Supramolecular organization of complexes I, III, and IV in control and *Lrpprc* heart knockout mitochondria from C57BL/6N mice. Mitochondria were solubilized in digitonin (6 g/g mitochondrial protein) followed by Coomassie Brilliant Blue staining and in-gel enzyme activity staining for complexes I and IV. The data are representative of three independent experiments.

(B) BN-PAGE analysis of heart mitochondria isolated from control and *Lrpprc* heart knockout C57BL/6N mice. The BN-PAGE conditions are identical to the ones used in (A). The left panel shows western blot analysis of complex III (anti-Core1). The right panels show western blot analyses with double fluorescent detection of complex IV (anti-COX2, red color) and complex III (anti-Core2, green color). The data are representative of three independent experiments.

specificity of the COX1 and COX2 antibodies (Mourier et al., 2014; Ruzzenente et al., 2012) and clearly showed that the observed complex IV in-gel enzyme activity assay indeed was dependent on the presence of complex IV (Figures 3A and 3B).

Complex IV Assembly Is Not Affected in Mice Containing the Short *COX7a2l* Allele

Next, we assessed complex IV assembly in CD1, C57BL/6J, and C57BL/6N mice by using an in vitro import assay on isolated mitochondria. For this purpose, we radiolabeled the COX6a subunit of complex IV and the UQCRFS1 subunit of complex III and performed import experiments in freshly isolated heart (Figures 4A–4C) and liver (Figure S2A) mitochondria. Import into the mitochondrial matrix is typically dependent on a potential across the inner mitochondrial membrane, and we therefore performed experiments in the presence and absence of the mitochondrial membrane potential in both heart (Figures 4B and 4C) and liver (Figure S2A) mitochondria. We followed the assembly pathway of imported UQCRFS1 and COX6a by performing BN-PAGE (Figures 4A–4C and S2A). There was no difference in the pattern of assembled complex III, complex IV, and related supercomplexes in CD1, C57BL/6J, and C57BL/6N mice (Figures 4A–4C). It has been argued that the detergent concentration is an important parameter when observing lack of supercomplexes in mice expressing the short *COX7a2l* isoform. To address this point, we investigated whether the supramolecular organization of complex IV is altered if mitochondria are solubilized at a wide range of digitonin concentrations prior to BN-PAGE analysis. In line with our previous results, we observed identical patterns of complex IV-containing supercomplexes in CD1 and C57BL/6J mice at various detergent concentrations (Figures 4C and S2B).

Conclusions

The use of inbred mouse strains allows reproducible experiments to be performed in independent laboratories and is an important tool to investigate regulation of metabolism in normal physiology, disease, and aging. Recently, it was claimed that mice harboring a short isoform of *COX7a2l* cannot form complex IV-containing supercomplexes and that this aberration leads to absence of respirasomes and clear bioenergetic aberrations (Lapuente-Brun et al., 2013). This finding would have profound implications for interpretation of results with mice on the C57BL/6J and C57BL/6N background, because both strains harbor the short *COX7a2l* isoform and are widely used in experimental mouse genetics. Here we demonstrate, using a variety of independent approaches, that the short *COX7a2l* gene isoform does not affect respiratory chain activity or respiratory chain supercomplex formation. Therefore, we conclude that mice harboring the short *COX7a2l* isoform unequivocally remain a suitable tool for metabolic and mitochondrial research. Furthermore, we provide evidence, using the *Lrpprc* knockout mouse, that complex IV is essential for respirasome formation in vivo.

EXPERIMENTAL PROCEDURES

BN-PAGE

For BN-PAGE, 75 μg isolated mitochondria were lysed in 50 μl solubilization buffer (20 mM Tris [pH 7.4], 0.1 mM EDTA, 50 mM NaCl, 10% [v/v] glycerol)

