

1 Functional role of respiratory supercomplexes in mice: 2 segmentation of the Q_{pool} and SCAF1

3 Enrique Calvo^{1†}, Sara Cogliati^{1,2†}, Pablo Hernansanz-Agustín^{1†}, Marta Loureiro-
4 López^{1†}, Adela Guarás¹, Rafael A. Casuso², Fernando García-Marqués¹, Rebeca Acín-
5 Pérez¹, Yolanda Martí-Mateos¹, JC. Silla-Castro¹, Marta Carro-Alvarellos¹, Jesús R.
6 Huertas², Jesús Vázquez^{1,3*} and J.A. Enríquez^{1,4*}

7 ¹ Centro Nacional de Investigaciones Cardiovasculares Carlos III. Madrid 28029, Spain.

8 ²Institute of Nutrition and Food Technology, Biomedical Research Centre, Department
9 of Physiology, University of Granada

10 ³ CIBERCV, Madrid, Spain

11 ⁴CIBERFES, Madrid, Spain

12 *Correspondence to: J. Vázquez (jvazquez@cnic.es) or J.A. Enríquez

13 (jaenriquez@cnic.es)

14 † these four authors contributed equally.

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25 **Summary**

26 Mitochondrial respiratory complexes assemble into different forms of supercomplexes
27 (SC). In particular, SC III₂+IV require the SCAF1 protein. However, the structural role of this
28 factor in the formation of the respirasome (I+III₂+IV) and the physiological role of SCs are
29 controversial. Here, we study C57BL/6J mice harbouring either non-functional SCAF1, the full
30 knock-out for SCAF1 or the wild-type version of the protein and found a growth and exercise
31 phenotype due to the lack of functional SCAF1. By combining quantitative data-independent
32 proteomics, high resolution 2D Blue Native Gel Electrophoresis and functional analysis of
33 enriched respirasome fractions, we show that SCAF1 confers structural attachment between III₂
34 and IV within the respirasome, increases NADH-dependent respiration and reduces ROS
35 production. Furthermore, through the expression of AOX in cells and mice we confirm that CI-
36 CIII superassembly segments the CoQ in two pools and modulates CI-NADH oxidative capacity.
37 These data demonstrate that SC assembly, regulated by SCAF1, modulates the functionality of
38 the electron transport chain.

39 **Introduction**

40 The mitochondrial cristae are the main site of biological energy conversion through the
41 respiratory complexes I to V known as oxidative phosphorylation system (OXPHOS). Our
42 understanding of the structure of the mitochondria electron transport chain was shaken in 2000
43 by Herman Schägger when proposing that respiratory complexes could form superstructures
44 called supercomplexes (SCs), among which the ones containing CI, CIII and CIV were named
45 respirasomes (Schagger, 2000). This proposal came from a novel electrophoretic methodology
46 mastered by that author named Blue Native Gel Electrophoresis (BNGE). First received with
47 extreme skepticism, SCs are nowadays generally accepted as true biological entities. They are
48 present in mitochondria from very different sources (Eubel et al., 2003; Schägger, 2002); they are
49 able to respire (Acín-Pérez et al., 2008); specific factors for the formation of some SCs have been
50 discovered (Lapiente-Brun et al., 2013); and the cryo-electron microscopy structures of the
51 respirasome (I+III₂+IV) and the supercomplex I+III₂ have been obtained (Gu et al., 2016; Letts

52 et al., 2016; Sousa et al., 2016). Although all these data demonstrate their existence, the
53 physiological role of SCs is still under strong debate. A line of thinking proposes that SCs have
54 no functional role (Milenkovic et al., 2017). Other authors indicate that SCs optimize electron
55 flux to gain efficiency in energy generation while minimizing reactive oxygen species production
56 (Lapiente-Brun et al., 2013; Lenaz and Genova, 2007; Maranzana et al., 2013). We proposed the
57 “Plasticity Model”, where individual and super-assembled complexes coexist in a regulated
58 equilibrium within the inner mitochondrial membrane (Acín-Pérez et al., 2008; Enríquez, 2016).
59 Thus, SCs rearrange in response to a shift in the metabolic source of electrons (Guarás et al.,
60 2016), in the metabolic adaptation of specialised cells in vivo (Garaude et al., 2016), or to adapt
61 the cell to different nutrients and physiological conditions (Greggio et al., 2017; Lopez-Fabuel et
62 al., 2016). For instance, under starvation the preferential use of fatty acids reduces the levels of
63 SCs containing CI, so that free III₂ is more accessible to electrons coming from FADH₂
64 (Lapiente-Brun et al., 2013).

65 The controversy was centered in the biological role of SCAF1 in respirasomes and in the
66 role of SCs in the functional segmentation of the CoQ pool. In mouse, SCAF1 is 113 amino acid
67 long and has a high homology in its carboxy terminus with one subunit of CIV, for which there
68 are two isoforms (COX7A1- 80 aa and COX7A2- 83 aa). The amino-terminal portion of the
69 protein has no homology with any known protein. SCAF1 is required to super-assemble CIII and
70 CIV by replacing COX7A2 in the structure of CIV and by binding CIII through its amino-terminal
71 portion (Cogliati et al., 2016). Interestingly, all inbred mouse C57BL/6 sub-strains investigated
72 to date (Enríquez, 2019), harbor a non-functional version of SCAF1 (named SCAF1¹¹¹) that, due
73 to a microdeletion that eliminate two amino acids, is unable to interact with CIV (Cogliati et al.,
74 2016). In the absence of functional SCAF1, the SC III₂+IV and the majority of SCs containing
75 CI, CIII and CIV cannot be formed (Cogliati et al., 2016; Lapiente-Brun et al., 2013; Lobo-Jarne
76 et al., 2018). However, in some instances, particularly in heart mitochondria from BL/6 mice, a
77 comigration between CI, CIII and CIV suggestive of the presence of respirasomes can be observed
78 (Cogliati et al., 2016; Lobo-Jarne et al., 2018). This raised doubts about the relevance of SCAF1
79 for the formation and function of the respirasome (Lobo-Jarne et al., 2018). In addition, we

80 observed, confirming the previous suggestions (Bianchi et al., 2004), that superassembly partially
81 segments CoQ in two pools, one predominantly dedicated to FAD and another to NAD(Lapunte-
82 Brun et al., 2013). Noteworthy, these data were obtained using isolated mitochondria. Elsewhere,
83 but using sub-mitochondrial particles, opposite conclusions were reported (Fedor and Hirst,
84 2018), calling the partial CoQ partitioning hypothesis into question.

85 In this work we demonstrate that the ablation of SCAF1 decreases the performance of
86 mice under rigorous metabolic stresses such as reduced food supply or intense exercise demand.
87 In addition, by combining data-independent proteomics, 2D BNGE-based structural analysis and
88 functional studies of the BNGE enriched respirasome fractions, we demonstrate that SCAF1 plays
89 a key role in the regulation of the structure of respirasome and its bioenergetics performance. In
90 addition, through the expression of AOX in several cell systems and animals, we have been able
91 to confirm the segmentation of the CoQ in two pools is a consequence of the superassembly
92 between CI and CIII. These findings demonstrate that SCs play a physiological role and provide
93 a molecular mechanism for the phenotype observed in animals.

94 **Results**

95 **The ablation of functional SCAF1 compromises growth under food restriction and impairs** 96 **exercise performance.**

97 One of the more recurrent arguments to challenge the proposal that SCAF1 and the
98 superassembly between complexes III and IV are bioenergetically relevant is the belief that all
99 C57BL/6 mice sub-strains, which harbor a non-functional SCAF1, lack an apparent phenotype.
100 Since this belief has never been experimentally addressed, we first investigated it in detail. We
101 compared the phenotype of C57BL/6 mice with full ablation of SCAF1 (SCAF1^{-/-} or SCAF1^{KO}),
102 with the non-functional version of SCAF1 (SCAF1^{111/111}), and with the wild-type and functional
103 version of this gene in homozygosis (SCAF1^{113/113}). We first confirmed that SCAF1^{113/113} liver
104 and heart mitochondria express high levels of SCAF1 that are associated substantially with the
105 respirasome and SC: III₂+IV (Fig. 1A). On the other hand, and as described before (Cogliati et
106 al., 2016), SCAF1¹¹¹ was very unstable and could only be found in minor amounts, interacting
107 with CIII either in the I+III₂ or in III₂ (Fig. 1A). In the full KO mice, no SCAF1 could be detected

108 (Fig. 1A). Work from our group revealed that the ablation of SCAF1 in zebrafish affects the
109 growth of male and female animals, the female fertility and the swimming performance of
110 otherwise healthy animals. We also showed that all these phenotypic features disappear when
111 overfeeding the animals (García-Poyatos et al., 2019). Therefore, we hypothesized that since in
112 the usual food regime of mouse facilities animals are fed ad-libitum, a situation that can be
113 considered overfeeding, C57BL/6 mice would be protected against similar manifestations of the
114 lack of functional SCAF1. Therefore, we first evaluated if the presence of functional SCAF1 in
115 mice had any physiological impact under restricted food administration. Animals were feed ad
116 libitum for 24 hours every three days and were fasted during the rest of the time (Fig. 1B). During
117 the first days the animals suffered a period of adaptation in which SCAF1¹¹³ males were able to
118 maintain or even increase their weight, whereas SCAF1¹¹¹ and KO males showed the opposite
119 trend (Fig. 1C). This period also resulted in the loss of some mice, without significant differences
120 between strains but with a clearly better survival rate of females vs. males (Fig. S1A). Once
121 adapted, all animals started gaining weight. These results indicate that the absence of SCAF1¹¹³
122 affects mice physiology and that SCAF1¹¹¹ does not retain any significant functionality (Fig. 1C).
123 Interestingly, females were insensitive to the presence of functional SCAF1 (Fig. 1C), probably
124 due to a different female mechanism of adaptation to fasting (Della Torre et al., 2018). Next, we
125 evaluated the impact of functional SCAF1 on exercise performance. Thus, we found that both
126 male and female SCAF1¹¹³ were able to reach a 30% higher maximum speed in the treadmill than
127 any other group of animals (Fig. 1D). These studies demonstrate that the presence of functional
128 SCAF1 has a direct impact on the mice phenotype, implying that supercomplex formation allows
129 a more efficient electron transport chain. This increased efficiency allows males to better extract
130 energy from aliments, and both sexes to respond to higher muscle work demands. The observation
131 that SCAF1 ablation causes such evident phenotype demands a deeper look into the molecular
132 role of this protein in the function of the mitochondrial electron transport chain, which could help
133 to solve the controversy on this issue. To that aim, we combined unbiased complexome analysis
134 by mass spectrometry, 2D-BNGE evaluation of the interaction of respiratory complexes within

135 supercomplexes, and functional analysis of enriched fractions of complexes and supercomplexes
136 in the presence or in the absence of functional SCAF1.

137 **The size distribution of OXPHOS components is tissue-specific as a consequence of**
138 **superassembly**

139 First, we applied Blue-DiS, a recently developed technology that takes advantage of the
140 reproducibility and sensitivity of data-independent mass spectrometry (Guarás et al., 2016), to
141 perform mitochondrial complexome profiling from a completely hypothesis-free perspective.
142 Thus, mitochondria-enriched preparations from heart, brain and liver from CD1 mice and from
143 the cell line L929 repopulated with mtDNA from C57BL/6 mice (L929^{C57}), having all of them the
144 wild type functional version of SCAF1, were separated by BNGE and each lane was cut into 26
145 slices; each slice was subjected to trypsin protein digestion and analyzed by MS using the DiS
146 method we previously developed (Guarás et al., 2016). We identified 1,134 proteins classified as
147 mitochondrial in the Mitocarta 2.0 (Calvo et al., 2016), which correspond to 98% of all Mitocarta
148 annotated proteins (Fig. S1B). Mitochondrial enrichment was different between samples, being
149 heart (81% of peptide identifications annotated in Mitocarta) and liver (71%) more enriched in
150 known mitochondrial proteins; while brain showed a lower enrichment (38%) (Fig. S1C). In all
151 cases an almost complete coverage of components of OXPHOS complexes was achieved (Dataset
152 1). Every subunit from CII and CIII was identified (Fig. S1D), together with 41 out of the 44
153 annotated proteins from CI (Fig. S1D). Concerning CIV, this complex is built out of 14 proteins
154 but several of them have isoforms making up a total of 21 possible components. However,
155 COX6B2 and COX7B2 are both expressed only in sperm, and COX4i2 only in lung, so that 18
156 proteins were potentially identifiable in our samples, from which we detected 16 (Fig. S1D).
157 Regarding CV, we could detect 17 out of the 20 structural proteins of this complex (Fig. S1D).
158 Apparent molecular weights could be accurately assigned to each one of the 26 BNGE slices from
159 the known masses of individual complexes and of SC (Fig. S1E).

