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1 **Impact of mutations within the [Fe-S] cluster or the lipoic acid biosynthesis pathways**
2 **on mitochondrial protein expression profiles in fibroblasts from patients.**

3

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24 **Abstract:**

25 Lipoic acid (LA) is the cofactor of the E2 subunit of mitochondrial ketoacid dehydrogenases
26 and plays a major role in oxidative decarboxylation. *De novo* LA biosynthesis is dependent
27 on LIAS activity together with LIPT1 and LIPT2. LIAS is an iron-sulfur (Fe-S) cluster-
28 containing mitochondrial protein, like mitochondrial aconitase (mt-aco) and some subunits of
29 respiratory chain (RC) complexes I, II and III. All of them harbor at least one [Fe-S] cluster
30 and their activity is dependent on the mitochondrial [Fe-S] cluster (ISC) assembly machinery.
31 Disorders in the ISC machinery affect numerous Fe-S proteins and lead to a heterogeneous
32 group of diseases with a wide variety of clinical symptoms and combined enzymatic defects.
33 Here, we present the biochemical profiles of several key mitochondrial [Fe-S]-containing
34 proteins in fibroblasts from 13 patients carrying mutations in genes encoding proteins
35 involved in either the lipoic acid (*LIPT1* and *LIPT2*) or mitochondrial ISC biogenesis (*FDX1L*,
36 *ISCA2*, *IBA57*, *NFU1*, *BOLA3*) pathway. Ten of them are new patients described for the first
37 time. We confirm that the fibroblast is a good cellular model to study these deficiencies,
38 except for patients presenting mutations in *FDX1L* and a muscular clinical phenotype. We
39 find that oxidative phosphorylation can be affected by LA defects in *LIPT1* and *LIPT2*

40 patients due to excessive oxidative stress or to another mechanism connecting LA and
41 respiratory chain activity. We confirm that NFU1, BOLA3, ISCA2 and IBA57 operate in the
42 maturation of [4Fe-4S] clusters and not in [2Fe-2S] protein maturation. Our work suggests a
43 functional difference between IBA57 and other proteins involved in maturation of [Fe-S]
44 proteins. IBA57 seems to require BOLA3, NFU1 and ISCA2 for its stability and NFU1
45 requires BOLA3. Finally, our study establishes different biochemical profiles for patients
46 according to their mutated protein.

47 **Introduction**

48 Lipoic acid (LA) is an important antioxidant used in human therapeutics [1, 2]. It is also
49 found as a covalent cofactor of the H subunit of the glycine cleavage system (GCS) and of
50 E2 subunits of mitochondrial ketoacid dehydrogenase complexes, namely pyruvate
51 dehydrogenase (PDHc), α -ketoglutarate dehydrogenase (α -KGDHc), branched-chain
52 ketoacid dehydrogenase (BCKDHc) and ketoadipate dehydrogenase (KADHc). Starting from
53 malonyl-coenzyme A, mammalian mitochondrial fatty acid synthesis type II enables the
54 modification of the acyl-carrier protein by a covalently linked octanoic acid. Then, the
55 octanoic acid is transferred to the H subunit of GCS with the help of the lipoyltransferase
56 LIPT2, and two thiolate groups are added to form LA. The next step is performed by the
57 lipoic acid synthase (LIAS), a [4Fe-4S] cluster-containing protein. Finally, LA is transferred by
58 LIPT1 to target E2 subunits of ketoacid dehydrogenase complexes [3].

59 LIAS, a key protein of the LA synthesis pathway, coordinates two [4Fe-4S] clusters,
60 which are critical for its activity [4]. The [Fe-S] cluster is a widespread prosthetic group found
61 in proteins localized in most mammalian organelles, including mitochondria, cytosol and
62 nucleus, but also in all the kingdoms of life. Their numerous functions vary from electron
63 transfer to sulfur donor or gene expression regulation [5]. They are composed only of iron
64 and inorganic sulfur and are found with different nuclearities. The two major [Fe-S] clusters
65 are the [2Fe-2S] and [4Fe-4S] clusters. In many cases, the [Fe-S] cluster is necessary for
66 enzymatic activity, as for LIAS [6]. Moreover, several subunits of respiratory chain (RC)
67 complexes as complex I (eight subunits), complex II (SDHB subunit) and complex III
68 (UQCRFS1 or Rieske protein) also assemble [Fe-S] clusters, which are essential for
69 OXPHOS electron transfer. Furthermore, ferrochelatase, a mitochondrial enzyme involved in
70 the heme synthesis pathway, carries a [2Fe-2S] cluster [7], and mitochondrial aconitase (mt-
71 aco), an enzyme of the tricarboxylic acid cycle, carries a [4Fe-4S] cluster [8].

72 The biogenesis of all mammalian [Fe-S] clusters starts in the mitochondrion,
73 independently of the subcellular localization of the [Fe-S] protein (Figure 1). Using inorganic
74 sulfur provided by the cysteine desulfurase NFS1 complexed to ISD11 and ferrous ions, a
75 [2Fe-2S] cluster is built on the scaffold protein ISCU with the help of ferredoxin (FDX1L) and
76 frataxin (FXN). In a second step, the [2Fe-2S] cluster is transferred from ISCU to the
77 glutaredoxin (GLRX5) with the help of the chaperone proteins HSPA9 and HSC20 [9].
78 Finally, the cluster is either transferred to target mitochondrial proteins or exported to the
79 cytosol for the maturation of extra-mitochondrial proteins through the iron-sulfur exporter
80 system. However, the exact nature of the exported component (ISC or sulfur) remains
81 unknown [5]. This last step of the maturation of mitochondrial [Fe-S] proteins involves
82 transfer factors, including IBA57, BOLA3, NFU1 and ISCA2 [10]. The specific roles of these
83 proteins are still debated. It is proposed that all of them are required only for the maturation
84 of [4Fe-4S]-containing mitochondrial proteins [11], with NFU1 and BOLA3 being more
85 specifically involved in the specific maturation of LIAS, mt-aco and SDHB [8, 9].

86 Since the first report of Cameron et al. in 2011 [12], various multiple mitochondrial
87 dysfunctions syndromes (MMDS) have been reported involving mutations in genes encoding
88 proteins involved in the Fe-S cluster biogenesis pathway, namely MMDS1 (NFU1), MMDS2
89 (BOLA3), MMDS3 (IBA57) and MMDS4 (ISCA2) [3, 12–17], or in the lipoic acid synthesis
90 pathway (LIAS, LIPT1, and LIPT2) [16, 18–21]. Recently, a new MMDS was described with
91 mutations in *ISCA1* [22]. The clinical phenotypes of all these patients are very similar to
92 those frequently observed in disorders concerning mitochondrial energy metabolism. In
93 general, the presenting symptoms occur during the neonatal period or infancy. Common
94 clinical presentations are neurological symptoms, including hypotonia, leukoencephalopathy,
95 and psychomotor regression, but extraneurological symptoms have also been reported [23].
96 Biochemical studies show low PDHc enzymatic activity, but defects in RC complex activities
97 are variably observed.

98 In this report, we describe 13 patients carrying mutations in genes encoding proteins
99 involved either in lipoic acid synthesis (*LIPT1* and *LIPT2*) or in mitochondrial [Fe-S] cluster
100 biogenesis (*NFU1*, *BOLA3*, *IBA57*, *ISCA2*, *FDX1L*). Ten of these patients are described here
101 for the first time. Using their cultured skin fibroblasts, we established biochemical profiles
102 (protein level and enzymatic activity) of several key mitochondrial proteins carrying either a
103 [Fe-S] cluster or bound to lipoic acid. Since the development of next-generation sequencing
104 technologies, molecular diagnosis of rare diseases is accelerating, but biochemical data are
105 needed to improve our knowledge of these proteins and to help the formulation of new
106 therapeutic approaches for present and future patients.