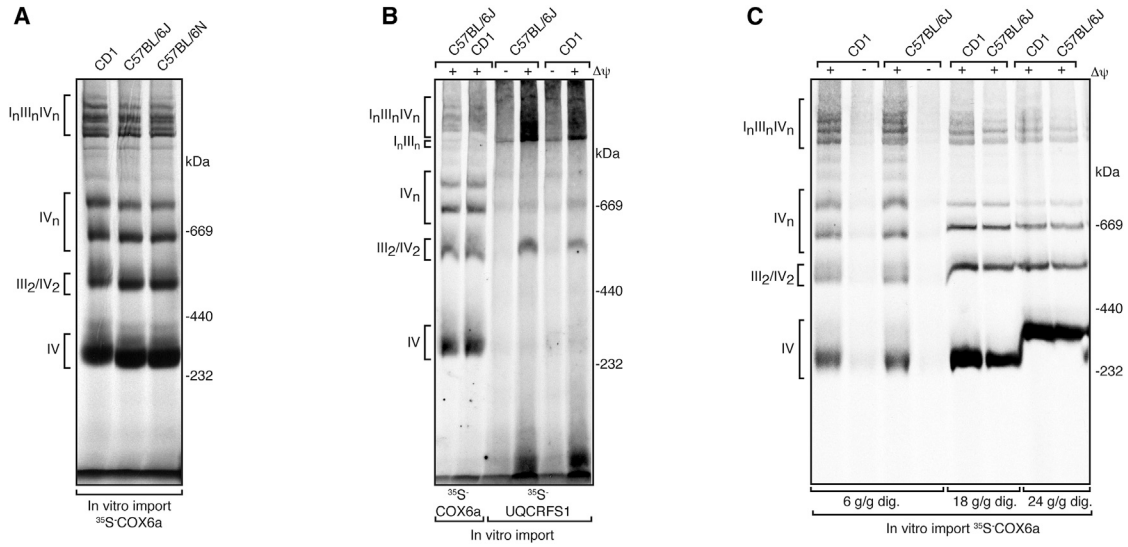

Figure 4. The Assembly of Complex IV Containing Respirasomes Is Unaffected in Mouse Strains Harboring the Short *Cox7a2l* Isoform

(A) Import of the radiolabeled Cox6a precursor and subsequent incorporation into complex IV and respirasomes in intact heart mitochondria isolated from different wild-type strains. After 60 min incubation, the mitochondria were solubilized in digitonin (6 g/g mitochondrial protein) and analyzed by BN-PAGE. A representative image from eight independent experiments is shown.

(B) Import of the radiolabeled UQCRFS1 precursor and subsequent incorporation into complex III and supercomplexes in intact heart mitochondria isolated from different wild-type strains. After 60 min incubation, the mitochondria were solubilized in digitonin (6 g/g mitochondrial protein) and analyzed by BN-PAGE. A representative image from three independent experiments is shown.

(C) Import of the radiolabeled Cox6a precursor and subsequent incorporation into complex IV and respirasomes in intact heart mitochondria isolated from different wild-type strains. After 60 min incubation, the mitochondria were solubilized in buffers containing different digitonin concentrations (6–24 g/g mitochondrial protein). Import experiments were performed in the presence or absence of the mitochondrial membrane potential ($\Delta\psi$). The data are representative of three independent experiments.

containing 1%–10% (w/v) digitonin (Calbiochem) and mixed with loading dye (5% [w/v] Coomassie Brilliant Blue G-250, 150 mM BIS-TRIS, and 500 mM ε-amino-n-caproic acid [pH 7.0]). BN-PAGE samples were resolved on 4%–10% gels and further subjected to Coomassie staining, western blot analysis, or in-gel activity staining for complexes I and IV. In-gel activity assays were performed as previously described (Wittig et al., 2007).

In Vitro Import

Radiolabeled human Cox6a and UQCRFS1 proteins were obtained by coupled transcription and translation in the presence of ^{35}S-methionine (PerkinElmer) using TNT SP6 Quick Coupled System (Promega). For import experiments, freshly isolated mitochondria from heart and liver tissue were incubated with the radiolabeled proteins at 37°C in potassium-acetate import buffer (250 mM sucrose, 5 mM Mg-acetate, 80 mM K-acetate, 20 mM HEPES [pH 7.4], 10 mM Na-succinate, 1 mM ATP, and 1 mM DTT). To dissipate the membrane potential before import, 1 μM valinomycin was added. Samples were lysed in solubilization buffer and further analyzed on 4%–10% gradient gels. Signals were visualized by digital autoradiography.