160 We performed a cross-correlation analysis of protein abundances across different slices
161 within the same mitochondrial type (Fig. S2A). This analysis showed that the relative abundances
162 of all proteins belonging to either CI, CIII, CIV or CV were constant across slices (Fig. S2A &

163 [Supplementary Table 1](#)), and that the most representative slice from each complex also had the
164 same protein proportions across the different mitochondrial types ([Fig. S2B & Supplementary](#)
165 [Table 2](#)). In striking contrast, the electrophoretic mobility protein distributions of CI, CIII and
166 CIV, but not of CV, were markedly different from one mitochondrial source to another ([Fig. S2C,](#)
167 [D](#)). Thus, complexes in different tissues have the same protein composition but form tissue-
168 specific high molecular weight structures.

169 Notably, the larger structures (slides 1-8) consistently contained identical proportions of
170 CI and CIII, or CIII and CIV proteins ([Fig. 2A, B](#)). Cross correlation analysis confirmed that the
171 protein composition in the I+III+IV, I+III and III+IV structures was maintained between the
172 adjacent slices ([Fig. 2C](#)) and between tissues ([Fig. 2D](#)), reflecting the formation of SC in an
173 unbiased manner. To obtain a quantitative estimate of the distribution of these structures in each
174 tissue we modelled the complete migration profiles of CI, CIII and CIV by unsupervised gaussian
175 deconvolution. We found that in all mitochondrial types the profile of CI and CIII could be
176 accurately explained as a superimposition of broad gaussian peaks corresponding to a ternary
177 complex (I+III+IV), to binary I+III and III+IV structures and to free CI, CIII and CIV forms
178 ([dashed red lines in Fig. 2E and Fig. S2D](#)), having these components the same electrophoretic
179 mobilities and similar peak widths across all mitochondrial sources ([Fig. 2F](#)). We also found that
180 the profile of CIV could not be adequately explained by these structures around slices 6-12 ([Fig.](#)
181 [2E, black arrows](#)), suggesting the presence of significant amounts of CIV dimers and multimers.
182 Thus, the addition of two more CIV structures (IV_2 and IV_m) was sufficient to model the migration
183 profile of this complex in all mitochondrial types ([dashed red lines in Fig. 2E and Fig. S2D](#)).

184 To further validate the predicted structures of OXPHOS molecular assemblies, we
185 performed a Blue-DiS analysis of mitochondria from cultured mutant cell lines that are unable to
186 assemble one or more of the complexes (Diaz et al., 2006; Moreno-Loshuertos et al., 2006;
187 Perales-Clemente et al., 2010). In the cell line ΔCI , which lacked the ND4 subunit from CI and
188 was unable to assemble CI (Moreno-Loshuertos et al., 2006), neither free CI nor SCs I+III₂ and
189 I+III₂+IV could be detected ([Fig. S3A, B](#)); however, free III₂ and IV and the SC III₂+IV were
190 detected at the expected sizes (green rectangles). In the case of the mutant cell line for Cox10

191 protein (Δ CIV), which lacks assembled CIV, all structures containing CIV were absent (red
192 rectangles in Fig. S3A, B). In addition, the structures containing CI could neither be detected, in
193 agreement with the fact that CIV is needed for the stabilization of CI (Diaz et al., 2006); hence in
194 this mutant only the free III₂ form was detectable (green rectangles in Fig. S3A, B). Finally, in
195 the Rho 0 cell line, which lacks mitochondrial DNA encoding for different subunits of the
196 respiratory complexes, none of these structures were observed (red rectangles in Fig. S3A).
197 However, CII, which is totally encoded in the nucleus, and a CV form of smaller size (V*)
198 remained detectable in these cells (Fig. S3A).

199 In summary, the complete size distributions of OXPHOS components could be explained
200 by structures of constant size and composition that were present in each mitochondrial type at
201 different relative proportions (Fig. 2F).

202 **Respiratory complexes are assembled into supercomplexes at fixed stoichiometries.**

203 The notion that SC contained complexes at fixed stoichiometries was reinforced by the
204 finding that the protein composition of SC was conserved in all mitochondrial types (Fig. 2D).
205 We took advantage of the good linear response of quantitative Blue-DiS protein values (Fig. 2A,
206 B; Fig. S2A) to estimate the relative molar stoichiometries of complexes within each slice (Fig.
207 S3C, D, see M&M for detailed explanation). From slices 2 to 6 the relative molar proportion
208 CIII:CI was 2:1, in the four mitochondrial types, implying that the proportion I+III₂ is constant in
209 the respirasome and in the SC formed by CI and CIII (Fig. S3E). Besides, the relative molar
210 proportions CI:CIV and CIII:CIV were 1:1 and 2:1, respectively, between slices 2 to 4, implying
211 that the respirasome has a composition I+III₂+IV (Fig. S3E).

212 To our knowledge, these are the first stoichiometry estimations of OXPHOS complexes
213 made by mass spectrometry, which agree with the structures analysed by cryo-electron
214 microscopy (Gu et al., 2016; Letts et al., 2016; Sousa et al., 2016), and contradict the
215 experimentally unsupported, but generalized tendency to assume that there are multiple types of
216 respirasomes that differed in their increasing content of 1-4 CIV monomers. The reason for the
217 split of the respirasome in several discrete bands in BNAGE, corresponding to a broad peak in
218 Blue-Dis analysis, remains to be clarified. In slices 5 and 6, CIV is still detected although at a

219 lower proportion, suggesting that in this area migrate CIV superstructures of unknown
220 composition (Fig. 3E). Slices 7 and 8 are enriched in CI, where it migrates as a free complex, but
221 again with significant presence of CIV in different high molecular weight structures. Finally, the
222 relative proportions of CIII and CIV in bands 9 and 10 are coherent with a stoichiometry 2:1 (SC:
223 III₂+IV) (Fig. 3E). In summary, unbiased Blue-DiS analysis reinforces the results obtained by
224 other techniques and provide complementary evidence that OXPHOS complexes are arranged
225 into SC at fixed stoichiometries.

226 **CIV forms novel supercomplexes**

227 Our complexome analysis predicts that several structures that contain CIV, not yet
228 molecularly characterized, should be present. These are CIV structures above and below SC:
229 III₂+IV but also some CIV structures of higher molecular weight migration around I+III₂ and
230 I+III₂+IV. The existence IV₂ is well documented, and high molecular weight entities containing
231 only CIV have been described elsewhere (Cogliati et al., 2016). To confirm whether the CIV
232 structures predicted by Blue-DiS analysis are true entities, we performed 2D-BNGE of CD1 heart
233 mitochondria using in the first dimension digitonin as detergent to preserve the integrity of
234 supercomplexes and n-Dodecyl-β-D-maltoside (DDM) in the second dimension to disaggregate
235 supercomplexes into their component complexes. This procedure partially preserves the
236 interaction between CIII and CIV and the IV₂ (Perales-Clemente et al., 2010). As shown in Fig.
237 3A this analysis confirmed the presence of IV₂ and two forms of CIV that migrate immediately
238 faster and slower than SC III₂+IV and that do not super-assemble with any other respiratory
239 complex (IV_m and IV_n in Fig. 3A). These three structures account for a significant proportion of
240 the CIV in heart samples. In addition, we detected the presence of low proportions of a putative
241 SC: I+IV₂, which migrates with SC I+III₂ and segregates into CI and dimer CIV (Fig. S3F), and
242 of a putative SC I+IV, which migrates between free CI and SC: I+III₂ (Fig. S3F).

243 More surprisingly, we found CI and CIV monomer but without CIII that migrate between
244 SC: I+III₂+IV and SC I+III₂, very close to the faster migrating respirasome band I+III₂+IV (Fig.
245 S3F, labelled as I+IV?). The reason why this putative SC migrates with the apparent molecular
246 weight of a respirasome is unclear and may indicate that this structure interacts with a yet to

247 discover mitochondrial inner membrane component. Therefore, 2D-BNGE analysis of heart
248 mitochondria reveals specific supercomplexes that contain CIV, confirming Blue-DiS
249 predictions, and also CIV and CI together and that need to be further investigated. Note that, due
250 to their relatively low abundances, these additional IV structures are fully compatible with the
251 gaussian models of CI and CIV determined from Blue-DiS profiles.

252 **SCAF1 confers structural attachment between III₂ and IV within the respirasome**

253 To identify potential interaction partners of specific SC structures from the Blue-DiS
254 analysis, we generated their reference migration profiles *in silico* by combining the SC profiles
255 obtained by deconvolution analysis and performing a correlation search for all identified proteins
256 displaying a similar profile. This analysis led to a natural, hypothesis-free detection of
257 Cox7a21/SCAF1 as the only candidate showing a significant correlation in all the mitochondrial
258 types with the structures containing CIII and CIV together (ED Fig. 4a left & b). Consistently,
259 deletion of CI inhibited the migration of SCAF1 in the position of the respirasome, but not in the
260 position of SC III₂+IV, and no traces of SCAF1 were detected in the mutant lacking CIV or in
261 Rho 0 cells (Fig. S4A right). These results obtained by unbiased complexome analysis provide
262 further experimental evidence that this protein regulates the interaction between these complexes,
263 as we postulate in previous reports (Cogliati et al., 2016; Lapuente-Brun et al., 2013).

264 Next, we analyzed by gaussian deconvolution the Blue-DiS profiles of mitochondria
265 prepared from SCAF1¹¹¹ heart and liver C57BL/6JOLA^{Hsd} mitochondria. As shown in Fig. 3B, in
266 BL6 liver the peaks corresponding to the respirasome and the SCs III₂+IV disappeared, while the
267 rest of structures, including SCs I+III₂ and the III₂ peaks, were maintained. However, while SC
268 III₂+IV was neither observed in BL6 heart, the presence of the respirasome remained clearly
269 detectable in this tissue (Fig. 3B). Therefore, BL6 and CD1 respirasomes are present in heart
270 mitochondria, but differ in the absence of SCAF1 in the former.

271 We reasoned that if the role of SCAF1 is to physically link CIII and CIV, and this function
272 is maintained in the respirasome, then mitochondria harboring SCAF1¹¹³ and SCAF1¹¹¹ should
273 have different types of respirasomes differing structurally in the interaction between CIII and
274 CIV. To experimentally evaluate this hypothesis, we took advantage of the fact that upon 2D-

275 BNGE analysis the second dimension used to disaggregate supercomplexes (which employs
276 DDM) does retain significant amounts of the III₂+IV association, allowing us to discern whether
277 CIII and CIV are physically linked or not in the respirasome in the absence of functional SCAF1.
278 We performed 2D-BNGE with liver mitochondria derived from CD1, C57BL/6JolaHsd mice and
279 also from C57BL/6JolaHsd harboring the wild type version of SCAF1 (SCAF1¹¹³) generated in
280 our laboratory (Cogliati et al., 2016). This approach confirmed again that heart mitochondria from
281 BL6 mice does not assemble SC III₂+IV (Fig. 3C) and clearly demonstrated that the III₂+IV
282 structure remains within the respirasomes containing functional SCAF1 (CD1 and SCAF1¹¹³) but
283 not in those with the non-functional form (BL6¹¹¹) (Fig. 3C). In conclusion, this analysis
284 demonstrates that SCAF1⁺ and SCAF1⁻ respirasomes are structurally different (Fig. 3D).