107

108 **1. Materials and methods**

109 **a) Patients and cell culture**

110 Thirteen patients with mutations in genes encoding proteins involved in either [Fe-S]
111 cluster biogenesis or the LA synthesis pathway were included. The genotype of each patient
112 is described in Table 1, patients P1, P2 and P8 having already been reported [19–21, 24].
113 The integrated software ALAMUT V.2.7 (Interactive Biosoftware; [http://www.interactive-](http://www.interactive-biosoftware.com)
114 [biosoftware.com](http://www.interactive-biosoftware.com)) was used for *in silico* analysis of missplicing effects. No novel mutations
115 were reported as SNP variants in Exome Variant Server, NHLBI GO Exome Sequencing
116 Project (ESP), Seattle, WA (<http://evs.gs.washington.edu/EVS/>) [March 2017 accessed].
117 Cultured skin fibroblasts from these patients and from individuals without mitochondrial
118 disease (controls) were grown in HAM'S F-10 medium (Eurobio, Les Ulis, France)
119 supplemented with antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin), 50 mg/L
120 uridine and 12% fetal bovine serum. Cells were cultured at 37°C in a humidified incubator
121 with 5% CO₂. Clinical and biochemical data and brain magnetic resonance imaging (MRI)

122 findings were collected retrospectively. Informed consent was obtained from all patients
123 before inclusion in the study.

124 **b) Biochemical studies**

125 All biochemical analyses were performed on cultured skin fibroblasts. PDHc activity was
126 measured as described [25]. RC complexes, α -KGDHc and citrate synthase activities were
127 measured by spectrophotometric assay [26]. Assays of complex I and of mt-aco activity were
128 performed on mitochondria-enriched fractions prepared from permeabilized cells with
129 digitonin, as described previously [27, 28]. RC complex and mt-aco activities were
130 normalized to citrate synthase activity. Glutathione measurement was carried out on total
131 fibroblast lysates [29].

132 **c) Western blot and BN-PAGE analyses**

133 Western blot analysis was performed on mitochondria-enriched fractions or on total fibroblast
134 lysates. About 30 μ g of protein extracts was separated by SDS-PAGE and transferred to
135 PVDF membranes, blocked in PBS-Tween containing 5% non-fat milk and then incubated
136 overnight with primary antibodies. Antibodies against lipoic acid (LA) (Abcam, ab18724),
137 PDH-E2 subunit (SantaCruz Biotechnology, sc-271352), NFU1 (kindly provided by Dr. T.
138 Rouault, NICHD, NIH, Bethesda, USA), IBA57 (kindly provided by Prof. R. Lill, Marburg,
139 Germany), SDHB (Abcam, ab14714), Rieske protein (Abcam, ab14746), LIAS
140 (ThermoFisher Scientific, PA5-22387), ferrochelatase (kindly provided by Dr. H. Puccio,
141 Strasbourg, France), mt-aconitase (kindly provided by Dr. R. B. Franklin, University of
142 Maryland, Baltimore, USA) and prohibitin (Novusbio, NBP2-37563) were used. Secondary
143 peroxidase-conjugated anti-mouse (Sigma-Aldrich, A4416) and anti-rabbit (Sigma-Aldrich,
144 NA934V) antibodies were used. Detection was achieved using the ECL chemiluminescence
145 kit (GE Healthcare) and G-Box (Sysgene) associated with GeneSNAP software. For some
146 experiments, goat anti-rabbit or anti-mouse IRDye® 800-conjugated secondary antibodies

147 (LI-COR, Lincoln, USA) were used. Membranes were scanned with the Odyssey infrared
148 imaging system and images were processed with the Image Studio Software (LI-COR,
149 Lincoln, USA).

150 Blue-native PAGE (BN-PAGE) analysis was carried out on mitochondria-enriched fractions
151 from fibroblasts [30]. Immunoblotting was performed using antibodies raised against the
152 complex I subunit GRIM19 (Abcam, ab10240), the 70kDa subunit of complex II (SDHA,
153 Abcam, ab14715) and the complex III subunit core2 (UQCRC2, Abcam, ab14745).

154

155 **2. Results**

156 *a) Patients*

157 Thirteen children from 12 unrelated families were included in our study. Patients P3 and P4
158 were first cousins. The clinical, MRI and biochemical data are summarized in Table 2:.
159 Clinical presentations were mostly severe encephalopathy features with hypotonia,
160 psychomotor delay since birth (n=2/13) or neurological regression (n=7/13). Epilepsy was
161 noted in five patients. Pulmonary arterial hypertension was also reported in two of the four
162 (P6 and P8) *NFU1* patients. The only patient with normal neurological development was an
163 *FDX1L*-deficient patient (P13), who presents recurrent acute episodes of rhabdomyolysis
164 and muscular weakness. Onset began between four and five years of age. In most patients,
165 first symptoms began during the first days or months of life. All the patients with *BOLA3* (P3
166 and P4), *NFU1* (P5 to P8), *IBA57* (P9 and P10) mutations and one *ISCA2* patient (P12) died
167 before 3 years of age. The second *ISCA2* patient (P11) has a mild disease with isolated and
168 non-progressive spastic paraplegia at 12 years of age. White matter abnormalities were
169 usually found in patients with encephalopathy. Magnetic resonance spectroscopy showed a
170 lactate peak in three (*LIPT1*, P1; *LIPT2*, P2; *NFU1*, P7) of the six tested patients (P1, P2, P5,
171 P7, P8 and P13).

Patient	Gene	Reference sequence	Allele 1			Allele 2		
			Nucleotide change	Protein change	Location	Nucleotide change	Protein change	Location
P1 [§]	<i>LIPT1</i>	NM_145199	c.535A>G	p.Thr179Ala	Exon 2	c.875C>G	p.Ser292*	Exon 2
P2 [§]	<i>LIPT2</i>	NM_001144869	c.89T>C	p.Leu30Pro	Exon 1	c.377T>G	p.Leu126Arg	Exon 1
P3	<i>BOLA3</i>	NM_212552	c.136C>T	p.Arg46*	Exon 2	c.136C>T	p.Arg46*	Exon 2
P4	<i>BOLA3</i>		c.136C>T	p.Arg46*	Exon 2	c.136C>T	p.Arg46*	Exon 2
P5	<i>NFU1</i>	NM_001002755	c.146del	p.Pro49Leufs*8	Exon 3	c.303-1988_c.369+1021del	p. ?	Intron 3 to intron 4
P6	<i>NFU1</i>		c.622G>T	p.Gly208Cys	Exon 7	c.629G>T	p.Cys210Phe	Exon 7
P7	<i>NFU1</i>		c.179G>T	p.Phe60Cys	Exon 3	c.179G>T	p.Phe60Cys	Exon 3
P8 [§]	<i>NFU1</i>		c.565G>A	p.Gly189Arg	Exon 7	c.622G>T	p.Gly208Cys	Exon 7
P9	<i>IBA57</i>	NM_001010867	c.335T>G	p.Leu112Trp	Exon 1	c.437G>C	p.Arg146Pro	Exon 2
P10	<i>IBA57</i>		c.316A>G	p.Thr106Ala	Exon 1	c.738C>G	p.Asn246Lys	Exon 3
P11	<i>ISCA2</i>	NM_194279	c.154C>T	p.Leu52Phe	Exon 2	c.154C>T	p.Leu52Phe	Exon 2
P12	<i>ISCA2</i>		c.313A>G	p.Arg105Gly	Exon 4	c.313A>G	p.Arg105Gly	Exon 4
P13	<i>FDX1L</i>	NM_001031734	c.1A>T	p.Met1?	Exon 1	c.1A>T	p.Met1?	Exon 1

Table 1: Mutational spectrum of the thirteen patients.

Mutations in bold are described for the first time in this publication. [§] P1, P2 and P8 were already reported [19–21, 24]. Nomenclature of variants is in accordance with the recommendations of the Human Genome Variation Society [31].

