



**HAL**  
open science

## Warburg-like effect is a hallmark of complex I assembly defects

Valerie Desquiret-Dumas, Geraldine Leman, Celine Wetterwald, Stephanie Chupin, Anaïs Lebert, Salim Khiati, Morgane Le Mao, Guillaume Geffroy, Mariame Selma Kane, Arnaud Chevrollier, et al.

### ► To cite this version:

Valerie Desquiret-Dumas, Geraldine Leman, Celine Wetterwald, Stephanie Chupin, Anaïs Lebert, et al.. Warburg-like effect is a hallmark of complex I assembly defects. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2019, 1865 (9), pp.2475-2489. 10.1016/j.bbadis.2019.05.011 . hal-02942827

HAL Id: hal-02942827

<https://hal.science/hal-02942827>

Submitted on 25 Oct 2021

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 **Warburg-like effect is a hallmark of complex I assembly defects**

2 Running title: **Mitochondrial Complex I assembly drives cell metabolic fate**

3 Valerie Desquiret-Dumas<sup>1,2</sup>, Geraldine Leman<sup>1</sup>, Celine Wetterwald<sup>2</sup>, Stephanie Chupin<sup>2</sup>,  
4 Anaïs Lebert<sup>1</sup>, Salim Khiati<sup>1</sup>, Morgane Le Mao<sup>1</sup>, Guillaume Geffroy<sup>1</sup>, Mariame Selma Kane<sup>1</sup>,  
5 Arnaud Chevrollier<sup>1</sup>, David Goudenege<sup>1</sup>, Cedric Gadras<sup>2</sup>, Lydie Tessier<sup>2</sup>, Magalie Barth<sup>1,2</sup>,  
6 Stephanie Leruez<sup>2</sup>, Patrizia Amati-Bonneau<sup>1,2</sup>, Daniel Henrion<sup>1</sup>, Dominique Bonneau<sup>1,2</sup>,  
7 Vincent Procaccio<sup>1,2</sup>, Pascal Reynier<sup>1,2</sup>, Guy Lenaers<sup>1</sup>, Naig Gueguen<sup>1,2</sup>

8

9 <sup>1</sup> UMR CNRS 6015-INSERM U1083, MitoVasc Institute, University of Angers, Angers,  
10 France

11 <sup>2</sup> Department of Biochemistry and Genetics, University Hospital of Angers, F-49000, France

12

13 Corresponding author: NaGueguen@chu-angers.fr

14

15

1 **SUMMARY**

2           Due to its pivotal role in NADH oxidation and ATP synthesis, mitochondrial complex  
3 I (CI) emerged as a crucial regulator of cellular metabolism. A functional CI relies on the  
4 sequential assembly of nuclear- and mtDNA-encoded subunits; however, whether CI  
5 assembly status is involved in the metabolic adaptations in CI deficiency still remains largely  
6 unknown. Here, we investigated the relationship between CI functions, its structure and the  
7 cellular metabolism in 29 patient fibroblasts representative of most CI mitochondrial diseases.  
8 Our results show that, contrary to the generally accepted view, a complex I deficiency does  
9 not necessarily lead to a glycolytic switch, i.e. the so-called Warburg effect, but that this  
10 particular metabolic adaptation is a feature of CI assembly defect. By contrast, a CI functional  
11 defect without disassembly induces a higher catabolism to sustain the oxidative metabolism.  
12 Mechanistically, we demonstrate that reactive oxygen species overproduction by CI assembly  
13 intermediates and subsequent AMPK-dependent Pyruvate Dehydrogenase inactivation are key  
14 players of this metabolic reprogramming. Thus, this study provides a two-way-model of  
15 metabolic responses to CI deficiencies that are central not only in defining therapeutic  
16 strategies for mitochondrial diseases, but also in all pathophysiological conditions involving a  
17 CI deficiency.

18

19 **KEYWORDS**

20 complex I deficiency; complex I assembly; mitochondrial metabolism; metabolic  
21 reprogramming; ROS production.

22

23

## 1 INTRODUCTION

2 A metabolic transition towards glycolysis in aerobic conditions, the so-called Warburg  
3 effect, is a hallmark of many pathological conditions not only in cancer cells but also in  
4 systemic diseases or in metabolic disorders. However, to date it remains unclear how cells  
5 engage the aerobic glycolysis at the expense of oxidative phosphorylation. In cancer cells,  
6 recent data converge to the concept that mitochondrial complex I (CI) is a crucial regulator of  
7 cellular metabolic fate [1-5]. Indeed, through its activity, consisting in NADH oxidation  
8 coupled to quinone reduction and protons pumping, CI contributes to the NAD<sup>+</sup>/NADH  
9 equilibrium, the ADP/ATP ratio and ROS production [6, 7], all key modulators of the cellular  
10 metabolic orientation [8-12].

11 CI deficiency is the most frequent defect encountered in mitochondrial disorders [13].  
12 Its clinical presentations (OMIM #252010) are highly variable among patients ranging from  
13 isolated organ failures, as the Leber Hereditary Optic Neuropathy (LHON OMIM #535000)  
14 affecting the optic nerve, to multivisceral failure as the Leigh syndrome (OMIM #256000). CI  
15 deficiency is also the pre-eminent feature of the MELAS syndrome (Myopathy,  
16 Encephalopathy, Lactic Acidosis and Strokes, OMIM #540000), mainly due to the  
17 m.3243A>G pathogenic variant in the mitochondrial tRNA (MT-TRNA) Leucine 1 (*MT-TL1*)  
18 [14]. Although it is commonly assumed that CI deficiencies are associated with an  
19 upregulation of aerobic glycolysis, this clinical heterogeneity increasingly challenges this  
20 assumption and questions whether the metabolic transition is really engaged proportionally to  
21 the degree of CI enzymatic defect. CI diseases are indeed commonly considered as a defect in  
22 CI activity. However, beyond these functional defects, increasing literature reports novel CI  
23 pathogenic variants compromising CI assembly [15, 16], a parameter yet underestimated in  
24 the pathophysiology of CI deficiency.

1 The structure of this large L-shaped complex of 44 subunits combines a matrix arm composed  
2 of two functional domains, the N (NADH binding) and Q (Quinone binding) modules and a  
3 membrane arm corresponding to the P (Proton pumping) module. The catalytic core subunits  
4 of the P-module are encoded by the mitochondrial DNA (MT-DNA), while the N and Q  
5 modules are exclusively encoded by the nuclear DNA (nDNA). Recent findings led to a  
6 consensus modular assembly model that involves the independent building of submodules,  
7 and their step-by-step association (Supplementary Figure 1A). This process is assisted by at  
8 least 13 assembly factors specific of each module [17, 18] that dissociate from the complex  
9 once finally assembled. Defects in this intricate assembly process would lead to the  
10 accumulation of CI assembly subcomplexes unconnected from each other's, which could be  
11 deleterious for the cell [19], therefore questioning the involvement of such a defect in  
12 metabolic rewiring.

13 Thus, a comprehensive analysis of the pathophysiological consequences of CI deficiencies is  
14 required for a better understanding of the mechanisms linking CI function, assembly and  
15 metabolic adaptations and is a pre-requisite for the development of therapeutic strategies. To  
16 address this question, we took advantage of a large skin fibroblast collection obtained from  
17 patients presenting CI deficiency, with impairment in CI functionality and/or assembly. Our  
18 results shed light on the CI assembly status as a highly discriminative parameter in the  
19 metabolic adaptation to CI deficiencies and position ROS and PDH phosphorylation as key  
20 mediators of the glycolytic switch related to CI assembly defects.

21

1 **MATERIAL AND METHODS**

2

3 **EXPERIMENTAL MODELS**

4

5 **Study approval**

6 This work was approved by the Ethics Committee of the Angers University Hospital (CPP  
7 Ouest II – Angers, France; Identification number: CPP CB 2014/02; Declaration number: DC-  
8 2011-1467 and Authorization number: AC-2012-1507). Written informed consent was  
9 obtained from all participants. The description of the mutations from each patient and their  
10 phenotype are detailed in Supplementary Figure 2.