285 **SCAF1 enhances respiratory performance and reduce ROS production by the respirasome.**

286 We then inquired if the structural difference linked to the presence of an active SCAF1
287 form in the respirasome has any impact on their respiratory capacity. We first evaluated the
288 suitability to perform these kinetic analyses by confirming in isolated individual complexes (I,
289 III₂ and IV) that it is possible to measure the rate of oxidation and reduction of their respective
290 donor/acceptor of electrons and that they generate the expected stoichiometries. All the rates of
291 the individual complexes were similar regardless the strain of origin, with the exception of a mild
292 reduction in CIII activity in BL6¹¹³ (Fig. S4C-E). Next, we excised BNGE bands containing SC
293 I+III₂+IV (respirasome) from SCAF1¹¹³ (CD1, BL6¹¹³ and 129 mice) and SCAF1¹¹¹ (C57BL/6J
294 mice) heart mitochondria and determined their respiratory capacity using NADH as substrate in
295 a Clark electrode (Acín-Pérez et al., 2008) and their NADH oxidation capacity by
296 spectrophotometry (Fig. 4A). For normalization purposes we determined the respiration capacity
297 induced by TMPD in the same samples after inhibition with antimycin and rotenone; we also
298 normalized by the protein content determined after eluting the respirasome from the gel. We found
299 that both SCAF1⁺ and SCAF1⁻-containing respirasomes were able to perform NADH-dependent
300 respiration without the need of adding either external CoQ or Cyt c (Fig. 4B). Interestingly, the
301 respiratory capacity of the respirasome was much lower than that of CIV-monomer and NADH
302 oxidation was also decreased in comparison to that of CI. These results suggest, in agreement

303 with a recent observation on amphipol-stabilized SC:I+III₂, that superassembly of respiratory
304 complexes modulate their activity (Letts et al., 2019). Moreover, the rate of respiration by
305 SCAF1⁺ respirasomes of different sources was about one order of magnitude higher than that of
306 the SCAF1⁻ ones regardless of whether they are normalized by CIV-dependent respiration (Fig.
307 4C) or by protein content (Fig. 4D). In agreement with this observation, the rate of NADH
308 oxidization by SCAF1⁺ respirasomes was between 3 and 4 times higher (Fig. 4E).

309 Lenaz's laboratory showed by reconstruction in liposomes that the association between
310 CI and CIII into SCs reduces the production of ROS (Maranzana et al., 2013). This observation
311 was validated in vivo by others (Lopez-Fabuel et al., 2016)(Huertas et al., 2017). The availability
312 of respirasomes with different degree of interaction between III₂ and IV allowed us to investigate
313 whether this interaction impacts on the production of ROS. For this purpose, we incubated the
314 BNGE-excised respirasome bands from CD1, BL6^{S111} and BL6^{S113} heart mitochondria
315 preparations in the presence of NADH and Amplex-red to monitor ROS production. We found
316 no differences in the level of ROS produced by the respirasomes from any of the investigated
317 strains (Fig. 4F, G). Considering that the rate of respiration and NADH oxidation of SCAF1¹¹¹
318 respirasome is lower than its wild type counterpart, we calculated that the respirasome harbouring
319 the mutant version of SCAF1 derived 0,185% of NADH electrons to ROS, whereas CD1 and
320 SCAF1¹¹³ derived 0,064 % and 0,067%, respectively. Next, we repeated the experiment but
321 substituting NADH by CoQ₁H₂ to donate electrons to CIII and in the presence of rotenone to
322 block CI interaction with CoQ. Under these conditions, which mimic a stress situation with an
323 abnormal rise in CoQH₂, SCAF1⁻ respirasomes produced significantly more ROS (Fig. 4F, G). In
324 all cases, ROS production was fully quenchable by the addition of superoxide dismutase and
325 catalase (Fig. 4F, G). Therefore, SCAF1⁺ respirasomes produced less ROS than SCAF1⁻ ones.

326 In summary, although SCAF1 can be dispensable for the formation of respirasomes it
327 confers structural attachment between complexes III₂ and IV within the respirasomes providing
328 more stability, significantly better functional performance and lower ROS production.

329 **Respiratory supercomplexes are unstable upon mitochondrial membrane disruption.**

330 The existence of two structurally distinct respirasomes was described recently in
331 ovine(Letts et al., 2016) and confirmed later in bovine models (Sousa et al., 2016). One form of
332 respirasome has complexes III₂ and IV tightly attached (named *tight respirasome*), while the other
333 is characterized by an increased distance between them (named *loose respirasome*). Our
334 observation that SCAF1 determines the interaction between III₂ and IV within the respirasome is
335 reminiscent of the existence of tight and loose respirasomes. Noticeably, both ovine and bovine
336 SCAF1 sequences match the mouse wild-type and functional 113 version.

337 Very intriguingly, Letts and co-workers observed that the proportion between both
338 respirasome forms in a given preparation is not stable. In fact, after incubation at 4° C the tight
339 respirasomes were transformed into the loose ones (Letts et al., 2016). We wondered whether this
340 phenomenon could be observed by BNGE analysis. Thus, we compared CD1-liver mitochondria
341 (which harbors the functional SCAF1 form), maintained several hours in the refrigerator, before
342 and after solubilization with digitonin. To our surprise, both SCs I+III₂+IV and III₂+IV, but not
343 SC I+III₂, disappeared in the samples maintained at 4 °C only if they were previously digitonized
344 (Fig. 5A). In addition, SCAF1 migrated as a bulk with the free CIV in the digitonized preparations
345 (Fig. 5A, B). Furthermore, 2D-BNGE/SDS-PAGE analysis revealed that the form of SCAF1 that
346 migrated with free CIV (marked with an asterisk) had a smaller molecular weight than the
347 canonical form (Fig. 5C). Proteomic analysis demonstrated that in slices corresponding to SC
348 I+III₂+IV, IV₂ and IV, a peptide spanning the sequence SSVTAYDYSYGK (originated from the
349 corresponding SCAF1-derived tryptic peptide LTSSVTAYDYSYGK) was unequivocally
350 identified exclusively in the non-fresh samples (Fig. 5E and Fig. S5A). This sequence was mapped
351 into the N-terminus region of the protein, which contains the CIII-interacting domain(Cogliati et
352 al., 2016) (underlined sequence in Fig. 5D). Further quantitative analysis revealed the coexistence
353 of the intact and proteolyzed forms of the peptide bound to CIV in the respirasome, while SC:
354 III₂+IV contained only the intact form (Fig. 5E). These results indicate that SCAF1 suffers a
355 partial proteolytic processing that disrupts SC: III₂+IV, destabilizes the respirasome and parallels
356 the loss of CIV-containing SCs. In silico analysis of the processing site in SCAF1 revealed a
357 putative cleavage site for calpain-1 (Fig. 5D). In agreement with this prediction the use of calpain

358 inhibitors was sufficient to prevent the cleavage of SCAF1 (Fig. 5F) and also to preserve the
359 integrity of CIV-containing SC after digitonin treatment (Fig. 5G). When similar experiments
360 were repeated with mitochondrial samples purified from CD1-heart we also observed the
361 relocation of SCAF1 with free CIV, but the reduction in the amount of CIV-containing SC was,
362 although evident, much milder (Fig. 5H). All these results indicate that SC are unstable or can
363 shift from tight (e.g. kinetically active) to loose (e.g. kinetically restrained) forms when
364 mitochondrial membrane integrity is compromised, likely as a consequence of the release of
365 proteases, such as calpain-1, and lipases that gain access to the inner mitochondrial membrane
366 and to the respiratory complexes.

367 It remains to be elucidated whether SCAF1 processing by calpain-1 is just an in vitro
368 artifact or a biologically relevant mechanism regulating SCs turnover dynamics. However, the
369 observation that respiratory complexes become unstable when the mitochondrial membrane
370 integrity is disrupted, raises serious concerns on the interpretation of the various published
371 experimental approaches used to investigate the function of the SCs. They include sub-
372 mitochondrial particles, where functional measurements were performed at 32 °C (Blaza et al.,
373 2014; Fedor and Hirst, 2018); isolated mitochondria, which were incubated at 37 °C (Guarás et
374 al., 2016; Lapuente-Brun et al., 2013); and partially isolated respirasomes (this report). To further
375 study this issue, we subjected digitonized and intact mitochondria from liver (Fig. S5B) and heart
376 (Fig. S5C) preparations to prolonged incubations at different temperatures. Liver digitonized
377 mitochondria completely lost the respirasome and the SC III₂+IV, after 72 hours at 4 °C, and all
378 SCs, including I+III₂ after 3h at 37 °C (Fig. 5A and Fig. S5B). Respirasomes from heart
379 digitonized mitochondria were more stable than those from liver at 4 °C (72h) but were completely
380 lost after 3h at 37 °C; at this temperature the SC III₂+IV and even the CIV monomer disappeared
381 (Fig. S5C). In contrast, all of these structures remained stable in intact organelles (Fig. S5B, C).
382 More worrying is the fact that the stability of both heart and liver SCs is not maintained if
383 digitonized mitochondria are incubated at 37 °C for just one hour (Fig. S5D). All these
384 observations call to caution when performing experiments in disrupted mitochondria. Very
385 importantly, the differential stability of SC in intact and broken mitochondria may well be the

386 cause of the apparently discrepant results regarding the physiological role of respiratory
387 supercomplexes between different groups.

388 Our unexpected results prompted us to evaluate if BNGE purified respirasomes were
389 stable enough during functional studies to trust the conclusions presented above. To address this
390 issue, we kept the first-dimension gel for 1 or 3 hours at 37°C after running the BNGE to inquire
391 if the respirasomes are preserved. After the incubation period we ran a second-dimension
392 electrophoresis also in the presence of digitonin to determine the intactness of structures, since
393 complexes and SCs are expected to migrate forming a diagonal unless some associations are
394 disrupted during incubation. Thus, after 1- or 3-hour incubation at 37 °C the respirasome and any
395 other detected complex or SCs remained substantially intact apart from comet tail-like shape of
396 the spots due to diffusion in the 1st dimension gel during the long period of incubation (Fig. S5E).
397 Moreover, 2D-BNGE analysis, using DDM in the second dimension to dissociate the
398 respirasomes, confirmed the stability of the interaction between III₂ and IV within the respirasome
399 (Fig. S5F). Therefore, BNGE allows the isolation of the respirasome maintaining its structure to
400 perform functional studies, validating our functional observations.

401 **Superassembly of OXPHOS complexes influences the delivery of electrons and the**
402 **respiratory capacity within the electron transport chain.**

403 The lack of stability of the respiratory supercomplexes upon disruption of mitochondrial
404 membranes raises serious concerns on the interpretation of recent experiments where recombinant
405 AOX was added to a preparation of sub-mitochondrial particles (Fedor and Hirst, 2018). The data
406 obtained suggested that the interaction of CI and CIII in supercomplexes does not modify the
407 delivery of electrons to the alternative oxidase AOX by CoQ. These results led to the conclusion
408 that there is a unique CoQ pool equally accessible to CI and CII within mitochondrial inner
409 membrane. Given the observation that the native structure of supercomplexes is preserved in
410 intact mitochondria, we performed similar experiments but expressing AOX in the mitochondria
411 of different cell models: a) wild-type cells expressing AOX (CIV^{WT}AOX and E9AOX); b) cells
412 lacking CIV which retain CI and SCI+III₂ superassembly due to the presence of AOX
413 (CIV^{KO}AOX); c) cells that lack CIII which preserve CI due to AOX expression but CI cannot

414 form supercomplexes with CIII (CIII^{KO}AOX). AOX expressed in mammalian mitochondria is
415 known to be functional and can recycle oxidized CoQ in cells lacking of mtDNA (Perales-
416 Clemente et al., 2008) or in cells lacking CIII or CIV (Guarás et al., 2016). We described
417 elsewhere that the expression of AOX prevent the degradation of CI in the absence of CIII or CIV
418 (Guarás et al., 2016) (Fig. S6A). We found that the level of AOX activity was similar between
419 the different cell lines (Fig. S6B). Following similar reasoning to that of Fan & Hirst, the delivery
420 of electrons from CI or CII to AOX should be independent of superassembly in the absence of
421 CIII. However, if the superassembly between CI and CIII has any effect on the activity of CI, in
422 the absence of CIV a change in the delivery of electrons from CI to AOX should be detected (Fig.
423 6A). In the two mutant cells we detected measurable CI-AOX and CII-AOX respirations, while
424 CIV-dependent respiration could not be recorded (Fig. 6B). Strikingly, CII-AOX dependent
425 respiration was insensitive to the presence of CIII, while CI-AOX dependent respiration was
426 significantly lower when CIII is present (Fig. 6B). This is true despite a higher NADH oxidation
427 capacity of CI in CIV^{KO}AOX cells (Fig. 6C). We also reasoned that if a CoQ pool was shared
428 between CII and CI, addition of succinate would outcompete with glutamate + malate-based AOX
429 respiration (Fig. 6D). We found that in either the wild type or in the CIV^{KO} cells, all expressing
430 AOX, the addition of succinate significantly increased the oxygen consumption over that
431 achieved by CI substrates (Fig. S6C, D and Fig. 6E). On the contrary, in CIII^{KO} AOX cells the
432 addition of succinate was unable to increase the AOX dependent oxygen consumption over that
433 reached by CI substrates (Fig.S6D and Fig. 6E). These results suggest that when CI is not
434 superassembled CoQ exist in a unique pool, whereas its superassembly triggers the formation of
435 two partially differentiated pools.