Ethics Statement

This study was performed in accordance with the guidelines of the Federation of European Laboratory Animal Science Associations. The protocol was approved by the Landesamt für Natur, Umwelt und Verbraucherschutz in Nordrhein-Westfalen in Germany.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2014.11.005.

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Supplemental Information

The Respiratory Chain Supercomplex Organization Is Independent of *COX7a2l* Isoforms

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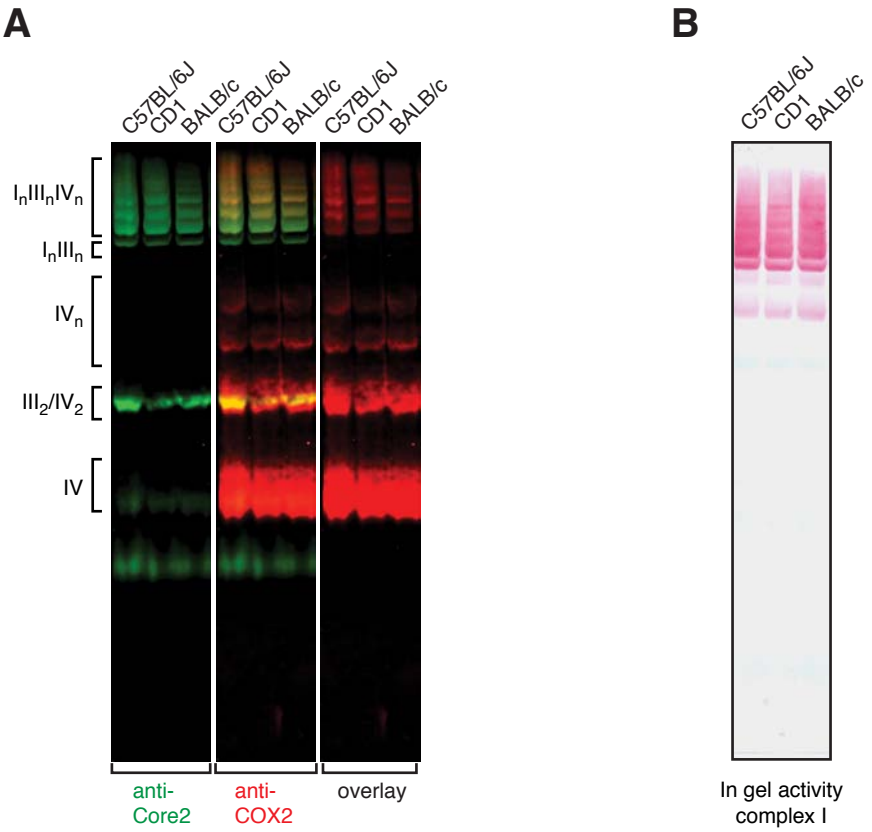
A
C57BL/6J
CD1
BALB/c
C57BL/6J
CD1
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CD1
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I_nIII_n
IV_n
III_2/IV_2
IV
anti-
Core2
anti-
COX2
overlay
B
C57BL/6J
CD1
BALB/c
In gel activity
complex I