11

12 **Cell lines and cell culture**

13 Fibroblasts obtained from skin biopsy from 40 individuals, 29 patients with CI deficiency and  
14 11 healthy controls (Supplementary Figure 2) were cultured in 2/3 Dulbecco's modified Eagle  
15 medium (DMEM-F12, PAN Biotech, Wimborne, UK), 1/3 Amniomax (Gibco, Invitrogen,  
16 Paisley, UK) supplemented with 10% fetal bovine serum (PAN Biotech, Wimborne, UK), 1  
17 mM sodium pyruvate, and 50 µg/ml uridine (Sigma Aldrich, Lyon, France) at 37°C, 5% CO<sub>2</sub>.  
18 One week before analyses, uridine and pyruvate supplementations were stopped to avoid any  
19 influence of high pyruvate concentration on the cellular metabolism. All experiments were  
20 conducted on fibroblast cultures between passages 6 and 25 to avoid artefacts due to  
21 senescence [20]. All fibroblast cell lines were routinely tested for the absence of mycoplasma  
22 contamination. For experiments, cells were washed with PBS, trypsinized, centrifugated (800  
23 rpm, 5 min), washed in PBS buffer and the cell pellets were either immediately used

1 (oxygraphic analysis, NAD<sup>+</sup>/NADH measurement) or immediately frozen in liquid nitrogen  
2 and stored at -80°C until use.

### 3 **METHODS**

#### 4 **Cell treatments and Small Interference RNA**

5 To induce CI assembly defect, fibroblasts from healthy controls were treated during 48h with  
6 1μM sodium Pyrithione (2-Mercaptopyridine-*N*-oxide sodium, NaPyr, Sigma Aldrich, Saint-  
7 Quentin Fallavier, France). NaPyr solutions in DMSO were diluted 1:5,000 in the cell culture  
8 medium (without uridine or pyruvate supplementation). Control cells were treated in parallel  
9 with DMSO 1:5,000 (“vehicle” condition).

10 To inhibit CI activity, cells were treated during 48h with Piericidin A, 5 nM (Sigma Aldrich,  
11 Saint-Quentin Fallavier, France). Piericidin A stock solutions in DMSO were diluted 1:5,000  
12 in the medium. Control cells were treated in parallel with DMSO diluted 1:5,000 in the  
13 medium (“vehicle” condition). Importantly, Piericidin A was chosen instead of the classical  
14 CI inhibitor, rotenone, due to the higher dynamic range between CI inhibition and induction  
15 of reactive oxygen species (ROS) production of piericidin A [21]. Dose-response titrations  
16 (2.5 nM up to 50 nM) with both rotenone and piericidin A were performed on control  
17 fibroblasts before cell treatment and CI-inhibition and CI-linked ROS production were  
18 analyzed simultaneously using O2K-fluorometry by measuring both the CI-linked maximal  
19 respiration and CI-linked Amplex red oxidation on the same sample (see below:  
20 Mitochondrial ROS production section). The dose of Piericidin A was chosen as the one that  
21 induced the closest inhibition of CI-linked respiration than the one observed on CI patient  
22 cells (i.e.  $-59.8 \pm 2.2\%$ ) without inducing a ROS production. At the chosen dose (5 nM),  
23 piericidin A inhibited both CI-linked respiration (-65%) and ROS production (-29%) while 5

1 nM of rotenone only inhibited CI-linked maximal respiration by -24% without inducing ROS  
2 production. At 50 nM, both inhibitors fully inhibited CI-linked respiration (-99 and -88% for  
3 piericidin A and rotenone, respectively) but strongly stimulated ROS production (+75 and  
4 +344% for piericidin A and rotenone, respectively).

5 Silencing of NDUFAF1 was performed on control fibroblasts by using a pool of three specific  
6 siRNA duplexes (Thermofisher, Waltham, USA). Control fibroblasts were reverse transfected  
7 using the Mission siRNA reagent (Sigma Aldrich, Saint Quentin Fallavier, France) and  
8 40pmol of NDUFAF1 or control siRNA (scramble). After 48 hours, cells were harvested and  
9 oxygraphic analysis was performed. A frozen pellet was kept at -80°C for Western blot and  
10 BN PAGE and an aliquot of cell culture medium was frozen for metabolite measurements  
11 (see below).

12 To induce ROS production by CI, control cells were treated during 48h with 5 µM  
13 mitochondria-targeted redox cycler, MitoParaquat (mt-PQ, Abcam, Cambridge, UK). Within  
14 the matrix, MitoPQ produces superoxide by redox cycling at the flavin site of complex I,  
15 selectively increasing superoxide production within mitochondria [22]. A 25 mM mt-PQ  
16 stock solution in DMSO was diluted 1:5,000 in the medium. Control cells were treated in  
17 parallel with DMSO diluted 1:5,000 in the medium (“vehicle” condition).

18 Cellular antioxidant treatments were conducted using N-Acetyl cysteine (NAC, 48H, 1mM,  
19 Sigma Aldrich, Saint Quentin Fallavier, France). A fresh 100 mM NAC solution was realized  
20 in DMEM-F12 medium for each experiment and diluted at 1:100 in the final cell culture  
21 medium. Control cells were treated in parallel with DMEM-F12 diluted 1:100 in the cell  
22 culture medium (“vehicle” condition).



1 AMPK inhibition in patient and control cells was performed using Compound C (Abcam,  
2 Cambridge, UK) (48H, 10  $\mu$ M). A 10 mM stock solution in DMSO was diluted 1:1,000 in the  
3 medium. Cells were treated in parallel with DMSO diluted 1:1,000 in the medium (“vehicle”  
4 condition).

5

### 6 **Mitochondrial enzymatic activities**

7 The activities of Complex I (NADH ubiquinone reductase, NUR, EC 1.6.5.3) and citrate  
8 synthase (CS, EC 2.3.3.1) were measured at 37°C with a UV $mc^2$  spectrophotometer (SAFAS,  
9 Monaco) on mitochondrial enriched fraction. Cells were re-suspended in cell buffer (250 mM  
10 saccharose, 20 mM tris[hydroxymethyl]aminomethane, 2 mM EGTA, 1 mg/mL bovine serum  
11 albumin, pH 7.2; 50  $\mu$ l/10<sup>6</sup> cells), disrupted by two freezing-thawing cycles, washed,  
12 centrifuged for one minute at 16 000 g to eliminate the cytosolic fraction, re-suspended in the  
13 cell buffer (250  $\mu$ l/10<sup>6</sup> cells) and sonicated (6 $\times$ 5 s) on ice. Complex I activity was  
14 immediately assayed according to [23, 24] in KH<sub>2</sub>PO<sub>4</sub> buffer (80 mM, pH 7.4) supplemented  
15 with 1mg/ml fatty acid free bovine serum albumin, 1 mM potassium cyanide (KCN), 2 mM  
16 sodium azide (NaN<sub>3</sub>) and 100  $\mu$ M DCPIP added to maintain the quinone acceptor in fully  
17 oxidized state, thus avoiding NUR reaction inhibition by reduced CoQH<sub>2</sub> accumulation [25].  
18 100  $\mu$ M Coenzyme Q1 (Sigma Aldrich, Saint Quentin Fallavier, France) were used as  
19 acceptor and 150  $\mu$ M NADH as substrate to initiate the reaction. The specificity of the NUR  
20 reaction was controlled on the same sample in the presence of its specific inhibitor, rotenone  
21 (5  $\mu$ M). Citrate synthase activity was assayed by a standard procedure [23, 24]. The protein  
22 content was determined with the bicinchoninic assay kit (Uptima, Interchim, Montluçon,  
23 France) using bovine serum albumin as standard. CI activity was normalized to CS as a  
24 mitochondrial content marker, after checking for the absence of difference in the

1 mitochondrial CS activity between groups. Enzymatic measurements were performed at least  
2 twice in duplicate, on two different cell pellets from different passages.