436 We then measured directly NADH or succinate dependent respiration (Fig. 6F, G) and
437 NADH oxidation (Fig. 6H) in mitochondrial membranes permeabilized by freeze & thawing in a
438 period of 4 min to prevent disruption of supercomplexes, and isolating mitochondria from wild
439 type, CIV^{KO} and CIII^{KO} cell models, all expressing AOX. This analysis offered a number of
440 interesting observations: (i) In the presence of CIII none of the substrates (NADH or succinate)
441 was able to reach the oxygen consumption levels obtained when both substrates were added

442 simultaneously (Fig 6F, G). This indicates that the delivery pathway of electrons to CoQ and
443 AOX is partially different for each substrate. (ii) NADH oxidation of the two wild-type cells lines
444 is different, being the E9 nuclear background significantly lower ($\approx 50\%$) (Fig. 6H). We described
445 elsewhere that this is due to the presence of a missense mutation in COI that reduce the activity
446 of CIV, and hence, respiration (Acin-Perez, 2003), a fact that can also be observed in ED Fig.5C
447 and D. (iii) The addition of succinate did not impact on the oxidation of NADH in any wild type
448 cell line while the inhibition of CIV by KCN almost completely abolished it in wild type cells
449 (Fig. 6H). (iv) The ablation of CIV dramatically reduced the rate of oxidation of NADH to levels
450 equivalent to those caused by KCN (Fig. 6H). (iv) The ablation of CIII significantly increased the
451 NADH oxidation with respect to the levels of their isogenic wild type cells -which harbor a
452 mutation in COI- (Fig. 6H). This result parallels what we have observed measuring oxygen
453 consumption in intact mitochondria (Fig. S6D), and fully confirms that the dependence of CIV
454 activity limits the NADH oxidation capacity in E9 derived cells. (v) In the absence of CIII but not
455 of CIV, the addition of succinate significantly reduced the NADH oxidation, which in these cells
456 is fully dependent of AOX (Fig. 6H). All these results demonstrate that the superassembly of CI
457 with CIII, either in SCs or in the respirasome, impacts dramatically in the NADH oxidation
458 capacity of CI by modifying the delivery of electrons to CIV or AOX. Moreover, they indicate
459 that CI and CII can potentially outcompete for delivering electrons to AOX, a phenomenon that
460 is minimized by the presence of CIII. Our results with AOX expression substantially disagree
461 with those published by Fedor and Hirst (Fedor and Hirst, 2018). The reason for this discrepancy
462 is likely due to the unnoticed disruption of the supercomplexes under their experimental set up,
463 as explained above.

464 There are, however, additional experimental differences between our analyses that may
465 contribute to those discrepancies. Fedor et al. utilized mitochondria extracted from hearts as
466 starting material for their experiments (Fedor and Hirst, 2018). In order to minimize the impact
467 of the mitochondrial source, we generated a novel mouse model expressing AOX in the heart and
468 muscle mitochondria (see material and methods for details) and repeated the experiments with
469 purified wild type muscle and heart mitochondria expressing AOX. We first performed the

470 analysis of respiration in intact mitochondria fed with CI substrates (Glut+Mal) and assessing the
471 distribution of electrons either to CIV or AOX by using specific inhibitors for each enzyme (Fig.
472 6I, left panel). As expected, more than 70% of CI-dependent respiration was sensitive to CIV
473 inhibition, with a significant respiration mediated by AOX (Fig. 6I, left panel). We confirmed
474 that this assay was specific for CI by monitoring NADH oxidation in intact mitochondria by
475 measuring changes in NADH auto-fluorescence during the assay in the absence or in the presence
476 of rotenone (Fig. 6I, right panel). We additionally performed experiments in freeze-thawed
477 mitochondria prepared from hearts expressing AOX and confirmed again that NADH-linked
478 respiration was completely inhibited by KCN, whereas CII-AOX respiration was maintained (Fig.
479 S6E). Monitoring NADH absorbance allowed us to confirm that KCN completely abolished
480 NADH oxidation, in contrast to SHAM, which did not have any significant effect (Fig. S6F).
481 Similar results were obtained with mitochondrial preparations from muscle expressing AOX (Fig.
482 S6G, H). In conclusion, our results based on AOX expression could be reproduced using different
483 sources of mitochondria and demonstrate that CI supercomplex assembly affects the delivery of
484 electrons to AOX.

485 Discussion

486 In this report we provide strong experimental support demonstrating that the
487 superassembly of respiratory complexes into supercomplexes induce novel characteristics that
488 deeply impact on the structure, kinetics and efficiency of the respiratory chain. We demonstrate
489 it using isolated supercomplexes, mitochondrial preparations and cell cultures, and also using
490 animal models. We also provide experimental data that explains why previously published results
491 apparently contradict the main conclusion of this paper.

492 The main findings supporting our conclusion can be summarized as follows: 1) The
493 absence of functional SCAF1 in mice causes a reduction in exercise performance both in males
494 and females and compromise growth under food restriction in males. 2) The absence of functional
495 SCAF1 eliminates the physical interaction between CIII and CIV in the SC III₂+IV or within the
496 respirasome (I+III₂+IV). 3) In the absence of functional SCAF1, the respirasome can be
497 assembled in some tissues despite the fact that the direct interaction between CIII and CIV is

498 absent. 4) The absence of SCAF1 destabilizes the respirasome, reduces both oxygen consumption
499 and NADH oxidation and increases ROS production. 5) The interaction between CI and CIII
500 decreases the rates of NADH oxidation and respiration by AOX, implying the existence of a
501 partially segmented CoQ pool driven by the superassembly of CI and CIII.

502 Overall, the more relevant conclusion of our work is that the formation of respiratory
503 supercomplexes modify the kinetics and the flux of electrons occurring with non-superassembled
504 complexes. In one side, the role of SCAF1 in the respirasome becomes now clear. After strong
505 discrepancies, there is a general consensus that SCAF1 is required for the superassembly of
506 SC:III₂+IV and to provide stability to the respirasome (Cogliati et al., 2016; Lobo-Jarne et al.,
507 2018). There is also agreement in that SCAF1 function is lost in the mutated form of SCAF1 that
508 naturally arise in the C57BL/6 sub-strains (Enríquez, 2019; Lapuente-Brun et al., 2013). In spite
509 of that, BL6 heart mitochondria still assemble bona-fide respirasomes, which are structurally
510 different, less stable and functionally impaired. These findings clarify the role of SCAF1 in the
511 respirasome and the relevance of the interaction between CIII and CIV. On the other side, the
512 expression of AOX in cells and in mice and the ability to genetically control the free or
513 superassembled status of CI, allowed us to confirm that the delivery of electrons from CI but not
514 CII to AOX or CIV is asymmetric only if the formation of supercomplex I+III₂ is occurring. These
515 results confirm that superassembly of CI and CIII allow a partially segregation of the CoQ pool.
516 Interestingly, a very recent paper from the group of Sazanov studying the kinetic properties of
517 NADH to Cyt c electron transfer of the isolated supercomplex I+III₂ also found that CoQ trapping
518 within isolated respiratory supercomplex I+III₂ limits CI turnover (Letts et al., 2019).

519 In addition to the major findings, we exhaustive re-evaluated the mitochondrial BNGE
520 profiles from a variety of murine sources and analyzed the stability of the sample under several
521 conditions using advanced data-independent proteomics. This analysis provided relevant new
522 knowledge to better interpret BNGE analysis. In one hand, the Blue-DiS technology allowed us
523 to confirm, from a true hypothesis-free perspective, the presence, composition and stoichiometry
524 of the OXPHOS CI, CIII, CIV and CV in free and superassembled forms. Thus, while the protein
525 composition of these complexes is maintained, the proportion of OXPHOS supercomplexes is

526 tissue-specific. Moreover we demonstrate that regardless of the different positions where the
527 respirasome migrate, it maintains a constant stoichiometry of I+III₂+IV, a result that is in full
528 agreement with the different cryo-electron-microscopy derived structures published to date (Gu
529 et al., 2016; Letts et al., 2016; Sousa et al., 2016; Wu et al., 2016). This estimation stresses the
530 necessity to correct the experimentally unsupported tendency to assume that the different bands
531 of the respirasome contain increasing amounts of complex IV and to define the band with the
532 faster migration rate as the I+III₂+IV respirasome and those with a higher apparent molecular
533 weight as successive megacomplexes (Lobo-Jarne et al., 2018). It is advisable to restrict the use
534 of the term megacomplex to define the association between SCs (Guo et al., 2017). Nevertheless,
535 it is remarkable that the respirasome migrate persistently in multiple bands, a phenomenon that
536 require further investigation of the potential partners. The Blue-DiS analysis also confirmed, from
537 a completely unbiased perspective, that SCAF1 is the main assembly factor which is present in
538 SC containing CIII and CIV. On the other hand, we describe unequivocally the existence of novel
539 SCs: I+IV that co-migrate between the free CI and SC: I+III₂ and I+IV₂ that co-migrate with SC:
540 I+III₂; and the co-migration of CI with CIV in the area of the respirasomes. CI and CIV
541 associations could be predicted from the evidence obtained by cryo-electron-microscopy that the
542 interaction between CI and CIII₂ is independent from that between CI and CIV (Letts et al., 2016;
543 Sousa et al., 2016). SC I+VI₂ was however unexpected. This novel SC, as well as some of the
544 already known associations may represent true SCs or partially disassembled elements from larger
545 complexes. In any case, their characterization in the BNGE is of major relevance because they
546 can lead to inaccurate interpretations. This may be the case of the proposed novel fast migrating
547 respirasome (I+III₂+IV) that comigrate with I+III₂, despite the different molecular weight, solely
548 based on the fact the CI, CIII and CIV co-migrate in the same band (Sun et al., 2016). The co-
549 migration of I+III₂ with I+IV₂ is a more plausible explanation, since they have a very similar MW.
550 The co-migration CI with CIV in the area of the respirasomes, well above SC: I+III₂, does not
551 contain IV dimers and the added molecular weight of CI and CIV does not justify its apparent
552 MW. The fact that 2D-BNGE/DDM splits CI and CIV suggest as the more plausible explanation
553 that this co-migration imply the presence of a SC I+IV that interact with an unknown partner.

554 This additional SC may again flaw our interpretation of the BNGE gels. Thus, when the gels are
555 performed with a steep gradient or short run (as it is the case of the more used pre-casted native
556 gels) the SCs I+III₂+IV (the faster migrating band) and this particular SC: (I+IV)* may overlap
557 (as a potential example see (Lobo-Jarne et al., 2018)) leading to the wrong interpretation solely
558 based on the position of CIV that this band correspond to a respirasome.

559 A third important discovery reported here is that the respirasome and the SC: III₂+IV, and
560 at less extent SC I+III₂, are unstable after disruption of the mitochondrial membranes even if
561 preserved at 4 °C. This previously unnoticed phenomenon raises concerns in the interpretation of
562 experiments aimed to measure the proportion and function of SCs when mitochondrial membrane
563 integrity is not preserved. More strikingly, the loss of respirasome and SC: III₂+IV is paralleled
564 by the specific proteolytic processing of SCAF1 by calpain-1 that causes the processed form of
565 SCAF1 to migrate together with CIV. If this cleavage is physiologically relevant or not need to
566 be further investigated, but at this point it explains discrepant results in the literature that
567 considered that SCAF1 as a shorter protein that acts as a mere isoform of CIV (Zhang et al., 2016).
568 By the same token it may explain the unexpected presence of variable amounts of SCAF1
569 associated with free CIV. Our observation is also coherent with the description of two respirasome
570 structures by several groups (Letts et al., 2016; Sousa et al., 2016) that differ in the connection
571 between III₂ and IV that is either present (tight respirasome) or absent (loose respirasome). Our
572 finding also explains the reported conversion of the tight respirasome into the loose one with time
573 (Letts et al., 2016). Therefore, these observations largely clarify the investigation of the
574 functional role of the respiratory supercomplexes using BNGE.

575 In summary, in this report we show that superassembly between respiratory complexes
576 substantially enhances the respiratory capacity of the electron transport chain while minimizing
577 ROS production. Superassembly establishes a segmented CoQ pool through the association
578 between CI and CIII, while SCAF1 plays a critical role in the III₂+IV interaction affecting the
579 structure and kinetic properties of the respirasome. Hence, SCAF1 is a key factor in the regulation
580 of the energy metabolism, optimizing efficiency under high energy demands or restricted
581 availability of nutrients.