Patient	Gene	Gender	Age at onset/ Age at death	Clinical presentation	Brain MRI	Lactate (mmol/L)		Glycine (µmol/L)		Other
						Plasma (RV <2)	CSF (RV<1.8)	Plasma (RV<300)	CSF (RV<16)	
P1	<i>LIPT1</i>	Male	15 months/ alive at 8 years	PMD, hypotonia, tetraparesis. Steady aortic root dilatation [19].	Cortical atrophy, delayed myelination. MRS: peak of lactate during decompensation.	8 at onset N at 8 years	6.6	<300	<16	increase of 2 keto-glutarate (urine)
P2	<i>LIPT2</i>	Male	Neonatal period/ alive at 7 years	PMD, hypotonia, microcephaly, epilepsy [20, 21].	Cortical atrophy, delayed myelination. MRS: peak of lactate.	4.5	<1.8	419	ND	
P3	<i>BOLA3</i>	Male	5 months/ 8 months	Neurological degradation, tetraparesis.	Diffuse demyelination.	3.2	2.4	600	43	increase of pyruvate (blood) and glycine (urine)
P4	<i>BOLA3</i>	Male	7 months/ 8 months	Hypotonia, pyramidal syndrome.	Diffuse demyelination. Leigh syndrome.	5.4	3.9	922	16	
P5	<i>NFU1</i>	Male	3 months/ 4 months	Hypotonia, seizures, apnea.	White matter abnormalities. MRS: no lactate peak.	3.6	<1.8	374	15 (RV 4-11)	increase of glycine and fumaric acid (urine)
P6	<i>NFU1</i>	Female	3.5 months/ 4 months	PMD, hypotonia, seizures, acute heart failure, PAHT.	No abnormalities.	>2	ND	>300	ND	increase of 2-amino-adipate (plasma) increase of 2 keto-adipate (urine)
P7	<i>NFU1</i>	Male	7 months/ <3 years	Progressive neurological degradation. PMD, spasticity and epileptoid trepidation.	Necrotizing leukodystrophy. MRS: lactate peak.	>2	ND	ND	ND	
P8	<i>NFU1</i>	Female	5 months/ 3 years	PMR, PAHT, hypotonia, tetraparesis, seizures [24].	Kystic leukoencephalopathy. MRS: no lactate peak.	2.7 (acute period)	<1.8	1178	20	
P9	<i>IBA57</i>	Male	8 months/ 11 months	PMR, hypotonia, episodes of general hypertonia, pyramidal syndrome.	Periventricular leukodystrophy.	7.7	1.9	<300	<16	
P10	<i>IBA57</i>	Female	3 weeks/ 2.5 months	Hypotonia, myoclonus, apnea.	Myelination delay.	2	5.5	ND	ND	
P11	<i>ISCA2</i>	Male	ND/Still alive at 12 years	Non-progressive spastic paraplegia.	White matter atrophy at 12 years.	<2	<1.8	ND	ND	
P12	<i>ISCA2</i>	Male	Neonatal/12 days	Hypotonia, apnea.	Leukodystrophy.	>2	5	ND	ND	
P13	<i>FDX1L</i>	Female	5 years/ Still alive at 18 years	Acute episodes of rhabdomyolysis, muscular weakness.	Normal	Up to 23 in acute episodes	3	<300	<16	increase of serum creatine kinase in acute episodes

Table 2: Clinical and biochemical features of the thirteen patients.

CSF, Cerebrospinal fluid; MRS, magnetic resonance spectroscopy; PAHT, pulmonary arterial hypertension; PMD, psychomotor delay; PMR, psychomotor regression; RV: reference values; ND, not determined.

Patient	Gene	Enzymatic activities (nmol/min/mg protein)			Relative mt-aco and respiratory chain complex activities					Glutathione (pmol/mg protein) (RV:7-21)
		PDHc	α -KGDHc	Citrate synthase (CS)	mt-aco/CS	Complex I/CS	Complex II/CS	Complex III/CS	Complex IV/CS	
		(RV:1.20-3.10)	(RV:3.7-7.1)	(RV:61-93)	(RV:0.40-0.59)	(RV:0.15-0.41)	(RV:0.20-0.32)	(RV:0.70-1.40)	(RV:1.00-1.50)	
P1	<i>LIPT1</i>	0.08	0.5	59	0.20	0.04	0.24	1.18	1.61	18
P2	<i>LIPT2</i>	0.28	0.5	58	0.33	0.07	0.30	2.28	1.05	11
P3	<i>BOLA3</i>	0.05	1.6	70	0.18	<0.01	0.08	0.82	0.93	13
P4		0.07	0.7	84	0.34	0.03	0.09	1.18	0.78	25
P5	<i>NFU1</i>	0.15	0.1	64	0.35	0.01	0.12	2.66	0.90	20
P6		0.04	0.6	64	0.13	0.03	0.02	1.69	0.85	11
P7		0.56	11.5	120	0.24	0.02	0.34	1.47	1.45	14
P8		0.35	<0.1	81	0.30	<0.01	0.06	0.86	1.38	9
P9	<i>IBA57</i>	0.39	2.3	81	0.44	0.04	0.15	1.48	1.0	17
P10		0.54	1.1	105	0.40	0.04	0.07	0.9	1.3	19
P11	<i>ISCA2</i>	0.95	2.8	66	0.33	0.05	0.21	2.67	2.03	20
P12		0.09	0.3	101	0.20	0.04	0.15	4.18	1.38	12
P13	<i>FDX1L</i>	0.95	8.4	89	0.46	0.09	0.33	1.48	1.43	19

Table 3: PDHc, α -KGDHc, mt-aco, respiratory chain complex activities and cellular glutathione concentration.

All the analyses were performed on cultured fibroblasts from the patients. Citrate synthase activity served as a reference for mitochondrial content in tested samples. mt-aco and RC complex activities were expressed relative to citrate synthase activity. Reduced glutathione measurement was determined in total lysates of fibroblasts. Gray shading indicates results in the normal value range. Other results were abnormal compared with reference values. CS, citrate synthase; RV, reference values.

168

169 *b) Biochemical results*

170 *Mitochondrial lipoylated proteins*

171 PDHc activity was decreased in fibroblasts of all patients (Table 3). It extended from 0.04 to
172 0.95 nmol/min/mg of protein, corresponding to 3% to 79% of the lowest reference value
173 (reference values: 1.20-3.10 nmol/min/mg of protein). α -KGDHc activity was decreased for all
174 patients except for one NFU1 patient (P7) and the FDX1L patient (P13). PDHc and α -KGDHc
175 activities were only moderately decreased in one ISCA2 patient (P11). We measured the level
176 of lipoylation of the E2 subunit of PDHc and α -KGDHc with an antibody against LA (Figure
177 2A). We found a severely altered lipoylation in all patients except for one NFU1 patient (P7),
178 one ISCA2 patient (P11) and the FDX1L patient (P13), with the E2 subunit of PDHc detectable
179 for all patients. This alteration of protein lipoylation is in accordance with decreased enzymatic
180 activity (Table 3).

181

182 *Mitochondrial Fe-S proteins*

183 We focused on two [4Fe-4S] proteins, LIAS and mt-aco, and two [2Fe-2S] proteins,
184 ferrochelatase and Rieske protein. LIAS activity is directly dependent on the assembly of its
185 [4Fe-4S] cluster, which is essential for incorporation of sulfur residues into octanoic acid. LIAS
186 protein was detectable for all patients and controls (Figure 2B). LIAS activity, which was
187 indirectly measured by the level of lipoylation of target proteins, was decreased in most
188 patients with defects in [Fe-S] biogenesis (Figure 2).