3

#### 4 **Mitochondrial respiration rates**

5 Mitochondrial oxygen consumption measurements were performed at 37°C and atmospheric  
6 pressure using a high-resolution oxygraph (O2K, Oroboros Instrument, Innsbruck, Austria).

7 Respiration rates on permeabilized cells were measured in respiratory buffer RB (10mM  
8  $\text{KH}_2\text{PO}_4$ , 300mM mannitol, 10mM KCl, 5mM  $\text{MgCl}_2$ , 0.5mM EGTA and 1 mg/ml serum  
9 albumin bovine, pH7.2) using substrates of CI, CI+CII and CII as followed: first, state 2 (non-  
10 phosphorylating) respiration was measured after adding 2.5mM pyruvate and 5mM malate.

11 Then, the CI-linked maximal phosphorylating respiration was stimulated by saturating ADP  
12 concentration (1.5mM) and 3mM  $\text{NAD}^+$ , added to avoid TCA limitation by  $\text{NAD}^+$   
13 availability. Succinate (10mM) was then added to measure the combined CI and CII-linked  
14 respiration with convergent CI+II electron flow into the Q-junction corresponding to the  
15 maximal stimulated phosphorylating respiration (OXPHOS capacity). Rotenone (5 $\mu\text{M}$ ) was  
16 used to inhibit CI activity and thus to obtain the maximal CII-linked respiration. Thirdly,  
17 oligomycin (F0F1-ATP synthase inhibitor, 4 $\mu\text{g/ml}$ ) and FCCP (carbonyl cyanide p-  
18 trifluoromethoxyphenylhydrazone, a mitochondrial uncoupler, 1 $\mu\text{M}$ ) were sequentially added  
19 to ensure that the cells were fully permeabilized. Finally, antimycin A addition (2 $\mu\text{g/ml}$ ) was  
20 used to check for the non-mitochondrial oxidation.

21 Respiration rates on intact cells were measured in DMEM-F12 medium (3g/l glucose, 1mM  
22 glutamine, 0.3mM pyruvate).  $4-5 \cdot 10^6$  cells were added in the oxygraphic chamber and the  
23 analysis started with routine respiration (R) measurement, which is defined as respiration in  
24 DMEM-F12 medium without additional substrates or effectors (cell endogenous respiration,  
25 corresponding to the cellular oxidative metabolism). Then, F0F1-ATP synthase was inhibited

1 with oligomycin (4  $\mu\text{g/ml}$ ), allowing the measurement of non-phosphorylating respiration  
2 (Leak respiration). This non-phosphorylating respiration (O) was subtracted from Routine (R)  
3 one to calculate the cellular phosphorylating respiration (R-O). This was followed by  
4 uncoupling of oxidative phosphorylation by stepwise titration of FCCP (carbonyl cyanide p-  
5 trifluoromethoxyphenylhydrazone) up to optimum concentrations (0.6 – 1.2  $\mu\text{M}$ ) allowing the  
6 measurement of the maximal endogenous respiration (cellular oxidative capacity). The part of  
7 the maximal capacity use for oxidative metabolism was calculated as R/F and the part of the  
8 maximal capacity use for oxidative ATP synthesis was calculated as (R-O)/F. Finally,  
9 respiration was inhibited by rotenone and antimycin A (2.5 $\mu\text{M}$  and 2  $\mu\text{g/ml}$ , respectively) to  
10 check for non-mitochondrial oxidation. All experiments were performed at least twice in  
11 duplicate on independent cell cultures.

12

### 13 **Mitochondrial ROS production**

14 ROS production was measured simultaneously to oxygen consumption at 37°C and  
15 atmospheric pressure in respiratory buffer RB using the O2k-Fluorometer (Oroboros  
16 Instrument, Innsbruck, Austria) equipped with two-channel fluorescence optical setup to  
17 monitor oxygen level and fluorescence.  $\text{H}_2\text{O}_2$  production was monitored according to [26]  
18 using the  $\text{H}_2\text{O}_2$ -sensitive probe Amplex® Red (Molecular Probes, Eugene, Oregon, USA),  
19 10 $\mu\text{M}$  (Excitation 525nm / Emission filter 580nm). 1U/mL horse radish peroxidase (HRP)  
20 and 5U/mL superoxide dismutase (SOD) were added to the chamber to convert superoxide  
21 into  $\text{H}_2\text{O}_2$ . Calibrations were carefully performed before, during and at the end of each  
22 experiment with stepwise additions of 0.1 $\mu\text{M}$   $\text{H}_2\text{O}_2$ . Dedicated CI and CI+CII-linked ROS  
23 production was sequentially analysed on the same sample using substrates of CI, CI+CII and  
24 CII as followed: first, NADH supply to CI was induced by adding 2.5mM pyruvate and 5mM  
25 malate (+ADP, 1.5mM). Then, NADH supply was further increased by addition of 5mM

1 glutamate. Finally, Succinate (10mM) was added to measure the ROS production with  
2 convergent CI+II electron flow into the Q-junction, corresponding to the maximal stimulated  
3 phosphorylating respiration. Specific H<sub>2</sub>O<sub>2</sub> fluxes were calculated in real-time using the  
4 DatLab software (OROBOROS Instruments, Innsbruck, Austria) from the positive time  
5 derivative of the resorufin signal over time.

6

### 7 **TCA cycle analysis**

8 Oxidation of CI substrates was followed on 6-7.10<sup>6</sup> permeabilized fibroblasts. 2.5mM  
9 pyruvate and 5mM malate were added and a 1ml aliquot was immediately removed for acid  
10 organic analysis. Five minutes after substrate addition, sub-saturating ADP concentration  
11 (0.5mM) was added. Precisely 30 minutes later, a new 1ml aliquot was withdrawn. Oxygen  
12 consumption was controlled during all the experiment. Both aliquots were treated with 200μl  
13 of 7% perchloric acid then rapidly stirred, frozen in liquid nitrogen and thawed at 37°C,  
14 centrifuged 5 minutes at 20,000g, 4°C. Supernatants were extracted by ethyl acetate (1/7 v/v),  
15 evaporated, silylated (45 minutes, 80°C) and injected in a gas chromatography/mass  
16 spectrophotometer (GCMS-2010C, Shimadzu, Marne la Vallée, France). Peaks were  
17 identified and quantified by the GCMS solution software (Shimadzu, Marne La Vallée,  
18 France).

19

### 20 **Western blotting and BN-PAGE analysis**

21 Cellular proteins solubilized in a Laemmli buffer were resolved by SDS-PAGE as described  
22 [27].

23 For complex I and supercomplexes assembly analyses, mitochondria were enriched by using  
24 differential centrifugation, according to a slightly modified version of the method described  
25 by Bonnet et al (2008). Briefly, the cell pellets were incubated 10 min on ice with cold

1 digitonin (4mg/ml in PBS, v/v; 0.2% w/v) to dissolve cell membranes. Then, digitonin was  
2 diluted by addition of cold PBS (5 v/v). Cells were centrifugated for 10 min, at 10,000g and 4  
3 °C to recover the mitochondria-enriched fraction. The pellet was washed once more in 1 ml  
4 cold PBS, centrifuged (10,000g, 4°C), resuspended at 10 mg/ml in AC/BT buffer (1.5 M  
5 aminocaproic acid and 75 mM Bis-Tris/HCl, pH 7.0, supplemented with Complete Mini  
6 Protease Inhibitor (Roche Diagnostics, Stockholm, Sweden)) and kept frozen at -80°C until  
7 analysis. For blue native (BN) PAGE analyses, 50 µg of the samples were diluted at 2 mg/ml  
8 in AC/BT buffer and mitochondrial membrane proteins were solubilized by incubation with  
9 the dedicated detergent for 10 minutes at 4°C. After centrifugation for 20 minutes, 20,000g at  
10 4°C, the supernatant was collected, and 5% Serva Blue G dye (Biorad, Marnes-la-Coquette,  
11 France) in 1 M aminocaproic acid/50 mM Bis-Tris/HCl, pH 7 was added (1/20 v/v) prior to  
12 loading. The following detergents were used (a) 3g/g protein n-dodecyl β-d-maltoside for  
13 preparation of native respiratory chain complexes and (b) 6 g/g protein digitonin (1.2% w/v)  
14 to detect the supercomplexes. Respiratory chain complexes or supercomplexes were separated  
15 on Native PAGE Novex 3-12% Bis-Tris gels (Invitrogen) for approximately 3 hours and  
16 transferred on PVDF membranes (GE Healthcare, Velizy-Villacoublay, France) in cold  
17 blotting buffer (25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol). Membranes were  
18 hybridized using dedicated monoclonal antibodies (NDUFS2, NDUF6, SDHA, III core2,  
19 COX5A, Abcam). Due to the reduced dynamic range of chemiluminescence, the high  
20 intensity of the CI holoenzyme band could blunt the CI intermediate detection. Thus, to avoid  
21 this limitation, we developed a new approach based on the detection of assembly factors that  
22 specifically bind to CI intermediates and are removed once the holoenzyme is fully  
23 assembled. Antibodies against assembly factors NDUF4 (Rabbit monoclonal antibody,  
24 Abcam) that specifically bind the Q and Q/Pp-a, NDUF1 (Rabbit monoclonal antibody,  
25 Abcam) which binds the membrane Pp-b and NDUF2 (Rabbit monoclonal antibody,