Figure S1

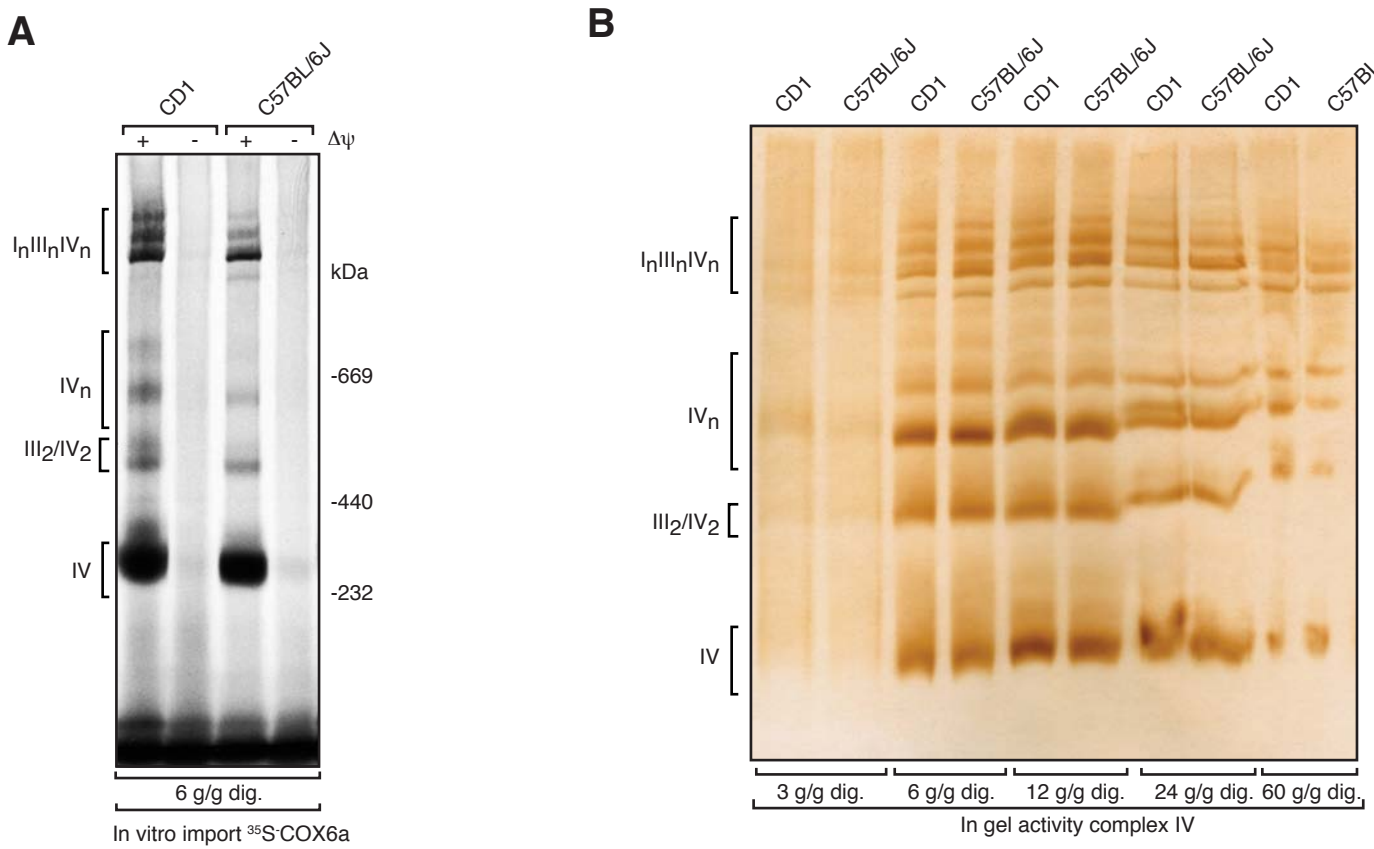
A
CD1
C57BL/6J
+
-
+
-
Δψ
$I_nIII_nIV_n$
IV_n
III_2/IV_2
IV
kDa
-669
-440
-232
6 g/g dig.
In vitro import ^{35}S-COX6a
B
CD1
C57BL/6J
$I_nIII_nIV_n$
IV_n
III_2/IV_2
IV
3 g/g dig.
6 g/g dig.
12 g/g dig.
24 g/g dig.
60 g/g dig.
In gel activity complex IV

Figure S2

SUPPLEMENTAL FIGURE LEGEND

Figure S1 (related to Figure 2) The supramolecular organization of the respiratory chain is not altered in BALB/c mouse strain

(A) Supramolecular organization of the respiratory chain in different wild type mouse strains. Western blot analyses with double fluorescent detection of complex IV (anti-COX2, red color) and complex III (anti-Core2, green color).

(B) In gel enzyme activities of complexes I in different wild type mouse strains.

Figure S2 (related to Figure 4). The short *Cox7a2l* isoform does not impair the formation of complex IV containing respiratory supercomplexes

(A) In vitro import of the radiolabelled precursor of Cox6a into isolated liver mitochondria from the CD1 and C57BL/6J mouse strains. After 60 minutes incubation in the presence or absence of mitochondrial membrane potential ($\Delta\psi$), the mitochondria were solubilized with digitonin (6g/g mitochondrial protein) and further analyzed by BN-PAGE.