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590 Author contributions:

591 EC, JV & JAE: conceived and designed the analysis, EC, ML-L, FG-M-M. and JS-C performed
592 the proteomic analysis. SC, AG, PH-A RA-P, YM and MC-A: performed the BNGE and the
593 respirasomes functional analysis. SC, MC-A, J.R.H and R.A.C performed the *in vivo* experiments.
594 EC, SC, ML-L, JV, PH-A, JV and JAE interpreted the results. EC, JV and JAE wrote the paper.

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712 **Figure Legends**

713 **Figure 1. SCAF1 deficiency induces a conspicuous phenotype in mice. A,** BNGE

714 followed by western-blot showing the absence or presence of SCAF1 in the indicated

715 tissue and mouse strain. 113: C57BL/6J0laHsd mice with the functional version of

716 SCAF1; 111: C57BL/6J0laHsd mice (it harbors a non-functional version of SCAF1); KO

717 C57BL/6J0laHsd mice without SCAF1. **B,** Scheme of the food restriction experiment

718 analyzed in **C. D,** Effect of SCAF1 on the maximum speed running in a treadmill by the

719 indicated mouse groups.

720 **Figure 2. Blue-DiS evidence of the formation of OXPHOS supercomplexes. A,** For each

721 complex, the number of protein PSMs was plotted against normalized protein abundances (see

722 Methods), showing that the relative proportions of proteins from CI (blue points), CIII (red points)

723 and/or CIV (green points) are constant in specific BNGE slices from liver mitochondria indicating

724 the presence of multimeric structures. Slices 2-4 correspond to a ternary structure (I+III+IV),

725 slices 5-6 and 9-10 to binary structures (I+III and III+IV, respectively) and slices 8 and 15 to

726 monomeric forms (I and IV, respectively). **B,** Similar results are obtained in heart and brain

727 mitochondria; for simplicity only one slice with the tertiary structure is shown. **C,** Cross-

728 correlation analysis of abundances of proteins forming part of each one of the supercomplexes,

729 showing that their relative proportions are tightly maintained across the consecutive slices. **D,**

730 Cross-correlation analysis of abundances of proteins of the indicated supercomplexes across the

731 four mitochondrial types, showing that their relative proportions are also constant independently

732 of the mitochondrial origin. **E,** Normalized profiles of CI, CIII and CIV from heart mitochondria

733 (black line) (see Methods) can be accurately explained as a superimposition (dashed thick red
734 line) of six gaussian peaks corresponding to monomeric and multimeric structures, as indicated.
735 The gaussian deconvolution was performed in a hypothesis-free manner by a simultaneous least-
736 squares fitting of the three normalized profiles adjusting the position, width and height of each
737 peak (thin lines) without any numeric constraint. In the case of CIV two additional peaks (CIV₂
738 and CIV_m) had to be added to the model in order to explain the normalized profile (arrows). **F**,
739 Gaussian components used to model the normalized profiles of the four mitochondrial types. For
740 simplicity, the two multimeric CIV structures are not represented.

741 **Figure 3. Structural consequences of SCAF1-deficiency in the formation of SCs. A**,
742 2DBNGE (Dig/DDM) analysis of heart mitochondria resolving complexes and supercomplexes
743 in the first dimension and disrupting SCs into their component complexes in the second
744 dimension. NDUFA9 immunodetection in red indicate the migration of CI, COI immunodetection
745 in red indicate migration of CIV and CORE2 immunodetection in green indicate migration of
746 CIII. **B**, Gaussian deconvolution of the normalized profiles of heart and liver mitochondria from
747 BL6 mice, where the function of SCAF1 protein is impaired. Red asterisks indicate the position
748 of SC III₂+IV, which is completely absent in both tissues, and of the respirasome, which is absent
749 in the liver but remains detectable in the heart. **C**, 2DBNGE (Dig/DDM) resolving complexes and
750 supercomplexes in the first dimension and disrupting SCs into their component complexes in the
751 second dimension. Samples from BL6, CD1 and BL6:S¹¹³ heart are compared highlighting the
752 area of migration of respirasomes, with the different traces at the bottom. Red asterisk indicates
753 absence of the respirasome-derived III₂+IV only in the BL6 sample indicating that III₂+IV are not
754 physically linked in SCAF1-deficient respirasomes. NDUFA9 immunodetection in red indicates
755 the migration of CI, COI immunodetection in red indicate migration of CIV and CORE2
756 immunodetection in green indicate migration of CIII. **D**, representation of the two structurally
757 different respirasomes.

758 **Figure 4. Functional consequences of SCAF1-deficiency in the activity of the respirasome.**
759 **A**, Scheme representing the experimental set up to analyze the function of the respirasomes. **B**,
760 Representative oxygen consumption traces obtained with heart respirasomes excised from the

761 BNGE and derived from either C57BL/6 or CD1 animals. The addition of different components
762 is indicated. **C & D**, NADH-dependent respiration rate normalized by TMPD-respiration rate (**C**),
763 or by mg. of protein (**D**) of heart respirasomes excised from BNGE from the indicated mouse
764 strain and measured in a Clark oxygen electrode. **e**, NADH oxidation rate by heart respirasomes
765 eluted from BNGE excised bands of the indicated mouse strain and measured by
766 spectrophotometry. **F & G**, Representative traces (**F**) and quantitative data (**G**) of H₂O₂
767 production upon NADH oxidation (upper-panel) or CoQH₂ oxidation (lower panel) by heart
768 respirasomes eluted from BNGE excised bands of the indicated mouse strain and estimated by
769 Amplex Red. When CoQH₂ oxidation was assayed rotenone was included to prevent the
770 interaction of CoQH₂ with CI, and the assay was performed.

771 **Figure 5. Supercomplexes are unstable upon mitochondrial membrane disruption.** **A**, BNGE
772 resolving complexes and SCs from CD1 liver from intact or digitonized mitochondria
773 preincubated during the indicated time at 4 °C, and probed with the indicated antibody. **B**, BNGE
774 resolving complexes and SCs from CD1 liver from digitonized mitochondria preincubated during
775 the indicated time at 4 °C, and probed with the indicated antibody. **C**, 2D-BNGE/PAGE resolving
776 complexes and SCs in the 1st dimension and protein components in the 2nd dimension from CD1
777 liver digitonized preparation preincubated 72 h at 4 °C showing that SCAF1 is processed
778 (SCAF1*). The membrane was immunoblotted with the indicated antibodies. **D**, Structure of
779 SCAF1 sequence, mapping CIII- (in green), and CIV- (in yellow) interacting regions. The
780 predicted calpain-1 processing site is indicated with a blue line. **E**, Quantitative analysis of the
781 SCAF1-derived tryptic (LTSSVTAYDYSYGK, in blue), and calpain 1-processed
782 (SSVTAYDYSYGK, in orange) peptides. Both peptides were quantified in the BNGE slices
783 corresponding to SCs I+III₂+IV and III₂+IV and to IV₂, and to CIV in fresh or in 4°C-incubated
784 liver mitochondria-enriched fractions. The calpain 1-processed peptide was only detected in the
785 non-fresh preparations, attached to CIV and IV₂ and also to the respirasome. A structural
786 interpretation of these results is presented at the left; the blue and orange shadows indicate
787 whether the SCAF1 peptide is tryptic or processed, respectively. **F**, 2D BNGE/PAGE showing
788 that the proteolytic cleavage of SCAF1 can be prevented by inhibition of Calpain-1. **G**,

789 BNGE/PAGE analysis of liver mitochondria showing that the stability of the respirasome and the
790 SC III₂+IV is preserved after digitonization in the presence of a calpain-1 inhibitor. **H**, BNGE
791 profile for CIV (COI-red) and SCAF1 (green) in heart samples maintained at 4°C after
792 digitonization during the indicated time.

793 **Figure 6. The superassembly between complexes I and III modulates the activity of CI and**
794 **functionally segments the CoQ pool. A**, Scheme representing the differential flux of electrons
795 to AOX from the indicated mutant cell line. **B**, Impact of the presence of CIII in the delivery of
796 electrons from CI and CII to AOX. **C**, DPI sensitive NADH oxidation capacity of the
797 mitochondrial preparation from the indicated cell line. **D**, Scheme representing the simultaneous
798 flux of electrons from either CI or CII to AOX from the indicated mutant cell line. **E**, Estimation
799 of the impact of the simultaneous addition of substrates for CII (succinate) on the CI-dependent
800 respiration with CI substrates (glutamate and malate) in the presence or absence of CIII. **F**, Impact
801 of CIII and CIV superassembly on the maximum respiration capacity with CII (succinate) or CI
802 (glutamate and malate) substrates vs. both substrates added simultaneously. **G**, Proportion of the
803 maximum respiration achievable by CI substrates in the presence or absence of CI and CIII
804 superassembly. **H**, Analysis of the flux of electrons from NADH and CI or succinate and CII to
805 AOX in the indicated freeze-thaw mitochondrial from wild type cells or mutant cells lacking CIV
806 or CIII all expressing AOX. **I**, Flux of electrons from NADH and CI to AOX or CIV in intact
807 heart mitochondria expressing AOX and monitored by oxygen consumption (left panel) or
808 autofluorescence of NADH (right panel).

809

810

811 **STAR METHODS**

812 **LEAD CONTACT AND MATERIALS AVAILABILITY**

813 Request of information and material should be made to José Antonio Enriquez. Mouse and cell
814 lines generated in this study are available upon request for a non-commercial use under a Material
815 Transfer agreement.

816 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

817 **Experimental Models** This study used mouse and cellular models which were generated in out
818 laboratory.

819 **Mouse generation:** 1) C57BL/6J^{OlaHsd} mice with the functional version of SCAF1 were
820 generated as previously described (Cogliati et al., 2016). C57BL/6J^{OlaHsd} mice knock out for
821 SCAF1 were generated by microinjection of ES cells knock-out in the first alleles from EuMMCR
822 repository in a C57BL/6J^{OlaHsd} blastocysts. Further, the blastocysts were implanted in a pseudo
823 pregnant female C57BL/6J^{OlaHsd}. 2) AOX expressing mice were generated by *genOway* by
824 targeted insertion of the AOX cDNA within the Rosa26 locus via homologous recombination in
825 embryonic stem cells originally derived from a 129 strain of mouse and injected into C57BL/6J
826 blastocysts and then re-implanted into OF1 pseudo-pregnant, and allowed to develop to term.
827 After removal the neo cassette flanked by FLP sites, the selected knock-in animals were then
828 systematically backcrossed for more than 20 generations to C57BL/6J^{OlaHsd} background. The
829 expression of the transgene is dependent upon the Cre recombinase mediated excision of a *LoxP*
830 flanked transcriptional “STOP” cassette upstream the AOX cDNA. For this study we induce the
831 expression of AOX in muscle and heart by breeding the AOX animals with expressing CRE under
832 the ACTA promoter.

833 **Mouse experimentation.** All animal procedures conformed to EU Directive 86/609/EEC and
834 Recommendation 2007/526/EC regarding the protection of animals used for experimental and
835 other scientific purposes, enforced in Spanish law under Real Decreto 1201/2005. Approval of
836 the different experimental protocols requires the estimation of the adequate sample size as well
837 as the definition of the randomization and blinding criteria. The mice were fed a standard chow
838 diet unless other food regime was required (5K67 LabDiet).

839 **Alternative fasting protocol.** Starting from weaning, mice undergo to 48h of fasting alternate
840 between 24h of controlled feeding (standard chow diet 5K67LabDiet Rod18-A; LASQCDiet) both
841 with free access to water for 40 days. The weight has been recorded at the end of feeding phase.

842 **Maximal incremental running test.** Mice was forced to run on a treadmill at 20° slope (LE8700
843 (76-0303), Treadmill Panlab, Harvard Apparatus). After a minute of acclimatization at 10 cm/s,
844 the speed was increased up to 16 cm/s for 5 minutes and further every 2 minutes by 3cm/s until
845 exhaustion, that was considered to reach when the mouse spent 3s in the back of the rack.

846 **Cellular Models:** All cell lines were grown in DMEM (GibcoBRL) supplemented with 5% FBS
847 (foetal bovine serum, Gibco BRL). mtDNA-less mouse cells were generated by long term growth
848 of L929 mouse cell line (ATCC CCL-1) in the presence of high concentrations of Ethidium
849 Bromide (EthBr) as previously described(Acin-Perez, 2003). Control cells (control cells) were
850 generated by transformation of ρ^0 929neo cells by cytoplasm fusion using NIH3T3 fibroblasts as
851 mitochondrial donors(Bayona-Bafaluy et al., 2008).