1 Abcam) which stacks the Q/P intermediates, were used after careful validation of the method  
2 (see Supplementary Figure 1). Acquisitions were performed using the Odyssey FC imaging  
3 system and analysed using Image Studio™ Lite (LI-COR Biosciences).

4

#### 5 **Metabolites measurements**

6 Cellular glucose consumption and lactate production were determined on cell supernatant  
7 after 48 hours of cell culture on an Architect C16000 apparatus using the Glucose assay for  
8 Architect C™ system and the Lactate assay for Architect C™ system (Abbott, Ringis,  
9 France). Cellular NAD<sup>+</sup>/NADH ratio and ADP/ATP ratio were measured on cell lysates  
10 according to the manufacturer instructions (NAD/NADH assay kit and ADP/ATP ratio assay  
11 kit, Abcam, Cambridge, UK).

12

#### 13 **QUANTIFICATION AND STATISTICAL ANALYSIS**

14 At least two biological replicates of each fibroblast cell line were analyzed in duplicate. All  
15 fibroblasts cell lines were systematically analyzed (Ctr, n=11; FA, n=19; AB, n=10), except  
16 when specified. Results are represented as mean  $\pm$  SEM. The nonparametric Mann–Whitney  
17 test (\*, \$ symbols) or its version for paired data (Wilcoxon test, # symbols) were used for  
18 means comparison (Statview, SAS Institute Inc., Cary NC, USA). All data were considered  
19 statistically significant at  $p < 0.05$ . For regression analyses, the best fitting model and the  
20 statistical significance were determined using SigmaPlot software (Systat Software, Inc., San  
21 Jose, USA) using  $t$  test and  $F$  test for the regression significance.

22 Principal component analysis (PCA) score plot was conducted to detect grouping of similar  
23 cell lines and outliers according to structural, biochemical and metabolic parameters (X  
24 matrix). An orthogonal partial least-squares discriminant analysis (OPLS-DA), a supervised  
25 pattern-recognition method, was further used to maximize the variation between groups and

1 determine the most significant variables contributing to this variation. The quality of models  
2 was validated by determining  $R^2$  (goodness-of-fit) and  $Q^2$  (goodness-of-prediction) values. In  
3 the model with the best predictive capabilities, the significant variables of orthogonal partial  
4 least-squares discriminant analysis models can be detected and selected by plotting VIP  
5 versus coefficient values (“volcano” plot). VIP values summarize the importance of each  
6 variable for the OPLS-DA model, while coefficient values summarize the relationship  
7 between the Y (groups) and X (matrix of measured parameters) variables. Variables with a  
8 VIP value  $\geq 1$  are considered important for group discrimination in predictive models. The  
9 multivariate data analysis was conducted using SIMCA-P software 13.0 (Umetrics, Umeå,  
10 Sweden).

11

## 12 **RESULTS**

### 13 **Different metabolic rewirings are set to face CI deficiencies.**

14 First, we checked the CI maximal activity (Supplementary information, Fig. S3A1) and  
15 maximal CI-linked respiration (St3 MP, Supplementary information, Fig. S3A2) of our cohort  
16 of 29 CI patient fibroblast cell lines and 11 controls (Supplementary information Fig. S2A).  
17 As expected, NADH Ubiquinone Reductase activity was decreased in all CI-mutated cell  
18 lines, except in the MT-ND4 and MT-TL cells with the Lowest heteroplasmy Levels (LL, i.e.  
19 MT-TL1.1 and MT-TL1.2, Supplemental information S2A Supplementary information, Fig.  
20 S3A1). By contrast, the maximal CI-linked respiration was reduced for all patient cell lines  
21 by at least 40% (Supplementary information Fig. S3A2), emphasizing a defect in CI function  
22 for all CI mutant cells. We then checked the maximal oxidative phosphorylation (OXPHOS)  
23 capacity by measuring the combined CI+CII phosphorylating respiration (St3 MPS,  
24 Supplementary information Fig. S3B). This parameter was not significantly reduced (-20 to -

1 30%), except for MT-TL and MT-TK cells (-60%). This latter particular feature of MT-  
2 TRNA mutated cells may be accounted for the additional decreased in both complexes IV and  
3 V activities (-60 and -70%, respectively, (data not shown), limiting the phosphorylating  
4 capacity. The relatively preserved maximal OXPHOS capacity witnessed that nearly all cells  
5 were able to compensate the CI deficiency by FADH<sub>2</sub> oxidation through CII, when its  
6 substrate is provided in excess.

7 Then, we characterized the balance between oxidative and glycolytic metabolism of the CI  
8 patient and control fibroblast cells (Fig. 1A, Supplementary information, Fig S3). Cellular  
9 oxidative metabolism was analysed by measuring on intact cells the routine (Routine,  
10 Supplementary information, Fig. S3C1) and the phosphorylating respirations (Routine -  
11 Oligomycin-insensitive respiration, R-O, Supplementary information, Fig. S3C2). Maximal  
12 respiratory chain capacity was measured after respiration uncoupling using FCCP (F,  
13 Supplementary information, Fig. S3D) and the relative use of the maximal capacity to sustain  
14 oxidative phosphorylation was calculated as the (R-O)/F ratio (Supplemental information,  
15 Fig. S3E). In parallel, we estimated the glycolytic metabolism by measuring the glucose  
16 conversion to lactate (L/G ratio, Supplementary information, Fig. S3F). Finally, these two  
17 parameters were represented on a same graph named metabolic orientation which represented  
18 one a same graph bar the use of glycolytic (L/G ratio) and oxidative ((R-O)/F) capacities in  
19 percent of control cells (Fig. 1A). Importantly, the metabolic balance was switched towards  
20 aerobic glycolysis only in a subset of mutated cells (Fig. 1A, Supplementary information Fig.  
21 S3E and S3F), suggesting different metabolic reprogramming among CI deficient cells.

22 To statistically process all the aforementioned data, we performed a non-supervised principal  
23 component analysis (PCA-X, Fig. 1B). The resulting model showed strong fitting ( $R^2X =$   
24 0.722) capabilities, distinguishing two groups among the CI-deficient cells which were



1 separated according to their metabolic orientation (x-axis: left side, L/G vs right side, R, R-O  
2 and R-O/F, Fig. 1B). The group A included all *MT-ND*, *MT-TL LL*, *MT-TP*, *NDUFV1.1*,  
3 *NDUFS4.1* and *NDUFA9* mutated cells while the group B encompassed all nDNA mutated  
4 cells, with the exception of *NDUFA9*, *NDUFV1.1* and *NDUFS4.1*, and the *MT-TK* and *MT-TL*  
5 with the Highest heteroplasmy Level (HL, *MT-TL1.3* and *MT-TL1.4*, Supplementary  
6 information S2A) (hierarchical clustering, Supplementary information Fig. S3G). To gain  
7 insights in the discriminating parameters, we performed a supervised analysis (OLPS-DA,  
8 Fig. 1C1) based on these two groups defined by PCA. The Volcano plot (Fig. 1C2) confirmed  
9 that the most discriminative parameters between the two groups were the oxidative  
10 metabolism (R, R-O, R-O/F, VIP >1) vs the glycolytic metabolism (L/G)), while CI  
11 functionality (St3 MP and CI/CS) only accounted for a minor weight in the model (VIP <0.9).  
12 In the light of these analyses, we reconsidered our data according to the different groups  
13 defined by the PCA analysis.