189 Then, we looked more closely at mt-aco, the stress-sensitive [4Fe-4S] cluster of which is also
190 crucial for its enzymatic activity and stability [28, 32]. Its activity was normalized to citrate
191 synthase activity to avoid bias due to between-sample variability in mitochondrial content
192 (Table 3). While mt-aco protein level was little affected (Figure 3), its activity was decreased
193 for all patients except for both IBA57 patients (P9 and P10) and the FDX1L patient (P13)
194 (Table 3). Thus, low mt-aco activity is a consequence of the alteration of its [4Fe-4S] cluster.

195 Given that glutathione (GSH) production is typically increased by severe oxidative stress [33],
196 we measured total reduced GSH in cell lysates of fibroblasts, but found no significant variation
197 between control and patient cells (Table 3).

198 Ferrochelatase assembles a [2Fe-2S] cluster, which is crucial for its stability and presents
199 some sensitivity to oxidative stress [32, 34]. We found that ferrochelatase level, and
200 consequently the presence of the cluster, was moderately reduced for patients 1, 9 and 11
201 only (Figure 4A).

202

203 *OXPHOS analysis*

204 Several subunits of RC complexes I, II and III carry [Fe-S] clusters that are essential to their
205 activities. We looked first at the activity of RC complexes relative to citrate synthase activity
206 (Table 3) and then at two [Fe-S] proteins of the RC complex, SDHB (complex II) (Figure 4C)
207 and Rieske protein (complex III) (Figure 4B). Finally, we checked the integrity of RC
208 complexes containing [Fe-S] clusters when activities were affected by BN-PAGE using
209 antibodies against subunits that do not coordinate an [Fe-S] cluster (Figure 5).

210 Complex I activity was decreased in all patients (Table 1) and its assembly was also clearly
211 affected in all of them except three patients, P1 (LIPT1 patient), P11 (ISCA2 patient) and P13
212 (FDX1L patient) (Figure 5). Complex II activity and integrity were normal in LIPT1 (P1) and
213 LIPT2 (P2) patients, in one NFU1 patient (P7) and in the FDX1L patient (P13) (Table 1 and
214 Figure 5). For these four patients, the amount of SDHB subunit was moderately decreased or
215 normal (Figure 4C). Concerning the other patients, complex II activity and assembly were
216 compromised for eight patients (the two BOLA3 patients, P3 and P4; three of the four NFU1
217 patients, P5, P6, P8; the two IBA57 patients, P9 and P10; one of the two ISCA2 patients, P12)
218 while one ISCA2 patient (P11) showed low normal activity of complex II and its assembly
219 seemed to be only moderately affected. For these nine patients, SDHB was undetectable
220 (Figure 4C). So, complex II activity and/or integrity seems to be affected by defects in NFU1,
221 BOLA3, IBA57 and ISCA2, proteins involved in the late step of mitochondrial [Fe-S] protein

222 maturation. Conversely, complex III activity was normal in all patients, as was the amount of
223 Rieske protein (Figure 4B). Complex IV activity was moderately affected by defects in BOLA3
224 or NFU1 (P3 to P6).

225 The biochemical profile of patient P7 with a mutation in *NFU1* differed from that of other
226 NFU1 patients. First, lipoylation of PDH-E2 and α -KGDH-E2 and α -KGDHc activity were
227 normal, while PDHc and mt-aco activities were clearly affected (Figure 3 and Table 3). RC
228 complex analysis showed only an effect on the activity and integrity of complex I (Table 3 and
229 Figure 5). These results contrasted with the findings concerning other NFU1 patients (P5, P6
230 and P8) and underscored the various biochemical profiles in the same deficiency despite
231 similar severe clinical features and a fatal outcome for all the NFU1 patients.

232

233 *NFU1 and IBA57 protein level*

234 Finally, we looked at the level of NFU1 and IBA57 proteins in the fibroblasts of patients
235 presenting a mutation in one protein involved in the late steps of mitochondrial [Fe-S] cluster
236 maturation (P3 to P12)(Figure 6). As expected, NFU1 protein was undetectable for the P5
237 NFU1 patient, who carries one intragenic deletion and one point deletion. NFU1 protein level
238 was decreased for the two other NFU1 patients (P6 and P8) presenting a point mutation in
239 their *NFU1* gene and, more surprisingly, for both BOLA3 patients (P3 and P4). NFU1 level was
240 not changed for the last NFU1 patient (P7) or for IBA57 (P9 and P10) and ISCA2 (P11 and
241 P12) patients. IBA57 protein was seriously decreased in all patients presenting a mutation in a
242 gene encoding a protein involved in the late steps of mitochondrial [Fe-S] cluster maturation
243 (P3 to P12).

244

245 **3. DISCUSSION**

246 We studied 13 patients with a mutation in a gene encoding a mitochondrial protein involved
247 either in LA biosynthesis (*LIPT1* and *LIPT2*) or in [Fe-S] cluster biogenesis (*BOLA3*, *NFU1*,

248 *IBA57*, *ISCA2* and *FDX1L*). Among these patients, ten are described for the first time in this
249 manuscript with mutations in *BOLA3* (P3 and P4), *NFU1* (P5, P6, and P7), *IBA57* (P9 and
250 P10), *ISCA2* (P11 and P12), and the second case with a mutation in *FDX1L* (P13).
251 Deficiencies in those proteins are known to cause diseases with severe encephalopathy due
252 to defects in mitochondrial OXPHOS and keto-dehydrogenases (PDHc and α -KGDHc) [23].
253 Conversely, *FDX1L* deficiency leads to a muscular phenotype with normal neurological
254 development [35]. In fact, PDHc and α -KGDHc are both active only when bound to LA, whose
255 synthesis involves LIAS, a [4Fe-4S] cluster-containing protein. As a consequence, LA
256 synthesis and keto-dehydrogenases activity are dependent on the mitochondrial [Fe-S] cluster
257 biogenesis pathway. We performed biochemical analysis to assess the precise impacts of
258 each mutation on both lipoylated proteins, but also on key mitochondrial [Fe-S] proteins
259 (several subunits of respiratory chain complexes, ferrochelatase and mt-aco). As the analyses
260 were carried out in the same conditions and tissue, i.e. skin fibroblast cultures, for all the
261 patients, biochemical profiles can be compared to each other.

262

263 *Fibroblasts from LIPT1 and LIPT2 patients*

264 Both patients present similar brain imaging and clinical features with early-onset
265 encephalopathy [19–21]. Up to now, four LIPT1 patients have been described: P1 of our study
266 [19] and three others [18, 36], and three LIPT2 patients have been reported one of whom was
267 included in our study (P2, corresponding to Patient 1 described by Habarou et al.) [20, 21]. P1
268 and P2 presented a decrease in PDHc and α -KGDHc activities due to a defect of lipoylation of
269 the E2 subunits (Table 3, Figure 2). Mt-aco is well known to be highly sensitive to cellular
270 oxidative stress, which degrades its [4Fe-4S] cluster to a [3Fe-4S] cluster, and, consequently,
271 decreases its enzymatic activity [37]. We observed an unexpected decrease in mt-aco activity
272 for both subjects (Table 3), despite a normal amount of the protein (Figure 3), indicating a
273 probable increase in oxidative stress in the fibroblasts of these patients. On the other hand,
274 the [2Fe-2S] cluster of ferrochelatase is not necessary for catalytic activity, but is required for

275 protein stability [34]. No strong variation in the level of ferrochelatase between patients and
276 controls was observed (Figure 4A). The hypothesis of increased oxidative stress in patients
277 with lipoylation defects has previously been suggested to explain their clinical pulmonary
278 hypertension [18]. There was no significant variation in the level of total reduced glutathione
279 (GSH) between patient and control fibroblasts (Table 3). While the amounts of the oxidized
280 form of glutathione (GSSG) were under the limit of detection of our assay, variations in the
281 ratio between GSH and GSSG cannot be excluded. Surprisingly, complex I was also affected
282 in both patients (Table 3, Figure 5). These results confirm those obtained by polarographic
283 study in both patients which showed low consumption of oxygen with pyruvate and malate [19,
284 21]. To our knowledge, none of the subunits of this complex are lipoylated. However, a
285 decrease of complex I following down-regulation of acyl-carrier protein, which is involved in
286 octanoic acid synthesis, has been observed [38]. Moreover, LA supplementation is known to
287 improve mitochondrial function [39], probably by increasing the efficiency of RNase P
288 processing and, consequently, tRNA^{Pro} processing, which is essential for mitochondrial protein
289 expression [40, 41]. Lastly, the decreased activity and stability of complex I could also be a
290 consequence of oxidative stress in these patients. Taken together, our results suggest that
291 moderate oxidative stress may be present in the fibroblasts of these patients and leads to
292 dysfunction of [Fe-S] proteins, which are highly sensitive to oxidative stress, like mt-aconitase
293 and complex I, but has no effect on other [Fe-S] proteins that are less sensitive to oxidative
294 stress, such as ferrochelatase. Clearly, LA deficiency induces OXPHOS dysfunction and more
295 studies are needed to elucidate the molecular mechanism.