852 **METHOD DETAILS**

853 **Blue-Native Gel Electrophoresis:** Supercomplex levels and compositions were analyzed in
854 isolated mitochondria from different tissues and cells by blue native electrophoresis
855 (BNGE)(Wittig et al., 2006). Mitochondrial proteins from heart tissue were solubilized with
856 10% digitonin (4g/g) (Sigma D5628) and run on a 3%–13% gradient Blue Native gel. The
857 gradient gel was prepared in 1.5 mm glass plates using a gradient former connected to a
858 peristaltic pump. After electrophoresis, the gels were further processed for proteomic

859 analysis, western blotting, 2D SDS-PAGE or 2D-BNGE (DDM) analysis. For 2D SDS-
860 PAGE, the first-dimension lanes were excised from the gel and incubated 1h at room
861 temperature in 1% SDS and 1% β -mercaptoethanol and run in a 16.5% second denaturing
862 gel. For 2D-BNGE (DDM) and 2D-BNGE Digitonin, first-dimension lanes were excised
863 from the gel and run in a 3-13% gradient gel in native condition adding 0.02% of DDM to
864 the cathode buffer.

865 ***Immunodetection of complexes and supercomplexes:*** After BNGE, 2DBNGE/DIG,
866 2DBNGE/DDM or 2DBNGE/PAGE, proteins were electroblotted onto PVDF transfer
867 membrane (Immobilon-FL, 0.45 μ m, Merck millipore, IPFL00010) for 1 h at 100 V in
868 transfer buffer (48 mM Tris, 39 mM glycine, 20 % EtOH). A Mini Trans- Blot Cell system
869 (BioRad) was used. Sea Blocking buffer (Thermo Scientific 37527) or PBS with 5 % BSA
870 was used for 1 hour at room temperature (RT) to avoid non-specific binding of antibodies.
871 For protein detection, antibodies were incubated with the membrane for 2 hours at RT.
872 Secondary antibodies were incubated for 45 minutes at RT. The membrane was washed with
873 PBS 0.1 % Tween-20 for 5 minutes three times between primary and secondary antibodies
874 and after secondary antibodies, the last wash was only PBS. To study supercomplexes
875 assembly, the PVDF membrane was sequentially probed with antibodies Complex I (anti-
876 NDUFA9, Abcam ab14713), Complex IV (anti-COI. Invitrogen 35-8100), Complex III (anti-
877 core2, Proteintech), SCAF1 (anti-COX7A2L, St. John's laboratory STJ42268. This antibody
878 was generated by immunization of rabbit with KLH conjugated synthetic peptide between
879 37-65 amino acids from the central region of human COX7A2L. It recognizes an epitope in
880 the common part of full size and processed SCAF1, allowing to visualize the processed
881 SCAF1 migrated with CIV after Calpain processing as in Fig.5c and Fig.5f. This antibody
882 was discontinued and substituted by the anti-COX7A2L, St. John's laboratory STJ110597
883 produced with a full length recombinant human CoX7A2L that, in the same experimental
884 conditions does not recognize the calpain-1 processed SCAF1 that need to be identified only
885 by MS. Anti-MIC10 (MINOS1 Novusbio NBP1-91587) and anti-CHCHD3 (Proteintech
886 25625-1-AP).

887 ***Proteomics by data-independent scanning (DiS) mass spectrometry (MS)***. DiS is a data-
888 independent acquisition method that covers all possible fragmentations of precursors in the 400-
889 1100 m/z range in two LC-MS runs and has already been successfully used to study the
890 mitochondrial proteome (Cogliati et al., 2016; Glytsou et al., 2016; Quintana-Cabrera et al., 2018).
891 DiS uses narrow MS/MS windows of 2 m/z, typical of data-dependent acquisition methods,
892 allowing direct peptide identification by database searching and FDR control by using a
893 conventional target/decoy competition strategy, without requiring peptide fragmentation libraries.
894 The Blue-DiS workflow generated a permanent, multi-dimensional, high-resolution time-
895 fragment mass map for all possible precursors present in each BNGE fraction and each
896 mitochondrial sample, from which quantitative protein maps can be straightforwardly obtained
897 with minimal computation. BNGE gels were excised in 26 slices taking as reference some discrete
898 Coomassie stained bands: slice 6 corresponds to a band that mainly contains SC I + III₂, slice 10
899 to CV, slice 12 to free CIII₂, and slice 15 to free CIV. All slices were cut into cubes (2x2 mm),
900 reduced with 10mM DTT (GE Healthcare), alkylated with 55mM iodoacetamide (Sigma-Aldrich)
901 and subjected to a standard overnight in-gel digestion at 37°C with 3 µg of sequencing grade
902 trypsin (Promega, Madison, WI, USA) in 100 mM ammonium bicarbonate, pH 7.8. After
903 desalting with C18 Omix cartridges (Agilent Technologies), the resulting tryptic peptide mixtures
904 were injected onto a C-18 reversed phase (RP) nano-column (75µm I.D. and 50 cm, Acclaim
905 PepMap, Thermo Fisher, San José, CA, USA) using an EASY-nLC 1000 liquid chromatography
906 system (Thermo Fisher, San José, CA, USA) and analysed in a continuous gradient consisting of
907 8–31% B for 130 min, 50–90% B for 1 min (B= 0.5% formic acid in acetonitrile). Peptides were
908 eluted from the RP nanocolumn at a flow rate of ~200nl min⁻¹ to an emitter nanospray needle for
909 real-time ionization and peptide fragmentation in either a Q-Exactive or a Q-Exactive HF mass
910 spectrometer (Thermo Fisher). Each sample was analysed in two chromatographic runs covering
911 different mass ranges (from 400 to 750 Da, and from 750 to 1,100 Da, respectively). The DiS
912 cycle consisted of 175 sequential HCD MS/MS fragmentation events with 2-Da windows that
913 covered the whole 350 Da range. HCD fragmentation was performed at 30 normalized collisional
914 energy, a resolution of 17,500 and a maximum injection time of 80 ms with the AGC set to a

915 target of 3×10^5 ions. The whole cycle lasted 30 s or less depending on ion intensity during
916 chromatography. Peptide identification was performed using Sequest running under Proteome
917 Discoverer 1.4 (Thermo Fisher Scientific), allowing two missed cleavages, and using 2 Da and
918 20 p.p.m. precursor and fragment mass tolerances, respectively. Met oxidation and Cys
919 carbamydomethylation were selected as dynamic and static modifications, respectively. FDR for
920 peptide identification was controlled using a separate inverted database and the refined
921 method(Navarro and Vázquez, 2009). Visualization, validation and quantification of MS/MS
922 spectra from specific peptides was performed using Vseq script, as described(Cogliati et al.,
923 2016).

924 ***Protein and complex profiling.*** Quantitative protein migration profiles from Blue-DiS analyses
925 were obtained by spectral counting, summing up the number of peptide-spectrum matches (PSMs)
926 of all peptides identified for each protein on each slice of the gel. In the case of protein complexes,
927 the total PSMs of the proteins contained in the complex was used for quantification. The
928 normalized BNGE profile of each complex was constructed by calculating the slope from the plot
929 of protein PSMs in a given slice versus protein PSMs in a slice used as reference (the one with
930 the highest number of PSMs). The normalized abundance of each protein within a complex was
931 calculated as the slope from the plot of PSMs of the protein in the different slices versus the PSMs
932 of the most abundant protein from the complex in the same slices.

933 ***Determination of stoichiometry between complexes in SC.*** We observed that the number of
934 PSMs for each protein, as calculated by DiS, was approximately proportional to the number of
935 tryptic peptides detectable by MS (NOP) (ED Fig. 3C, left). This finding agrees well with the use
936 of the “protein abundance index” by other authors, which normalizes spectral counts by the
937 number of detectable peptides(Ishihama, 2005). This consideration allowed us to calibrate the
938 individual MS response of each protein, generating an estimation of the effective NOP (ED Fig.
939 3C, right). By plotting, per each complex, the PSMs of the proteins against their effective NOP,
940 we could estimate from the slopes the molar stoichiometries of complexes within each slice (ED
941 Fig. 3d).

942 ***Activity of complexes or respirasomes from BNGE eluted bands.*** CD1, BL6:S¹¹¹ and BL6:S¹¹³
943 heart mitochondria were extracted, processed and run in BN-PAGE as described above. CI, CIII₂,
944 CIV-monomer or respirasome bands were quickly excised from gels and minced on ice. The grist
945 was immediately resuspended in Medium MAITE and respirasomes eluted by twirling for 4 hours
946 at 4°C. Elution was collected and mixed with the appropriate volume of MAITE + 2.5 mg/ml
947 BSA at 37 °C to reach 1 mL. Reaction was started by adding 100 μM NADH, 130 μM CoQ₁,
948 reduced DQ 130 μM, oxidized Cyt c 100 μM. NADH, oxidized CoQ₁ and Cyt c levels were
949 tracked by recording absorbance at 340 nm, 289 nm and 550 nm, respectively for 240 sec in a
950 UV/VISJASCO spectrophotometer. Optimal absorbance values were calculated by titration of
951 each reactive in MAITE + 2.5 mg/ml BSA. DQ maximal absorbance peak could not be estimated
952 due to the consequent turbidity of its dissolution in an aqueous environment. Baseline NADH
953 oxidation and CoQ₁ reduction from the same elution was recorded after addition of 1 μM
954 rotenone. Baseline Cyt c reduction from the same elution was recorded after addition of 2.5 μM
955 antimycin A. Baseline Cyt c oxidation from the same elution was recorded after addition of 1 mM
956 KCN.

957 ***Oxygen consumption by BNGE respirasome bands.*** CD1, BL6:S¹¹¹ and BL6:S¹¹³ heart
958 mitochondria were extracted, processed and run in BN-PAGE as described above. CIV-monomer
959 or respirasome bands were quickly excised from gels and minced on ice. The grist was
960 immediately resuspended in MAITE + 2.5 mg/ml BSA at 37°C and introduced in an Oxytherm
961 System S1/MINI. Reaction was started by adding 100 μM reduced Cyt c for CIV-monomer or
962 100 μM NADH for respirasomes. Oxygen levels were tracked for at least 180 seconds and
963 baseline oxygen consumption was recorded after addition of 1 mM KCN for CIV-monomer or 1
964 μM rotenone for respirasomes.

965 ***H₂O₂ production by respirasomes from BNGE eluted bands.*** CD1, BL6:S¹¹¹ and BL6:S¹¹³ heart
966 mitochondria were extracted, processed and run in BN-PAGE as described above. Respirasome
967 bands were quickly excised from gels and minced on ice. The grist was immediately resuspended
968 in Medium MAITE and respirasomes eluted by twirling for 4 hours at 4°C. Elution was collected

969 and mixed with Amplex Red solution (Molecular probes), following manufacturer's instructions.
970 Reaction was started by adding 100 μ M NADH or 100 μ M CoQH₂+1 μ M rotenone. Amplex Red
971 levels were tracked by recording fluorescence at 540/590 nm (exc/emm) for 2400 sec in a
972 Fluoroskan Ascent fluorimeter (Thermo LabSystems). Baseline Amplex Red fluorescence from
973 the same elution was recorded after addition of 5U/mL of SOD and Catalase.

974 ***NADH oxidation monitoring in intact mitochondria by autofluorescence.*** AOX expressing mice
975 heart mitochondria were extracted as described above and immediately resuspended in medium
976 MAITE at 4°C. Mitochondria were mixed with the appropriate volume of MAITE+ 2.5 mg/ml
977 BSA at 37 °C to reach 100 μ l. Reaction was started by adding 5 mM Glu + 5 mM Mal \pm 1 mM
978 KCN, 5 mM SHAM or \pm 1 μ M rotenone. NADH levels were tracked by recording
979 autofluorescence at excitation/emission of 340/475 nm for 20 min in a Fluoroskan Ascent
980 fluorimeter (Thermo LabSystems)

981 ***Oxygen consumption measurement.*** AOX expressing heart mitochondria of mitochondria from
982 AOX expressing cell lines were extracted as described above and immediately resuspended in
983 medium MAITE at 4°C. To permeabilize mitochondrial membranes, mitochondria were subjected
984 to a freeze-thaw step. Intact or freeze-thawed mitochondria were mixed with the appropriate
985 volume of MAITE + 2.5 mg/ml BSA at 37 °C and placed in an Oxytherm System S1/MINI.
986 Reaction was started by adding 5 mM Glu + 5 mM Mal \pm 10 mM succinate \pm 1 mM KCN, 5 mM
987 SHAM or \pm 1 μ M rotenone. Permeabilized mitochondria were provided with 100 μ M NADH or
988 100 μ M CoQ₁H₂. Baseline levels were recorded after addition of 1 μ M rotenone or 5 mM SHAM.