14 In the group A, the oxidative metabolism (Routine and phosphorylating (R-O) respirations  
15 Fig. 1D1 and 1D2) was maintained thanks to the use of a higher part of the spare capacity ((R-  
16 O)/F, +30%, Fig. 1D3, Supplemental Figure 3H) and the glycolysis was barely increased  
17 (increase in glucose consumption and lactate production, Fig. 1E1 and increase in L/G: +27%,  
18 Fig. 1E2). By contrast, the oxidative metabolism was significantly decreased in the group B  
19 (Routine - 47% and phosphorylating respiration, R-O, - 60% compared to Ctr, Fig. 1D1 and  
20 1D2, Supplemental Figure 3H). Surprisingly, these cells did not used and even reduced the  
21 use of their respiratory capacity to maintain oxidative phosphorylation, as illustrated by the  
22 decreased (R-O)/F ratio (-40%, Figure 1D3, Supplemental Figure 3H), substantiating a clear  
23 shutdown of the oxidative metabolism in the group B. Simultaneously, in these cells, glucose  
24 was almost exclusively converted to lactate (Fig. 1E1), as indicated by a L/G ratio close to 2

1 (+77%, Fig. 1E2, Supplemental Figure 3H). In addition, we found a low cellular ATP/ADP  
2 ratio (-55% compared to Ctr and group A, Fig. 1F) that was not modified by the inhibition of  
3 the phosphorylating respiration (+ Oligomycin), confirming that the ATP synthesis in the  
4 group B is mainly supported by the glycolytic metabolism. Altogether, these results revealed  
5 two different metabolic adaptations: in the group A, both the oxidative and glycolytic  
6 metabolisms are slightly stimulated, while the equilibrium between them is maintained  
7 (metabolic orientation, glycolytic vs oxidative metabolisms, i.e. L/G vs (R-O)/F, Fig. 1G),  
8 evidencing a hyper-catabolism. Conversely, in the group B, the metabolism is drastically  
9 switched toward aerobic glycolysis at the expense of the oxidative phosphorylation, namely  
10 the Warburg effect (Fig. 1G).

11 It is generally thought that the glycolytic switch is linked to the degree of CI deficiency.  
12 However, while CI activity was more decreased in B than in the A group, no difference in the  
13 degree of CI-linked respiration deficiency was observed between the two patient groups (Fig.  
14 1H1 and 1H2). Moreover, neither the CI activity nor the CI supported maximal respiration  
15 rate correlated with the metabolic orientation, confirming that these parameters are not  
16 discriminant in the observed metabolic adaptations (Fig. 1I1 and 1I2). CI activity is also a  
17 crucial regulator of  $\text{NAD}^+/\text{NADH}$  ratio [23]. This ratio was decreased in both groups,  
18 compared to Ctr (Fig. 1J) but was neither different between the two groups nor correlated to  
19 the CI holoenzyme quantity (Fig. 1K), excluding a regulation of metabolic rewiring by  
20  $\text{NAD}^+/\text{NADH}$  ratio.

### 21 **CI assembly is a key determinant of metabolic rewiring in CI deficient cells.**

22 The location of the mutated subunits within CI could not only affect its activities (NADH  
23 oxidation, proton pumping) but also its structure. Thus, we evaluated by Blue Native PAGE  
24 the impact of the different mutations on CI assembly (Fig. 2A and Supplementary

1 information, Fig. 4A, B). CI holoenzyme was detected by using antibodies targeting structural  
2 CI subunits (NDUFS2 and NDUF6). CI intermediates were revealed with chaperon targeted  
3 antibodies (NDUFA4, NDUFA1 and NDUFA2) that bind the matrix arms (Q and Q/Pp-  
4 a), the membrane arm (Pp-b) and the 830kDa intermediates, respectively (Supplementary  
5 information, Fig. 1B). The analysis revealed two CI assembly profiles (Figure 2A, 2B and  
6 Supplementary information, Fig. S4A). Firstly, in group A, CI holoenzyme quantity was not  
7 decreased by more than thirty percent (Fig. 2A, 2B1 and Supplementary information, Fig.  
8 S4A1) while no significant accumulation of CI intermediates was evidenced (Fig. 2A, 2B1  
9 and Supplementary information, Fig. S4A2) and the equilibrium between CI holoenzyme and  
10 assembly intermediates was maintained (Fig. 2B2). This group was thus renamed FA for  
11 Fully Assembled. Secondly, in group B, CI holoenzyme quantity was markedly reduced (Fig.  
12 2A, 2B1 and Supplemental information, Fig. S4A) and either matrix (Q/Pp-a, MT-TRNA  
13 cells), membrane (Pp-b, nDNA) or membrane and late-stage (Q/P, N-module mutated cells)  
14 intermediates accumulated (Supplemental information, Fig. 4B). Whatever the blocking step  
15 involved, these data emphasized a strong CI assembly defect in B group (Fig. 2B2). This  
16 group was renamed AB for Assembly Blockage. Importantly, the level of assembly defects  
17 strongly correlated with the metabolic rewiring (Fig. 2C,  $R^2=0.83$ ), meaning that when the  
18 quantity of the CI holoenzyme is reduced, the metabolic orientation is proportionally switched  
19 towards glycolysis. Moreover, the supervised analysis according to the defined groups and the  
20 corresponding Volcano plot (Supplemental information, Fig. 4C) confirmed that the most  
21 discriminative parameters between the two groups are the CI assembly status (CI holoenzyme  
22 quantity (CI) vs the accumulation of assembly intermediates (A.I)).

23 As our results question the common idea that the CI functions dictate the metabolic  
24 adaptations, we strengthened our analysis by selecting all the cell lines that displayed strictly

1 the same CI functionality (CI maximal activity and CI-linked maximal respiration) and  
2 comparable maximal phosphorylating capacity, thus differing only by their CI assembly level  
3 (Fig. 2D). Among them, only the ones with a CI assembly blockage showed a decreased use  
4 of respiratory spare capacity ((R-O)/F, -50%) and an increased use of glycolytic metabolism  
5 (L/G +50%, Supplementary information, Fig. 2D), witnessing a metabolic switch towards  
6 glycolysis. Thus, the metabolic shift observed in AB cells is clearly not related to the severity  
7 of CI deficiency but rather to the level of CI assembly defect.

8

### 9 **Targeted inductions of CI assembly defects reproduce the metabolic impairment of AB** 10 **cells**

11 To reinforce the link between the CI assembly defect and the metabolic switch, we induced  
12 either a CI functional inhibition or a CI assembly defect in Ctr fibroblasts, using  
13 pharmacological agents or siRNA. First, piericidin A, a CI inhibitor that has no effect on its  
14 assembly (Fig. 3A1 and Supplementary information, Fig. 5A) was used to mimic the FA  
15 group. As expected, piericidin A significantly reduced the CI activity (-62±4%,  
16 Supplementary information, Fig. S5B) and all respiratory parameters (F, R and R-O  
17 respirations (- 53%, - 41% and - 72% compared to vehicle treated cells, Fig. 3A2), thus  
18 substantiating a decrease in the maximal respiratory chain capacity in accordance with the  
19 reduced CI enzymatic activity. However, the part of the respiratory capacity dedicated to the  
20 ATP synthesis was preserved ((R-O)/F, Fig. 3A3) thanks to a higher use of the maximal  
21 respiration capacity (R/F, Fig. 3A3). Piericidin A did not induce a glycolytic switch, as shown  
22 by a conserved metabolic orientation and L/G ratio (1.110 for vehicle vs 0.974 for piericidin  
23 A-treated cells, Fig. 3A4 and 3A5). Then, in order to induce the accumulation of CI assembly  
24 intermediates, we treated Ctr cells with either sodium pyruvate (NaPyr, Fig. 3B) or with a