296

297 *Fibroblasts from the FDX1L patient*

298 Here we report the second case of ferredoxin deficiency. Both patients (P13 and [35]) carry
299 the same homozygous mutation c.1A>T and present similar clinical features, with late onset of
300 metabolic myopathy, recurrent episodes of rhabdomyolysis, hyperlactacidemia and muscular
301 weakness. Neurological development is normal. FDX1L is clearly involved in the first steps of

302 mitochondrial [Fe-S] cluster biogenesis, steps that are necessary for the maturation of all the
303 [Fe-S] clusters independently of their subcellular localization. The FDX1L patient (P13)
304 presented with mild biochemical abnormalities (Table 3, Figure 2) and a moderate decrease in
305 SDHB protein level (Figure 4C). Spiegel et al. observed an SDHA defect in fibroblasts and
306 muscle of their patient with stronger OXPHOS deficiencies in muscle, with lower activity of
307 complex I and II in muscle than in fibroblasts [35]. Similar observations were reported
308 previously with patients with *FXN* and *ISD11* defects [44, 45], patients with motor deficiency as
309 the major clinical feature, as observed in FDX1L patients. Muscle biopsy was not available for
310 our FDX1L patient and we could not check whether deficiencies, particularly lipoylation of
311 proteins, were also stronger in muscle than in fibroblasts. In conclusion, for FDX1L patients it
312 would be appropriate to look at the deficiencies not only in fibroblasts but also in muscle to
313 obtain a more significant biochemical signature.

314
315 *Patients with defects in the late steps of the mitochondrial ISC machinery fibroblasts*

316 We looked at 10 patients carrying mutations in four different proteins involved in the delivery of
317 the [Fe-S] cluster to mitochondrial target proteins, namely NFU1, BOLA3, IBA57 and ISCA2.
318 As with other patients previously described, they presented encephalopathy with early-onset
319 and a fatal outcome for nine of them. One ISCA2 patient (P11) was more moderately affected
320 and still alive at 12 years (Table 2). In the current model for eukaryotic cells [10, 41, 46], NFU1
321 and BOLA3 serve for the maturation of some [4Fe-4S]-containing proteins such as LIAS, mt-
322 aco and SDHB. Interestingly, all the patients we have studied are affected in complex I activity
323 (Table 3) and integrity (except one ISCA2 patient: P11) (Figure 5). As for the LIPT1 and LIPT2
324 patients, the amount of ferrochelatase (Figure 4A) and the GSH concentration (Table 3) are
325 not significantly affected. Thus, for ISCA2, IBA57, and BOLA3, these results are in accordance
326 with the model proposed by Lill's group, which positions these proteins downstream of IND1 in
327 the maturation of complex I [10]. But, according to this model, NFU1 is only dedicated to the

328 maturation of SDHB and LIAS. The effects we observed in *NFU1* patients indicate that *NFU1*
329 is involved in complex I maturation, as proposed by Cameron et al. [12].

330 Interestingly, all *BOLA3*, *NFU1* and *ISCA2* patients have a decreased amount of IBA57
331 (Figure 6). It was shown previously that, in yeast, Iba57p physically interacts with Isa1p and
332 Isa2p [47]. Due to the high homology of sequences between yeast and mammals, it is
333 probable that IBA57 interacts with *ISCA2* in mammals too, an interaction that would explain
334 the effects of mutation in *ISCA2* on IBA57 protein level. The effects of mutations in *BOLA3* on
335 IBA57 protein level indicate a link (cellular or physical interaction) between these two proteins.
336 Concerning [2Fe-2S] protein maturation, our results suggest that mutations in these four
337 genes would not affect the maturation of the [2Fe-2S]-containing proteins (Rieske protein,
338 ferrochelatase). This is in agreement with the model for eukaryotic cells, which does not
339 involve Nfu1 in the delivery of [2Fe-2S] clusters [9, 48–50]. It seems that *BOLA3*, *ISCA2* and
340 IBA57 are not involved in this function either.

341 *NFU1 and BOLA3 patients*

342 Six *BOLA3* patients [12, 16, 17] and more than 25 patients with *NFU1* mutations have been
343 reported to date [12, 13, 24, 51, 52]. The clinical and biochemical phenotypes of these two
344 deficiencies are quite similar, with early onset of severe encephalopathy, variable
345 cardiomyopathy, PDHc and mitochondrial RC defects (Tables 2 and 3). Our study includes two
346 new *BOLA3* patients (P3 and P4) carrying the known homozygous mutation p.Arg46* [12] The
347 four *NFU1* patients presented early-onset encephalopathy and died before the age of three
348 years. Two of them had pulmonary hypertension (P6 and P8), as is usually observed in
349 patients carrying the same mutation p.Gly208Cys [51]. Patient P5 carries two heterozygous
350 deletions in the *NFU1* gene, leading to undetectable amounts of *NFU1* protein in his
351 fibroblasts and this can be considered as an almost complete *NFU1* deficiency (Figure 6A).
352 This patient presents severe RC and mt-aco dysfunctions, and keto-dehydrogenases
353 deficiency, secondary to lipoylation defects, resulting in severe clinical encephalopathy. This

354 confirms that NFU1 is clearly involved in at least the maturation of [4Fe-4S] proteins such as
355 LIAS, mt-aco and SDHB. In the case of patient P7, we observed a discrepancy between
356 biochemical profiles (with unexpected normal α -KGDHc activity and protein lipoylation) and
357 clinical features as previously reported for some patients [52]. But, our patient presents a point
358 mutation (p.Phe60Cys) that does not affect NFU1 protein level. This mutation in the β 1 strand
359 of the protein is predicted to be deleterious by *in silico* predicting softwares (SIFT,
360 MutationTaster and PolyPhen-2). The recent NMR structure of NFU1 highlights the formation
361 of a trimer of dimers of holo-NFU1 and the authors proposed that the β 1 strand might be
362 involved in interaction with chaperones, which would help cluster transfer [49]. Thus, this
363 mutation, which does not affect the stability of NFU1, might decrease the efficiency of cluster
364 delivery to target proteins, which would explain the mild effects on biochemical profiles of the
365 patient.

366 Both BOLA3 patients (P3 and P4) present low levels of NFU1 protein (Figure 6A). These
367 results are in accordance with studies in yeast showing that Bol3 forms a complex with Nfu1,
368 stabilizes it and is involved in [4Fe-4S] cluster delivery [41]. Thus, in these patients, the
369 mutation in *BOLA3* affects both BOLA3 and NFU1 proteins. This explains the very severe
370 clinical phenotype of all reported BOLA3 patients, with a cumulative effect of lack of both
371 BOLA3 and NFU1 [17].