989 ***Activity of complexes in freeze-thaw mitochondria.*** AOX expressing heart mitochondria of
990 mitochondria from AOX expressing cell lines were extracted as described above. Mitochondria
991 were immediately resuspended in medium MAITE and subjected to a freeze-thaw step. Freeze-
992 thaw mitochondria were mixed with the appropriate volume of MAITE + 2.5 mg/ml BSA at 37
993 °C to reach 1 mL. Reaction was started by adding 100 μ M NADH and in some cases 1 mM
994 Fe(CN)₆, 1 μ M rotenone + 1 μ M antimycin A, 100 μ M succinate or 1 mM KCN was added to the

995 reaction mixture. Since the accessibility of CoQ-analogs could be differential across the different
996 AOX-cell models (i.e. with or without supercomplexes), we performed DPI-sensitive
997 NADH:Fe(CN)₆ activity (i.e. an activity for FMN in CI) which allowed us to quantify CI-content.
998 NADH and Fe(CN)₆ levels were tracked by recording absorbance at 340 nm and 412 nm,
999 respectively for 240 sec in a UV/VISJASCO spectrophotometer. Baseline NADH consumption
1000 from the same sample was recorded after addition of 1 μM rotenone. Baseline Fe(CN)₆
1001 absorbance from the same sample was recorded after addition of 5 μM diphenyliodonium (DPI).

1002 **QUANTIFICATION AND STATISTICAL ANALYSIS**

1003 *Statistical analysis.* Unless specified, statistical analyses and graphics were produced with
1004 GraphPad Prism 8 software. Data sets were compared by t-test, ANOVA or non-parametric
1005 analysis when corresponded and with p-values adjusted for multiple test. Differences were
1006 considered statistically significant at P values below 0.05. **p-value* < 0.05; ** *p-value* < 0.01;
1007 *** *p-value* < 0.001; **** *p-value* < 0.0001. All results are presented as mean ± SD or mean ±
1008 SEM.

1009 **DATA AND CODE AVAILABILITY**

1010 Some of the datasets supporting the current study have not been deposited in a public repository
1011 yet, but will be done when possible, meanwhile they are available from the corresponding author
1012 on request.

1013

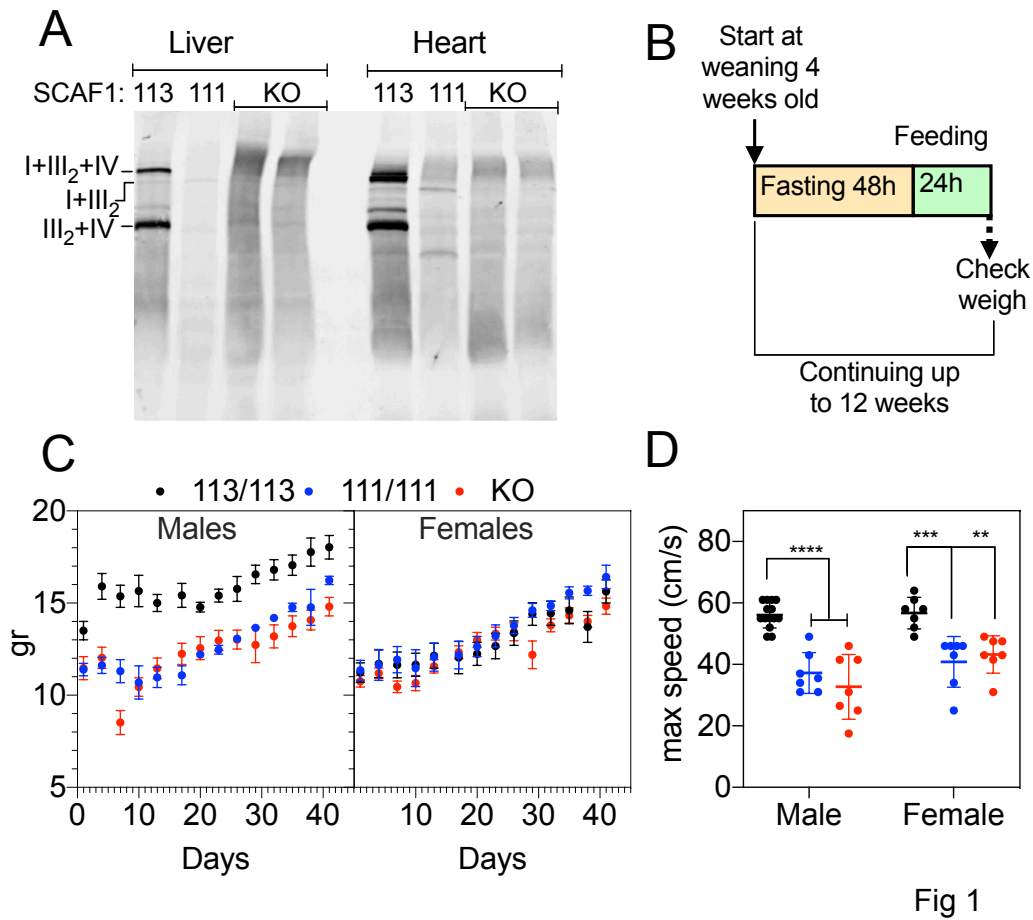
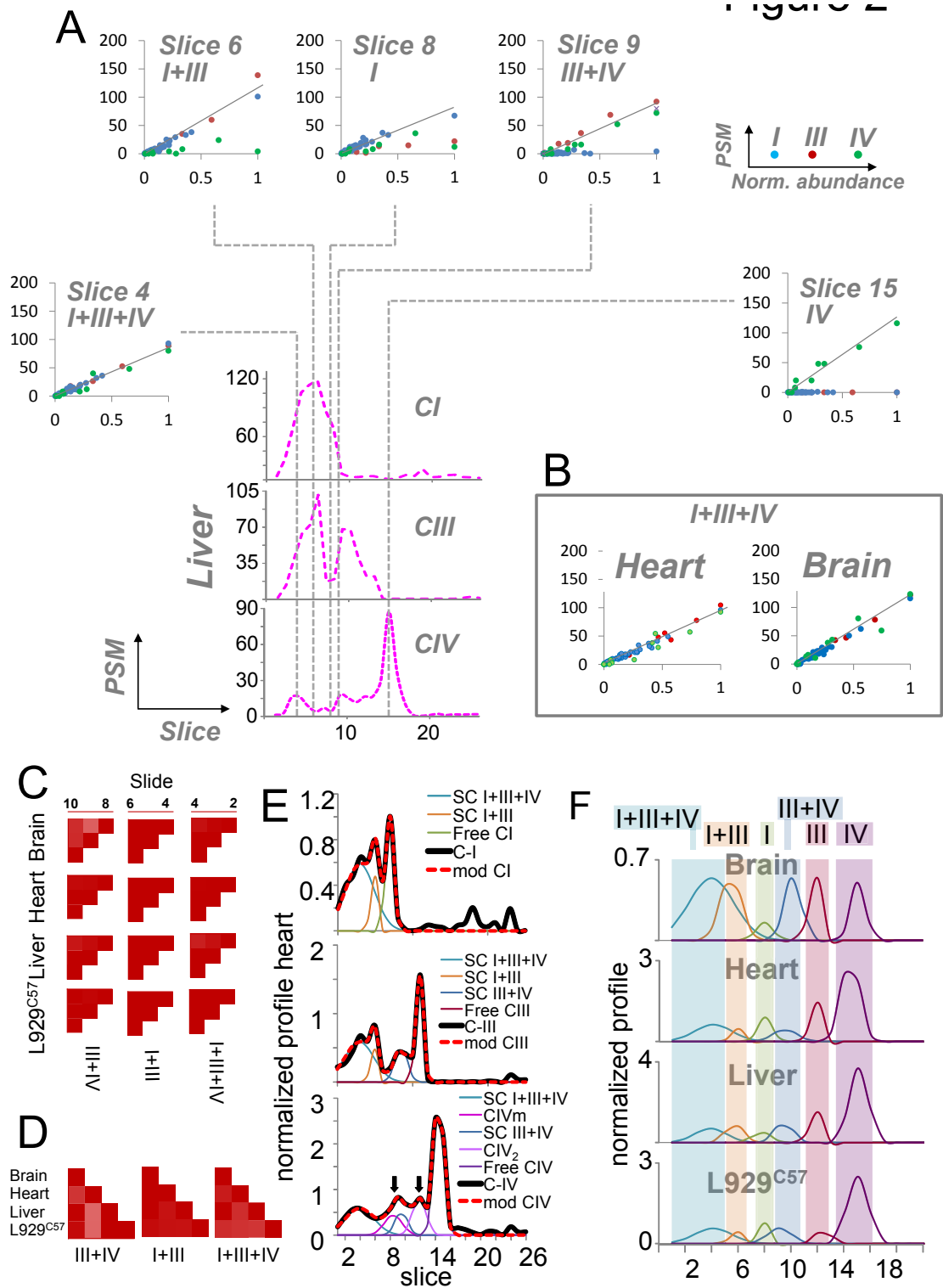