1 siRNA targeting the assembly factor *NDUFAF1* (Fig. 3C). NaPyr is a TIM23 modulator that  
2 favors the membrane sorting of nuclear-encoded respiratory chain complex subunits [28] and  
3 is thus expected to induce membrane intermediate accumulation. In NaPyr treated cells, a  
4 60% increase of the membrane Pp-b intermediate together with a small decrease in CI  
5 holoenzyme quantity (-20% compared to vehicle, Figure 3B1 and Supplementary Figure 5A)  
6 were observed inducing a 46% decrease in R (routine) respiration and a 64% decrease in  
7 phosphorylating (R-O) respiration without any significant modification of the respiratory  
8 complex activities (Supplementary Figure 5C). This led to a decrease in oxidative  
9 metabolism as evidenced by the 39% decrease in (R-O)/F ratio. *NDUFAF1* is an assembly  
10 factor stabilizing the membrane Pp-b intermediate, thus its silencing by siRNA is expected to  
11 induce the accumulation of matrix Q intermediates. Inhibition of *NDUFAF1* expression  
12 (Supplementary information, Fig. S5D) decreased Pp-b intermediate amount, leading to a  
13 consistent accumulation of the Q/Pp-a intermediate (Fig. 3C1 and Supplementary  
14 information, Fig. S5A). Neither the accumulation of Pp-b (NaPyr treatment) nor of Q/Pp-a  
15 (*NDUFAF1* siRNA) intermediates significantly modified the maximal respiratory capacity (F,  
16 Fig. 3B2, 3C2). However, both the routine and phosphorylating respirations were decreased  
17 (R (-48% compared to vehicle treated cells) and R-O (-63% compared to vehicle treated  
18 cells), Fig. 3B2, 3C2), indicating an oxidative metabolism inhibition. Moreover, the  
19 accumulation of both Pp-b and Q/Pp-a intermediates decreased the part of the respiratory  
20 capacity used for ATP synthesis ((R-O)/F: -56% and -40%, respectively, Fig. 3B3, 3C3) and  
21 led to a higher conversion of glucose to lactate (L/G ratios: +77% and +39%, respectively,  
22 Fig. 3B4, 3C4). These results confirmed that CI disassembly slows down the oxidative  
23 metabolism and triggers a metabolic reprogramming towards glycolysis (Fig. 3B5, 3C5).

24

1 **ROS overproduction due to CI misassembly is involved in the metabolic rewiring.**

2 We previously demonstrated that CI assembly defect induced ROS overproduction linked to  
3 the presence of unconnected active N module [29]. In the present study, the presence of  
4 unassembled N module was evidenced by BN-PAGE in both control and mutated cells (Fig.  
5 4A1), but did accumulate relative to CI holoenzyme quantity only in AB cells (Fig. 4A2).  
6 Maximal ROS production was measured in parallel to the maximal respiration rate, on  
7 permeabilized fibroblasts in the presence of CI and CI+CII (Fig. 4B) substrates. CI ROS  
8 production was somehow increased in the FA group (+54%), while drastically increased in  
9 the AB group (+421%). Furthermore, the ROS production increased with the NADH supply  
10 (St3 MPG *vs* St3 MP) in AB, but not in FA cells and remained higher than in Ctr cells even in  
11 the presence of the CII substrate (+100% for CI+CII, St3 MPGS, Fig. 4B). Regression  
12 analyses (Fig. 4C) indicated strong correlations between ROS production and both CI  
13 holoenzyme and unassembled N module quantities on one hand, and ROS production and  
14 metabolic orientation on the other hand. The ROS production by CI was also stimulated in  
15 NaPyr-treated cells (+60%, Fig. 4D), further emphasizing that CI disassembly is associated  
16 with increased ROS production. The cellular impact of ROS overproduction was assessed by  
17 measuring the lipid peroxidation adduct-content (4-hydroxynonenal, HNE) by Western  
18 blotting. HNE content was higher in AB than in FA and Ctr cells (+150%, Supplemental  
19 information, Fig. S6A1 and S6A2), revealing a systemic presence of oxidative stress  
20 specifically in the AB cells.

21 To further explore the involvement of ROS production in the metabolic switch, we induced in  
22 Ctr cells a CI-driven ROS overproduction using mito-Paraquat (mt-PQ) treatment [22]. mt-PQ  
23 treatment increased ROS production by 485% (Fig. 4E1) while inducing decrease in CI-linked  
24 respiration (-66%, Fig. 4E2) and CI maximal activity (-55%, Fig. 4E3). Additionally, mt-PQ

1 treatment reduced the routine (R, -55%, Fig. 4E4) and the phosphorylating respiration (R-O, -  
2 90%, Fig. 4E4), while it increased the L/G ratio (+38%, Fig. 4E5), thus emphasizing the onset  
3 of a glycolytic switch. Surprisingly, the treatment of Ctr cells with mt-PQ significantly  
4 reduced the amount of CI and II, with a particularly marked effect on the CI assembly (Fig.  
5 4E6). Indeed, mt-PQ induced a drastic reduction in the amount of CI holoenzyme paralleled  
6 by the accumulation of an intermediate of about 700 kDa. This suggests a close interrelation  
7 between the levels of ROS production and CI assembly.

8 Alternatively, we inhibited ROS production with the anti-oxidant N-Acetyl Cysteine (NAC)  
9 [30, 31]. NAC treatment specifically increased both the routine (Routine, +50%) and the  
10 phosphorylating (R-O, +79%) respirations in AB cells (Fig. 4F1) and also significantly  
11 decreased the glucose conversion to lactate (L/G, -20%, Fig. 4F2). While NAC treatment  
12 increased CI holoenzyme quantity in Ctr cells, in AB cells, it did not alleviate the assembly  
13 defect of CI caused the mutations in nuclear-encoded CI subunit (Fig. 4F3 and supplemental  
14 information Fig. S6B1, B2). Despite a CI that remains disassembled, cellular metabolism  
15 returns to a more oxidative one with the antioxidant treatment (Fig. 4F1). This result indicates  
16 that the effects of NAC on cellular metabolism do indeed involve the decrease in ROS  
17 production and not a correction of the CI assembly defect in AB cells, placing ROS between  
18 the CI disassembly and the metabolic switch.

19

20 Altogether, these results emphasized that ROS overproduction is involved in a signaling  
21 pathway linking CI disassembly to the glycolytic switch.

22

1 **A ROS/AMPK/PDH pathway is involved in the metabolic rewiring induced by CI**  
2 **disassembly**

3 AMPK is a main sensor of mitochondrial energetic stress. In patient cells, AMPK  
4 phosphorylation was increased in AB group only (+74% compared to Ctr, Fig. 5A1).  
5 Interestingly, mt-PQ treatment of Ctr cells also induced an increase in AMPK  
6 phosphorylation (+ 71%, Fig. 5A2). Because the PDH-phosphorylation is an essential actor of  
7 the metabolic rewiring [32, 33] we monitored the phosphorylation status of the PDH E1 $\alpha$   
8 subunit. We found a 2.5-fold increase of its phosphorylation in the AB group, compared to the  
9 FA and control groups (Fig. 5B1). Furthermore, mt-PQ treatment enhanced the PDH  
10 phosphorylation in Ctr cells (+79%, Fig. 5B2). To decipher this signaling cascade, we  
11 inhibited either ROS production with NAC or AMPK activity using Compound C.  
12 Remarkably, both treatments decreased PDH phosphorylation in AB cells, with a higher  
13 extent for Compound C (-80%) than for NAC (-20%, Fig. 5C). Altogether, these results  
14 disclose a signaling pathway involving ROS overproduction due to CI disassembly, which  
15 induces the PDH over-phosphorylation in an AMPK dependent manner. To confirm the  
16 functional role of PDH inhibition in the decreased oxidative metabolism in AB cells, we  
17 assessed the mitochondrial pyruvate oxidation by measuring the formation of the tricarboxylic  
18 acid (TCA) cycle intermediates on permeabilized cells. In Ctr and FA cells, citrate and  $\alpha$ -  
19 ketoglutarate were generated from pyruvate and malate oxidation (Fig. 5D) and only 5% of  
20 the pyruvate was converted to lactate. By contrast, in the AB group, pyruvate oxidation was  
21 strongly reduced and citrate formation was not detected, while a high level of lactate  
22 production was evidenced (+150%, Fig. 5D), confirming a blockage of pyruvate oxidation  
23 upstream of the TCA cycle, linked with the inhibition of the Pyruvate Dehydrogenase (PDH).  
24 We confirmed this observation by adding, during oxygraphic measurement, Glycerol-3-