372 *IBA57 and ISCA2 patients*

373 In our study, both IBA57 patients carry two heterozygous missense mutations, have no
374 detectable IBA57 protein (Figure 6B), and present a severe clinical phenotype with early onset
375 and a fatal outcome. Of the 18 IBA57 patients already described, some suffer from severe
376 diseases (n=4), while others, all members of the same family, present chronic spastic
377 paraplegia [14, 53, 54]. Clinical presentations of our two ISCA2 patients (P11 and P12), who
378 both present novel missense mutations, are quite different, with neonatal onset and early
379 death for patient P12, whereas patient P11, at 12 years of age, suffers from non-progressive

380 spastic paraplegia (Table 2). Only 7 cases, from six unrelated families, of patients with *ISCA2*
381 mutations have been described before [15, 55]. They presented spasticity and psychomotor
382 delay, a clinical presentation quite similar to that observed for P11 (Table 1). One *ISCA2*
383 patient (P12) showed marked decreases in the enzymatic activities of PDHc, α -KGDHc and
384 RC complexes and in SDHB protein level, while the other one (P11) showed more limited
385 effects in accordance with their respective clinical phenotypes. The molecular relation between
386 IBA57 and ISCA proteins is still debated. Initially, it was considered that IBA57 acts with
387 ISCA1 and ISCA2 in the maturation of mitochondrial [4Fe-4S] proteins [11]. Then, it was
388 proposed that ISCA proteins might form heterocomplexes without the help of IBA57 [56].
389 Finally, proteomic studies proposed that ISCA2 interacts with ISCA1, but also with GLRX5 and
390 IBA57 [57]. As for patients with *NFU1* and *BOLA3* disorders, IBA57 patients have impaired
391 lipoylation (Figure 2) with decreased activities of PDHc and α -KGDHc (Table 3). RC complex
392 defects were reported in most of the previous published cases of IBA57 deficiencies [14, 53,
393 58] and dysfunction of complexes I and II was observed in our two IBA57 patients. As for
394 *BOLA3* and *NFU1* disorders, they are likely due to abnormal assembly of the [Fe-S] clusters
395 held by subunits of these complexes. But, as previously observed in IBA57-depleted HeLa
396 cells [11], mt-aco is not affected in these patients (Table 3 and Figure 3). Thus, IBA57 protein
397 may not be essential for [4Fe-4S] transfer to mt-aco protein, even if one IBA57 patient,
398 carrying mutations compared with our patients, was recently reported with decreased mt-aco
399 protein in fibroblasts [58].

400

401 In conclusion, we report two patients with mutations in either *LIPT1* or *LIPT2*, one new patient
402 with a mutation in *FDX1L*, a protein of the early steps of mitochondrial [Fe-S] cluster
403 biogenesis, and ten patients, including nine new patients with deficiency in proteins (*IBA57*,
404 *BOLA3*, *NFU1*, *ISCA2*) involved in the late steps of this same pathway. Clinical presentation
405 was extremely severe with early fatal outcome for eight of them. Ferredoxin deficiency leads to

406 severe myopathy with moderate mitochondrial protein dysfunction in fibroblasts. In all other
407 deficiencies, PDHc and α -KGDHc activities and lipoylation of these enzymes were affected.
408 OXPHOS was also affected. Interestingly, in all the patients, the integrity of complex I was
409 affected and its activity was greatly reduced even in LIPT1 and LIPT2 patients. Similar
410 observations were made for mt-aco activity. These decreases in activity of several
411 mitochondrial [Fe-S] proteins in patients with mutations in either LIPT1 or LIPT2 may reflect an
412 increase in oxidative stress, which degrades stress-sensitive [Fe-S] clusters. We confirm that
413 ISCA2, NFU1, BOLA3 and IBA57 are involved only in the maturation of mitochondrial [4Fe-
414 4S]-containing proteins and not [2Fe-2S]-containing proteins. Moreover, IBA57 does not seem
415 to be involved in mt-aco maturation, as previously proposed using HeLa cells as model.
416 BOLA3, NFU1 and ISCA2 are required for IBA57 stability and BOLA3 is required for NFU1
417 stability. These findings, summarized in a proposed scheme (Figure 7), indicate the formation
418 of multiple heterodimers between these four proteins, as already proposed for IBA57 and
419 ISCA2 [11, 56], and BOLA3 and NFU1 [41]. We cannot exclude that they form a larger
420 complex including all four proteins.

421

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431

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608

609 **Figure 1: Simplified scheme of human mitochondrial [Fe-S] protein biogenesis** [5, 9, 23].
610 Starting from iron atoms and inorganic sulfur provided by cysteine desulfuration, a [2Fe-2S]
611 cluster is built on the scaffold protein ISCU, with the help of NFS1 and ISD11. Ferredoxin
612 serves as electron donor. In the second step, chaperone proteins transfer a [2Fe-2S] cluster
613 from ISCU to GLRX5. The cluster can then be exported to the cytosol to integrate cytosolic Fe-
614 S assembly (CIA) machinery or transferred to mitochondrial apoprotein, which needs one or
615 several [2Fe-2S] or [4Fe-4S] clusters. For this, ISC targeting factors (ISCA1, ISCA2, IBA57,
616 NFU1, BOLA3) are essential, but the exact role of each one is not yet clearly defined. Among
617 [4Fe-4S] target proteins, lipoic acid synthase (LIAS) is a key enzyme in lipoic acid synthesis,
618 and is covalently linked to mitochondrial ketoacid dehydrogenases such as PDHc and
619 α KGDHc. Mutated proteins (green) found in the patients included in this study. Mitochondrial
620 [4Fe-4S]- and [2Fe-2S]-containing proteins (salmon and blue, respectively) and lipoylated
621 proteins (yellow) studied in this report.
622

623 **Figure 2: Protein level of lipoylated proteins and lipoic acid synthase in fibroblasts.**
624 Fibroblasts of the 13 patients and 2 controls (C1 and C2) were analyzed by immunoblot after
625 mitochondrial enrichment. Antibodies against lipoic acid (detection of lipoic acid bound to E2
626 subunits of PDHc and α -KGDHc), E2 subunit of PDHc (A) and LIAS (B) were used.

627

628 **Figure 3: Protein level of mt-aconitase in fibroblasts.** Fibroblasts of the 13 patients and 2
629 controls (C1 and C2) were analyzed after mitochondrial enrichment by immunoblot with
630 antibody against mt-aconitase. Prohibitin is shown as the loading control of mitochondrial
631 content.

632 **Figure 4: Protein level of mitochondrial [Fe-S] proteins in fibroblasts.** Fibroblasts of the
633 13 patients and 2 controls (C1 and C2) were analyzed after mitochondrial enrichment using
634 antibodies against the [2Fe-2S]-containing ferredoxin (panel A) and Rieske protein (panel
635 B). Prohibitin served as the loading control. Panel C shows the level of the [4Fe-4S] protein
636 SDHB, subunit of RC complex II. Antibody against SDHA, another subunit of respiratory chain
637 complex II, without [Fe-S] clusters, is shown as the loading control of mitochondrial content.

638 **Figure 5: Blue-native PAGE analysis of respiratory chain complexes I and II in**
639 **fibroblasts.** BN-PAGE analysis of mitochondria-enriched fibroblasts of the 13 patients and
640 three controls (C1, C2 and C3) was performed using antibodies against respiratory chain
641 complex subunits that do not coordinate an [Fe-S] cluster (CI: GRIM19, CII: SDHA). Complex
642 III (subunit UQCRC2) served as the loading control, while the activity of complex III was not
643 affected in all patients.

644 **Figure 6: NFU1 and IBA57 protein levels in fibroblasts.** Fibroblasts of the 10 patients
645 (patients with mutations in *LIPT1*, *LIPT2* and *FDX1L* were not included) and two controls (C1
646 and C2) were analyzed after mitochondrial enrichment using antibodies against NFU1 (A) and
647 IBA57 (B). Prohibitin was used as the loading control of mitochondrial content. Unfortunately,
648 the tested antibodies against BOLA3 and ISCA2 were not suitable for immunoblotting.