Fig 1

1014

1015 **Figure 1. SCAF1 deficiency induces a conspicuous phenotype in mice.**

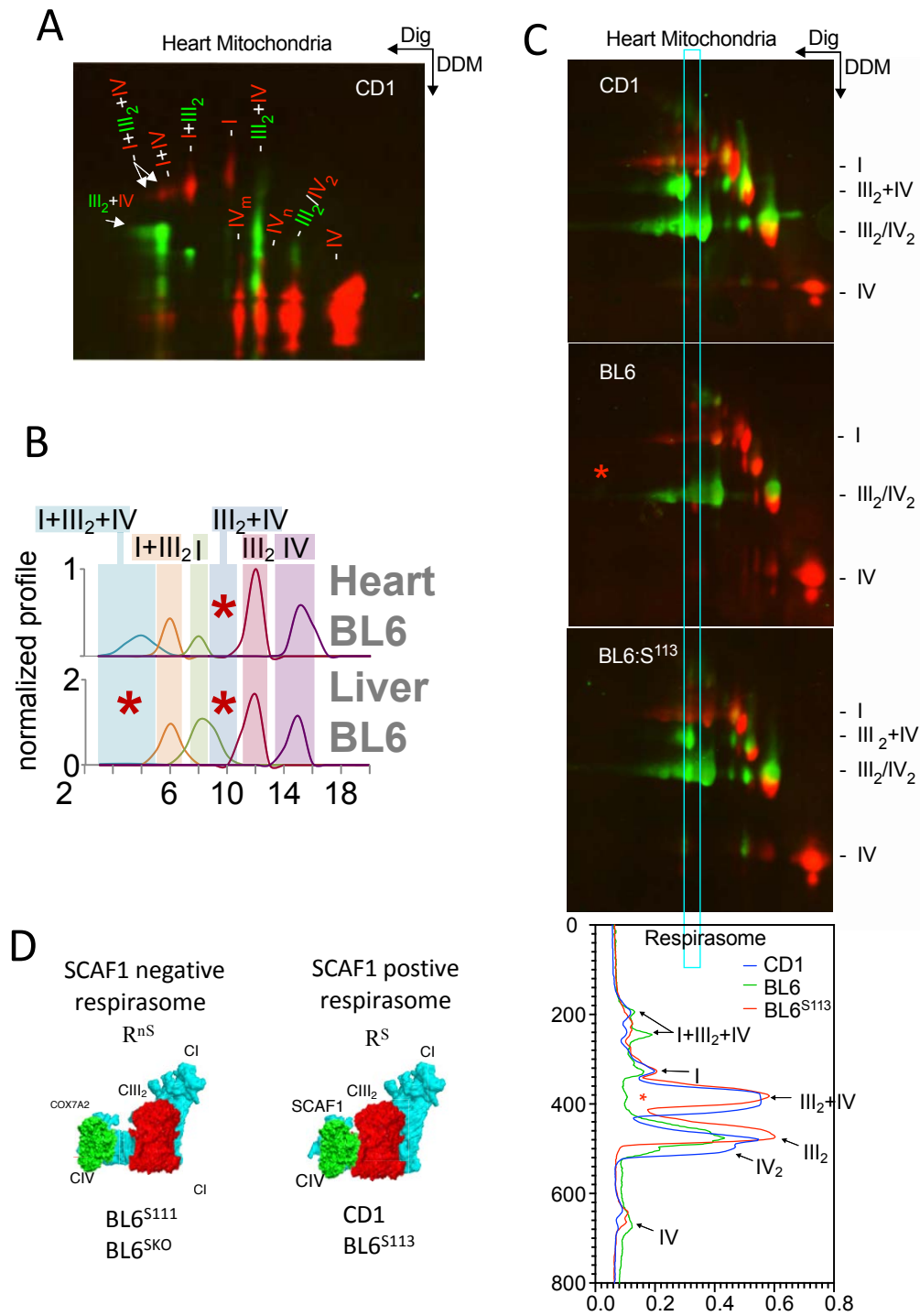
1016



1017

1018 **Figure 2. Blue-DiS evidence of the formation of OXPHOS supercomplexes.**

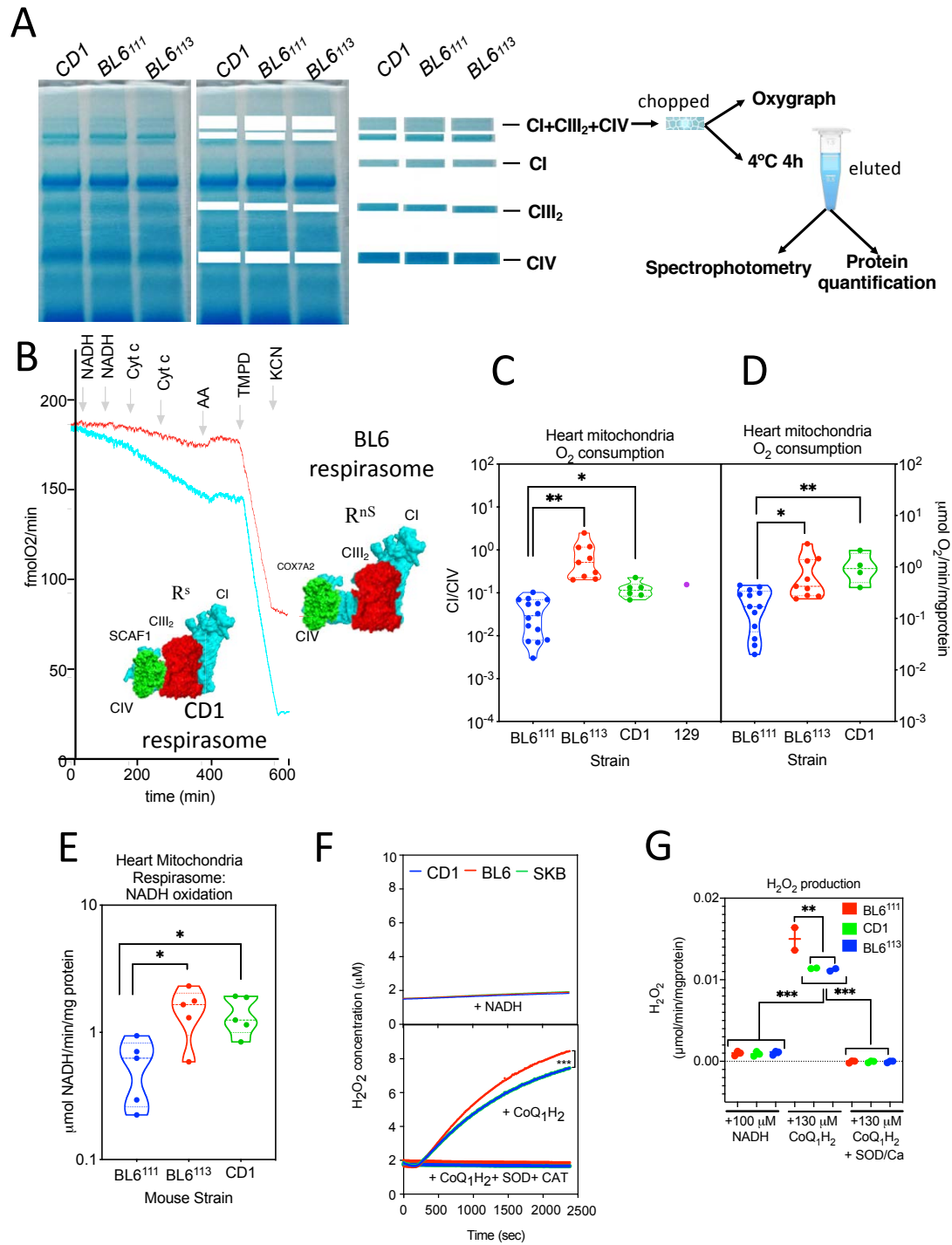
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1020

1021 **Figure 3. Structural consequences of SCAF1-deficiency in the formation of SCs.**

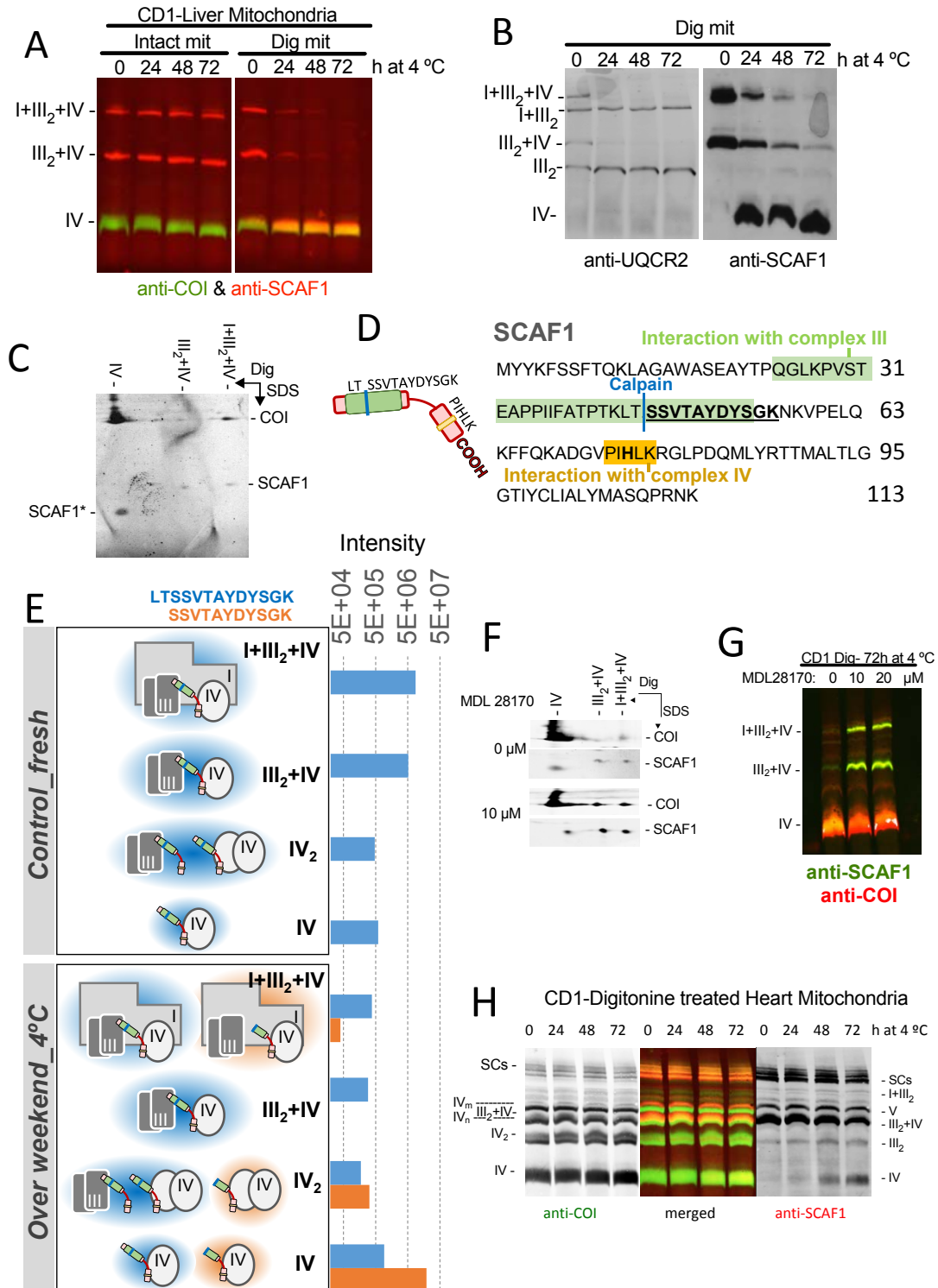
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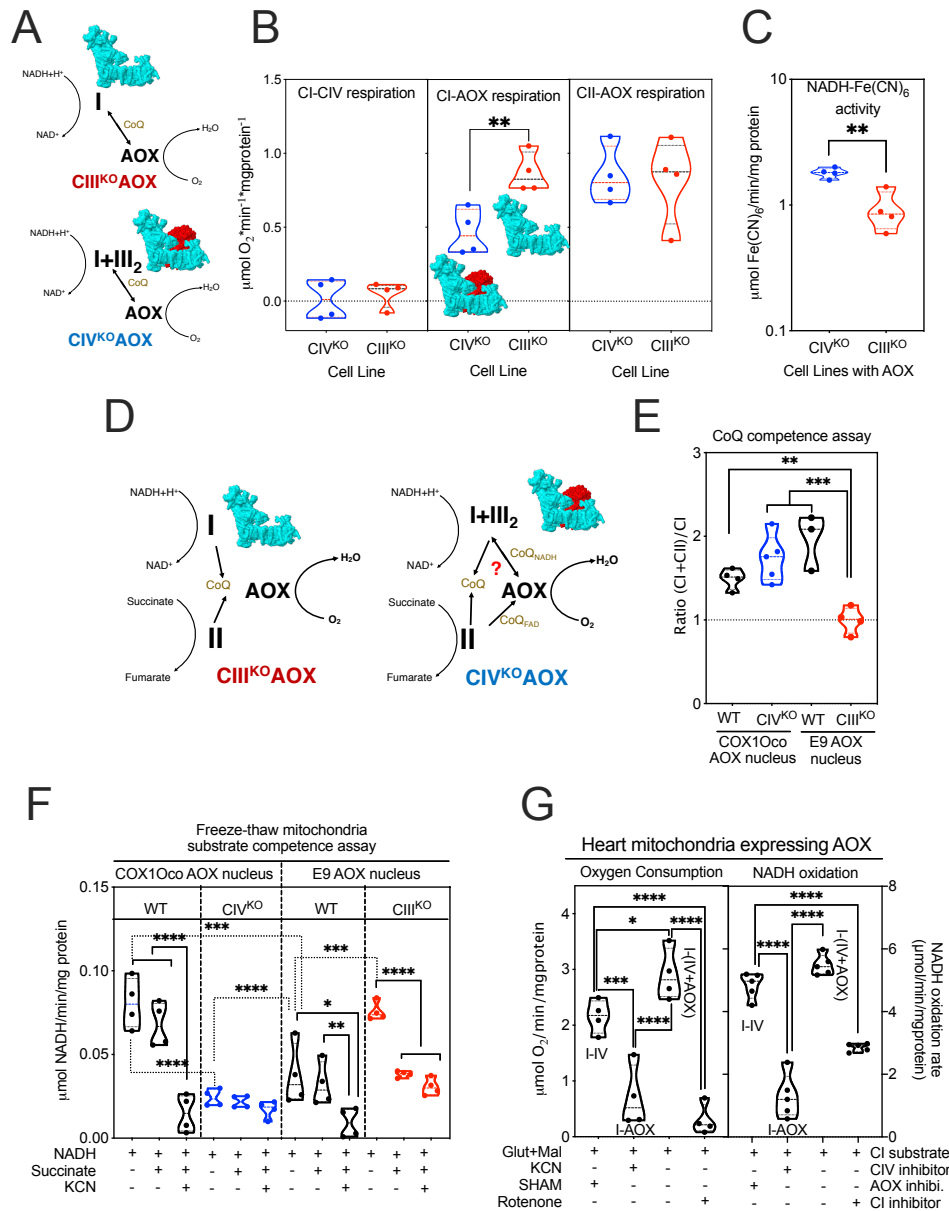
1024 **Figure 4. Functional consequences of SCAF1-deficiency in the activity of the respirasome.**

1025



1026

1027 **Figure 5. Supercomplexes are unstable upon mitochondrial membrane disruption.**



1028

1029 **Figure 6. The superassembly between complexes I and III modulates the activity of CI and**
 1030 **functionally segments the CoQ pool.**