1 Phosphate (G3P) which directly increases FADH<sub>2</sub> supply to the respiratory chain, bypassing  
2 the PDH regulation (Supplementary information, Fig. S7). G3P stimulated the oxidative  
3 metabolism, particularly in the AB group (+10%, +30% and +70%, in Ctr, FA and AB,  
4 respectively, Supplementary information, Fig. S7A1), with the routine respiration reaching  
5 the maximal capacity in the AB cells (R/F ratio close to 1, Supplementary information, Fig.  
6 S7A2) and led to a complete restoration of the part of the respiration dedicated to ATP  
7 synthesis ((R-O)/F, Supplementary information, Fig. S7A3). These results show that, in AB  
8 cells, the inhibition of mitochondrial pyruvate supply can be overcome by sustaining the  
9 FADH<sub>2</sub> production.

10 Our results, summarized in Fig. 6 A and B demonstrated that ROS overproduction was  
11 negatively correlated to CI assembly status and positively correlated to both PDH  
12 phosphorylation and metabolic rewiring placing this triad as a key determinant of the  
13 glycolytic switch.

14

15

16

## 17 **DISCUSSION**

18 Mitochondrial CI is the largest integral membrane enzyme of the respiratory chain. Its  
19 biogenesis is a highly intricate process involving the assembly of both nDNA- and MT-DNA-  
20 encoded proteins into subcomplex intermediates, finally combined into the functional  
21 holoenzyme. While defects in its assembly are a frequent cause of mitochondrial disorders,  
22 their relative contributions in the pathophysiology of CI-linked diseases have not been  
23 precisely studied. It has been shown that CI assembly is down-regulated under oxidative [34]

1 or hypoxic stress [35], and that unbalanced CI assembly causes premature senescence [19].  
2 However, the effect of CI assembly defects and in particular the accumulation of assembly  
3 intermediates on cellular metabolism remains largely unknown.

4 In this study, we provide the first evidences demonstrating the central role of CI assembly,  
5 rather than CI activity, in the onset of the metabolic reprogramming in CI deficiency. Indeed,  
6 the glycolytic switch is not observed in cells with a pure CI catalytic defect, neither in patient  
7 cells nor in piericidin A-treated control cells. Moreover, the PCA-based statistical analysis  
8 clearly evidenced that the level of the metabolic switch is tightly correlated to the degree of  
9 CI disassembly. Our results suggest that rather than CI holoenzyme quantity, it is the  
10 accumulation of CI assembly intermediates that is involved in metabolic reprogramming. In  
11 this respect, a metabolic switch, evidenced by an increase in L/G ratio and a shutdown of  
12 oxidative metabolism ((R-O)/F) can be triggered by NaPyr treatment or NDUFAF1  
13 downexpression in control cells by inducing a CI assembly intermediates overrepresentation,  
14 without impairing CI function. This further supports the fact that CI misassembly, but not its  
15 activity, is the culprit mechanism responsible for the metabolic switch and confirms a  
16 previous study suggesting that CI assembly could be involved in the switch of energy  
17 metabolism towards glycolysis, in smooth muscle cells in pulmonary hypertension [36].

18 This process occurs through a decrease in substrate inputs into the respiratory chain through  
19 the inhibition of the PDH and TCA cycle. CI not only acts as a metabolism hub essential to  
20 energy production but it is also one of the major source of ROS production [37]. In our study,  
21 although all the CI deficient cell lines displayed an increase in CI-related ROS production, the  
22 level of CI disassembly was the main contributor to ROS overproduction, a relationship  
23 already reported in Parkinson disease [38] and ageing [19]. We propose that this ROS  
24 overproduction is related to the toxicity of the CI intermediates, as we and others previously

1 demonstrated that, even when disconnected from the CI, the matrix N-module retains a  
2 NADH dehydrogenase activity [29, 39, 40] and participates to superoxide generation and  
3 oxidative stress [29]. Although known as damaging molecules, ROS are also increasingly  
4 considered as important signalling mediators [41, 42], notably involved in the AMPK  
5 dependent activation [43] of the PDH kinase [44] and glycolytic switch [45]. Here, we found  
6 that NAC antioxidant treatment partly reversed the PDH phosphorylation and the glycolytic  
7 switch, whereas increasing ROS production stimulated the AMPK and PDH phosphorylation  
8 and downstream the glycolytic metabolism. Our findings suggest that ROS production  
9 represents a tipping point, below which cells increase the catabolism to sustain the oxidative  
10 metabolism and over which cells shutdown the respiratory chain through PDH inhibition,  
11 impeding the metabolic flexibility necessary to meet the energetic cell requirements. Such a  
12 threshold of ROS overproduction is reached when a significant accumulation of CI assembly  
13 intermediates occurs. By contrast, in cells with a pure CI catalytic defect, a compensatory  
14 hyper-catabolism allows to maintain the oxidative metabolism at the expense of an increased  
15 fuel use. One can suppose that this hyper-catabolism copes with cellular metabolic  
16 requirements in basal conditions, but will fail to face an energetic challenge as exercise,  
17 infections or fasting.

18 Our conclusions demonstrating the importance of CI assembly in ROS production and in  
19 metabolic adaptations should prompt the improvement and refinement of antioxidant  
20 strategies and define targeted therapeutic routes for treating the two specific groups of CI  
21 patients that we defined in this work. Moreover, as CI deficiency is observed in an increasing  
22 number of pathophysiological situations as aging, cancer and neurological disorders, our data  
23 not only shed light on the importance of assessing CI assembly in these clinical conditions,

1 but also identified molecular pathways to be targeted for the design of future therapies of  
2 these diseases.

3

4

5

1 **AUTHORS CONTRIBUTION**

2 Conceptualization, methodology and writing - original draft: V.D.D., N.G.; validation: Ge.L,  
3 G.G; investigations: V.D.D., C.W., S.C., A.L., M.L.M., C.G., L.T., N.G.; resources: M.B.,  
4 S.L., P.A.B., D.B.; data curation: D.G., writing-review and editing: M.S.K., S.K., A.C., V.P.,  
5 P.R., G.L.; supervision: D.H., P.R., G.L.; funding acquisition: D.B., V.P., P.R., G.L.

6

7 **ACKNOWLEDGMENTS**

8 This work was supported by the following institutions and patient associations : Région Pays  
9 de la Loire, Angers Loire Métropole, Université d'Angers and CHU d'Angers, Fondation  
10 pour la Recherche Médicale, l'Union Nationale des Aveugles et Déficients Visuels, Retina  
11 France, Ouvrir les Yeux, Association contre les Maladies Mitochondriales, Fondation  
12 Maladies Rares. We are indebted to the partnership between the UNADEV and the ITMO  
13 NNP (l'Institut Thématique Multi-Organisme Neurosciences, sciences cognitives, neurologie,  
14 psychiatrie) / AVIESAN (alliance nationale pour les sciences de la vie et de la santé), for their  
15 involvement in research on diseases affecting the vision.