(B) Analysis of complex IV in gel enzyme activity after BN-PAGE of heart mitochondria isolated from CD1 and C57BL/6J mice. The mitochondria were solubilized in buffers containing different digitonin concentrations (6-60 g/g mitochondrial protein).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mouse breeding

About 20 mice per genotype (CD1, C57BL/6) and 3 BALB/c mice were obtained directly from Charles River and Jackson laboratory and sacrificed for analyses a few weeks after maintenance in our mouse breeding facility.

Mitochondria isolation

Isolation of mitochondria was performed by differential centrifugation as previously described (1). Briefly, mice were sacrificed by cervical dislocation, and tissues were quickly collected in

ice-cold DPBS (Gibco), minced and homogenized with few strokes of a Potter S homogenizer (Sartorius) in 5 ml of ice-cold mitochondria isolation buffer (MIB; 310 mM sucrose, 20 mM Tris-HCl, 1 mM EGTA, pH 7.2). Mitochondria were purified by differential centrifugation (1200g for 10 minutes) and supernatants were subsequently centrifuged at 12000g for 10 minutes. The crude mitochondrial pellet was resuspended in an appropriate volume of MIB. The mitochondrial protein concentration was determined using the Protein DC Lawry based assay (Bio-Rad).

Oxygen consumption measurements:

Mitochondrial oxygen consumption flux was measured as previously described (2) at 37°C using 65–125 µg of crude mitochondria diluted in 2.1 ml of mitochondrial respiration buffer (120 mM sucrose, 50 mM KCl, 20 mM Tris-HCl, 4 mM KH_2PO_4, 2 mM $MgCl_2$, 1 mM EGTA, pH 7.2) in an Oxygraph-2k (OROBOROS INSTRUMENTS, Innsbruck, Austria). The oxygen consumption rate was measured by using either 10 mM pyruvate, 5 mM glutamate and 5 mM malate or 10 mM succinate and 10 nM rotenone. Oxygen consumption was assessed in the phosphorylating state with 1 mM ADP (state 3) or in the non-phosphorylating state by adding 2.5 µg/ml oligomycin (pseudo state 4). In the control mitochondria, the respiratory control ratio (RCR) values were >10 with pyruvate/glutamate/malate as substrates. Respiration was uncoupled by successive addition of carbonyl cyanide m-chlorophenyl hydrazone (CCCP) up to 3 µM to reach maximal respiration. ETC maximal activities were obtained by incubating permeabilized mitochondria with saturating concentration of cytochrome c (2.5 mg/ml) and NADH (10 mM) and/or succinate (30 mM). The complete permeabilization was obtained after several freeze and thaw cycles and controlled by the absence of additional effects after addition of CCCP or alamethicin (10 μg/ml).

Western blot analysis

Proteins were separated by SDS–PAGE or BN–PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes (Milipore). Immunodetection was performed according to the standard techniques using enhanced chemiluminescence (Immun-Star HRP

Luminol/Enhancer Bio Rad) or fluorescence. To perform the fluorescent detection of complex III and IV, PVDF membranes were blocked using the Rockland blocking buffer (MB-070). After incubation with the primary antibody, an Alexa Fluor 680 goat anti-rabbit or an IRdye800 antimouse secondary antibody was used. The detection was performed using the Li-COR Odyssey system. Monoclonal antibodies specific for NDUFA9 (complex I) were obtained from Invitrogen. F1α (complex V) monoclonal antibodies and mitoprofile total OXPHOS cocktail antibodies were obtained from Abcam. COX1, Core1 and Core2 monoclonal antibodies were obtained from Mitoscience. Rabbit polyclonal antisera were used for detection of COX2 (3).

Polymerase chain reaction:

For microdeletion analysis, we used PCR on genomic DNA isolated from heart and liver tissue of CD1, C57BL/6J, C57BL/6N mice with the following primers Cox7a2l-fw (CTTTCTTGCTTTGCAGAAGGC and Cox7a2l-rev (GAAGGCCTCGTTTCAGGTGG). The products were analyzed on 6% TBE urea gels (Invitrogen).

SUPPLEMENTAL REFERENCES

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