649 **Figure 7: Proposed scheme of human mitochondrial [Fe-S] protein biogenesis.** The
650 scheme summarizes the results obtained in our study. Arrows between BOLA3, NFU1, ISCA2,
651 IBA57 symbolize the stabilizing effects we observed between these four proteins.

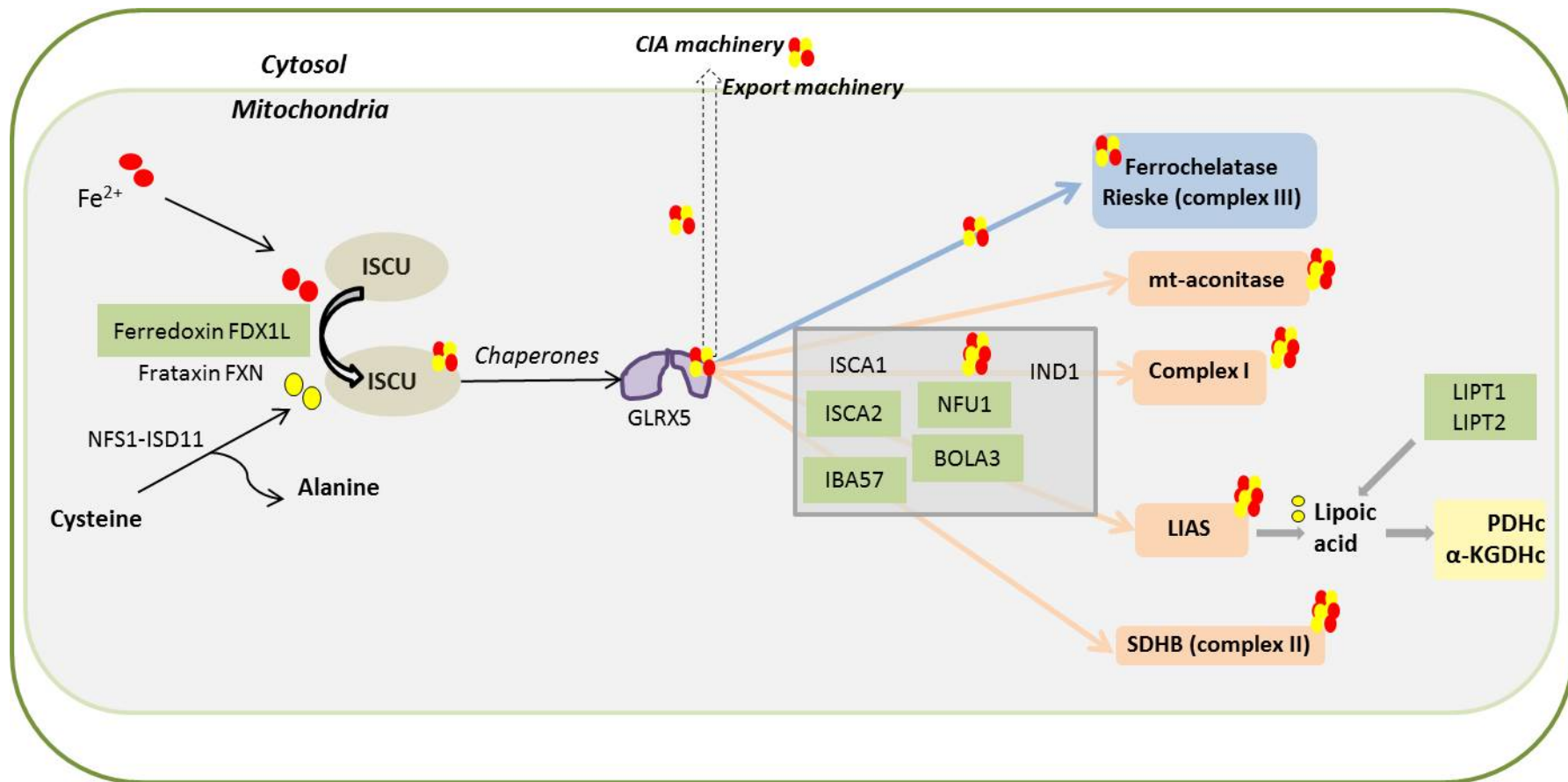
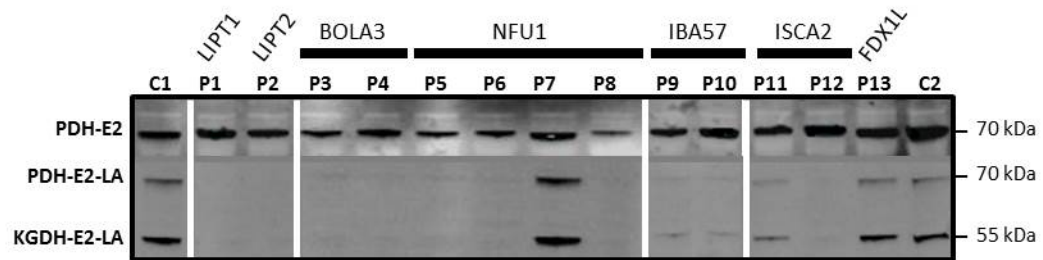


Figure 1

A



B

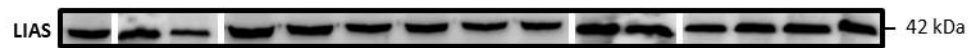


Figure 2

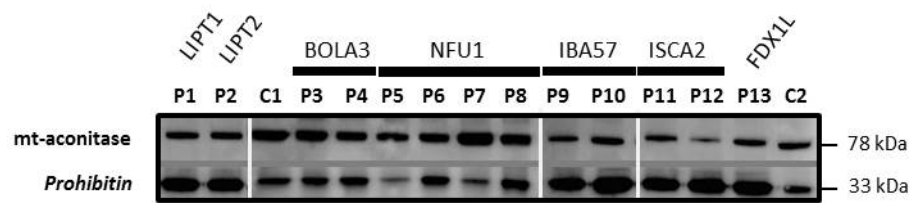


Figure 3

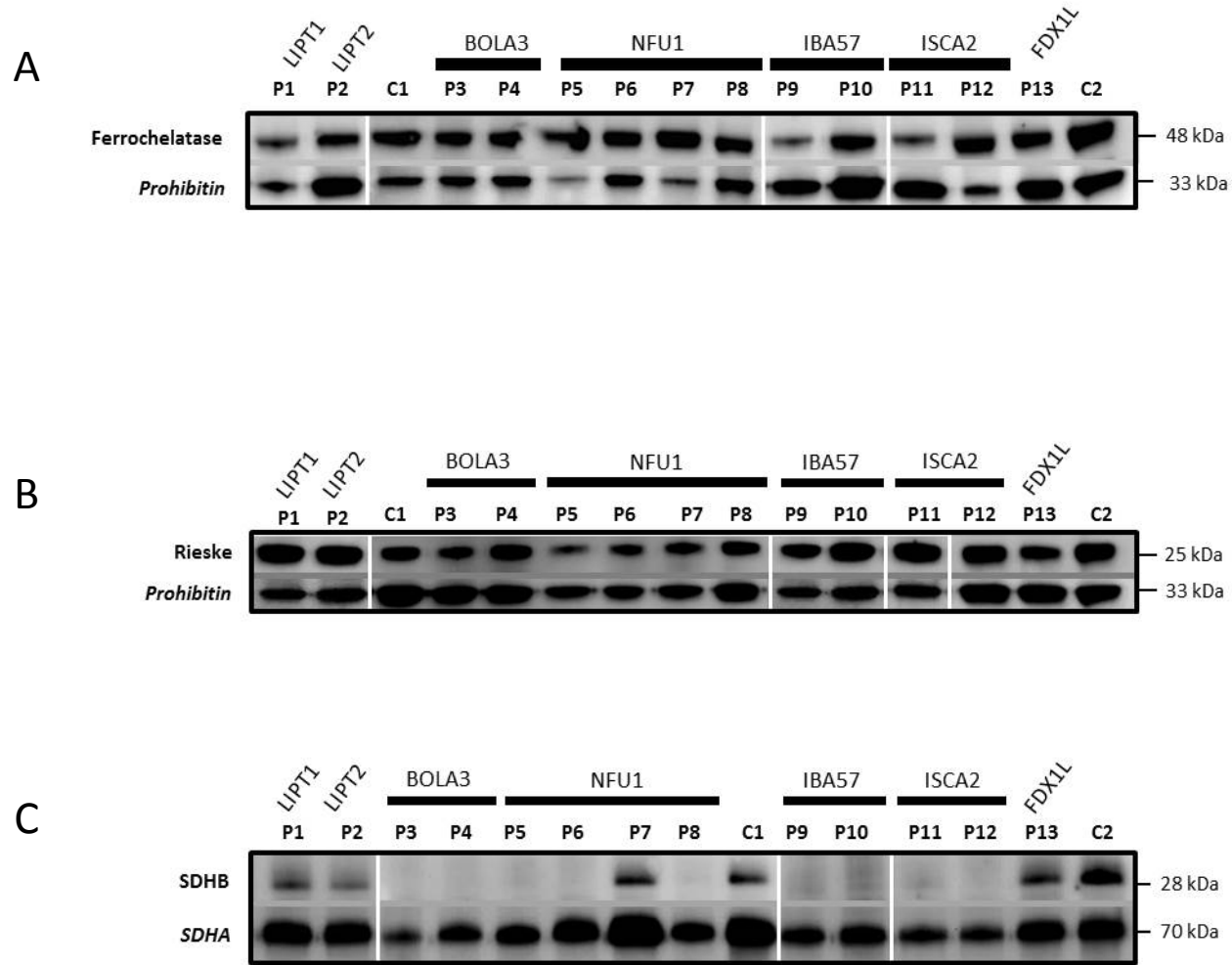


Figure 4

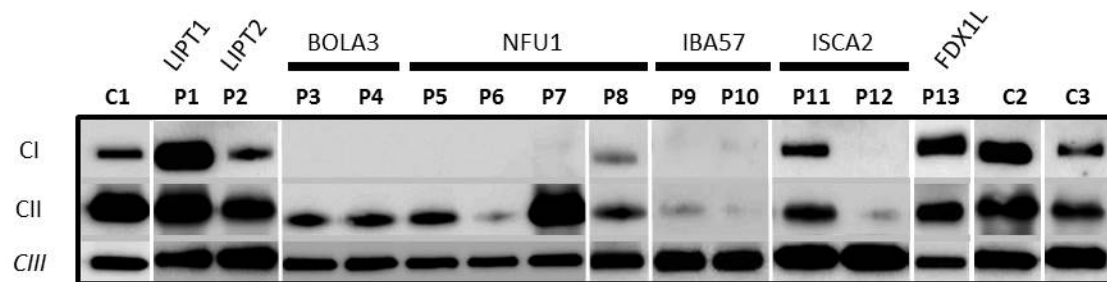
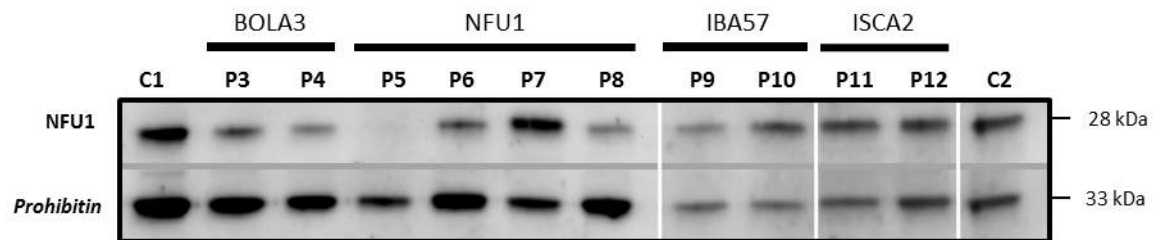


Figure 5

A



B

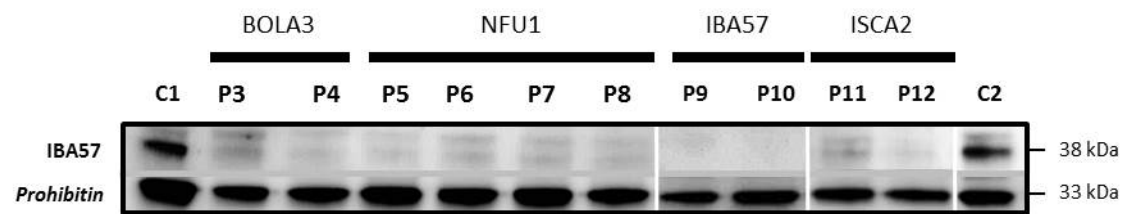


Figure 6

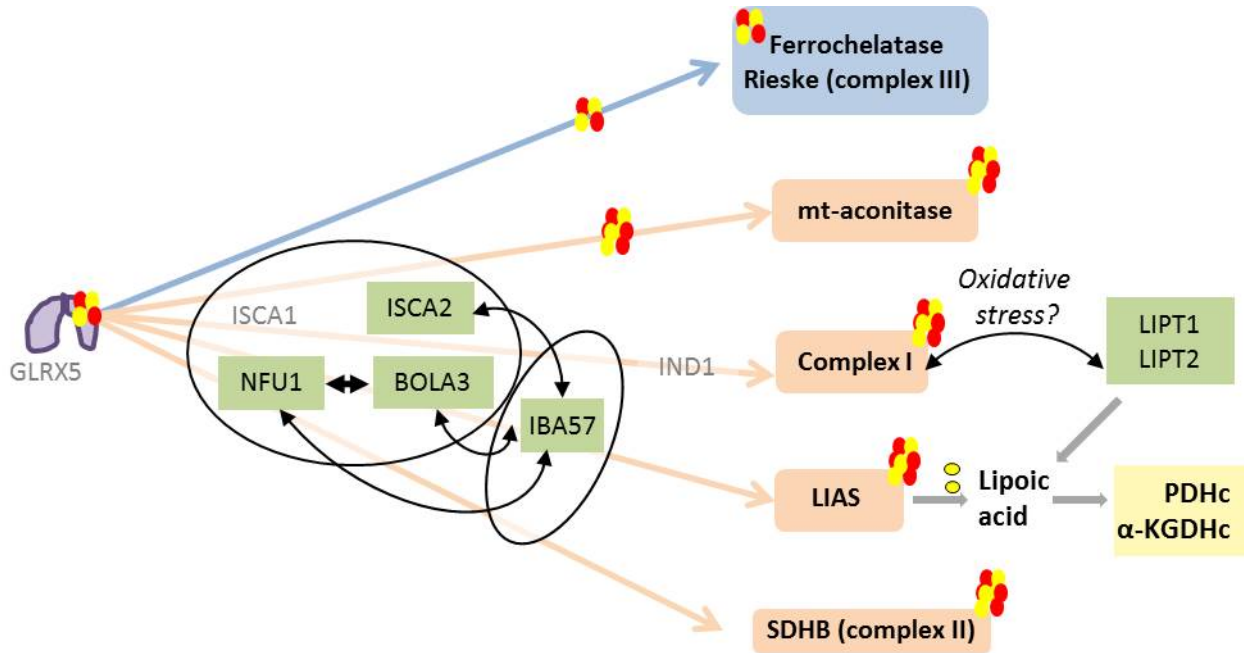


Figure 7