16

17

## 1 REFERENCES

- 2 [1] L. Iommarini, M.A. Calvaruso, I. Kurelac, G. Gasparre, A.M. Porcelli, Complex I  
3 impairment in mitochondrial diseases and cancer: parallel roads leading to different outcomes,  
4 *Int J Biochem Cell Biol*, 45 (2013) 47-63.
- 5 [2] L. Iommarini, I. Kurelac, M. Capristo, M.A. Calvaruso, V. Giorgio, C. Bergamini, A.  
6 Ghelli, P. Nanni, C. De Giovanni, V. Carelli, R. Fato, P.L. Lollini, M. Rugolo, G. Gasparre,  
7 A.M. Porcelli, Different mtDNA mutations modify tumor progression in dependence of the  
8 degree of respiratory complex I impairment, *Hum Mol Genet*, 23 (2014) 1453-1466.
- 9 [3] P. Peruzzo, M. Comelli, E. Di Giorgio, E. Franforte, I. Mavelli, C. Brancolini,  
10 Transformation by different oncogenes relies on specific metabolic adaptations, *Cell Cycle*,  
11 15 (2016) 2656-2668.
- 12 [4] L.K. Sharma, H. Fang, J. Liu, R. Vartak, J. Deng, Y. Bai, Mitochondrial respiratory  
13 complex I dysfunction promotes tumorigenesis through ROS alteration and AKT activation,  
14 *Hum Mol Genet*, 20 (2011) 4605-4616.
- 15 [5] P. Wang, M. Song, Z.L. Zeng, C.F. Zhu, W.H. Lu, J. Yang, M.Z. Ma, A.M. Huang, Y.  
16 Hu, P. Huang, Identification of NDUFAF1 in mediating K-Ras induced mitochondrial  
17 dysfunction by a proteomic screening approach, *Oncotarget*, 6 (2015) 3947-3962.
- 18 [6] P. Jezek, L. Hlavata, Mitochondria in homeostasis of reactive oxygen species in cell,  
19 tissues, and organism, *Int J Biochem Cell Biol*, 37 (2005) 2478-2503.
- 20 [7] I.S. Kil, K.W. Ryu, S.K. Lee, J.Y. Kim, S.Y. Chu, J.H. Kim, S. Park, S.G. Rhee, Circadian  
21 Oscillation of Sulfiredoxin in the Mitochondria, *Mol Cell*, 59 (2015) 651-663.
- 22 [8] C. Canto, K.J. Menzies, J. Auwerx, NAD(+) Metabolism and the Control of Energy  
23 Homeostasis: A Balancing Act between Mitochondria and the Nucleus, *Cell Metab*, 22 (2015)  
24 31-53.
- 25 [9] C. Canto, J. Auwerx, NAD<sup>+</sup> as a signaling molecule modulating metabolism, *Cold Spring*  
26 *Harb Symp Quant Biol*, 76 (2011) 291-298.
- 27 [10] E.N. Maldonado, J.J. Lemasters, ATP/ADP ratio, the missed connection between  
28 mitochondria and the Warburg effect, *Mitochondrion*, 19 Pt A (2014) 78-84.
- 29 [11] E. Panieri, M.M. Santoro, ROS homeostasis and metabolism: a dangerous liason in  
30 cancer cells, *Cell Death Dis*, 7 (2016) e2253.
- 31 [12] L.R. Stein, S. Imai, The dynamic regulation of NAD metabolism in mitochondria, *Trends*  
32 *Endocrinol Metab*, 23 (2012) 420-428.

- 1 [13] R.J. Rodenburg, Mitochondrial complex I-linked disease, *Biochim Biophys Acta*, 1857  
2 (2016) 938-945.
- 3 [14] G. Geffroy, R. Benyahia, S. Frey, V. Desquiret-Dumas, N. Gueguen, C. Bris, S. Belal, A.  
4 Inisan, A. Renaud, A. Chevrollier, D. Henrion, D. Bonneau, F. Letournel, G. Lenaers, P.  
5 Reynier, V. Procaccio, The accumulation of assembly intermediates of the mitochondrial  
6 complex I matrix arm is reduced by limiting glucose uptake in a neuronal-like model of  
7 MELAS syndrome, *Biochim Biophys Acta*, 1864 (2018) 1596-1608.
- 8 [15] K. Fiedorczuk, L.A. Sazanov, Mammalian Mitochondrial Complex I Structure and  
9 Disease-Causing Mutations, *Trends Cell Biol*, 28 (2018) 835-867.
- 10 [16] D. Ghezzi, M. Zeviani, Human diseases associated with defects in assembly of OXPHOS  
11 complexes, *Essays Biochem*, 62 (2018) 271-286.
- 12 [17] M. McKenzie, M.T. Ryan, Assembly factors of human mitochondrial complex I and their  
13 defects in disease, *IUBMB Life*, 62 (2010) 497-502.
- 14 [18] L. Sanchez-Caballero, S. Guerrero-Castillo, L. Nijtmans, Unraveling the complexity of  
15 mitochondrial complex I assembly: A dynamic process, *Biochim Biophys Acta*, 1857 (2016)  
16 980-990.
- 17 [19] S. Miwa, H. Jow, K. Baty, A. Johnson, R. Czapiewski, G. Saretzki, A. Treumann, T. von  
18 Zglinicki, Low abundance of the matrix arm of complex I in mitochondria predicts longevity  
19 in mice, *Nat Commun*, 5 (2014) 3837.
- 20 [20] E. Hutter, K. Renner, G. Pfister, P. Stockl, P. Jansen-Durr, E. Gnaiger, Senescence-  
21 associated changes in respiration and oxidative phosphorylation in primary human fibroblasts,  
22 *Biochem J*, 380 (2004) 919-928.
- 23 [21] R. Fato, C. Bergamini, M. Bortolus, A.L. Maniero, S. Leoni, T. Ohnishi, G. Lenaz,  
24 Differential effects of mitochondrial Complex I inhibitors on production of reactive oxygen  
25 species, *Biochim Biophys Acta*, 1787 (2009) 384-392.
- 26 [22] E.L. Robb, J.M. Gawel, D. Aksentijevic, H.M. Cocheme, T.S. Stewart, M.M.  
27 Shchepinova, H. Qiang, T.A. Prime, T.P. Bright, A.M. James, M.J. Shattock, H.M. Senn,  
28 R.C. Hartley, M.P. Murphy, Selective superoxide generation within mitochondria by the  
29 targeted redox cyler MitoParaquat, *Free Radic Biol Med*, 89 (2015) 883-894.
- 30 [23] V. Desquiret-Dumas, N. Gueguen, G. Leman, S. Baron, V. Nivet-Antoine, S. Chupin, A.  
31 Chevrollier, E. Vessieres, A. Ayer, M. Ferre, D. Bonneau, D. Henrion, P. Reynier, V.  
32 Procaccio, Resveratrol induces a mitochondrial complex I-dependent increase in NADH

1 oxidation responsible for sirtuin activation in liver cells, *J Biol Chem*, 288 (2013) 36662-  
2 36675.

3 [24] F. Medja, S. Allouche, P. Frachon, C. Jardel, M. Malgat, B. Mousson de Camaret, A.  
4 Slama, J. Lunardi, J.P. Mazat, A. Lombes, Development and implementation of standardized  
5 respiratory chain spectrophotometric assays for clinical diagnosis, *Mitochondrion*, 9 (2009)  
6 331-339.

7 [25] P. Benit, A. Slama, P. Rustin, Decylubiquinol impedes mitochondrial respiratory chain  
8 complex I activity, *Mol Cell Biochem*, 314 (2008) 45-50.

9 [26] M. Makrecka-Kuka, G. Krumschnabel, E. Gnaiger, High-Resolution Respirometry for  
10 Simultaneous Measurement of Oxygen and Hydrogen Peroxide Fluxes in Permeabilized  
11 Cells, Tissue Homogenate and Isolated Mitochondria, *Biomolecules*, 5 (2015) 1319-1338.

12 [27] V. Guillet, N. Gueguen, R. Cartoni, A. Chevrollier, V. Desquiret, C. Angebault, P.  
13 Amati-Bonneau, V. Procaccio, D. Bonneau, J.C. Martinou, P. Reynier, Bioenergetic defect  
14 associated with mKATP channel opening in a mouse model carrying a mitofusin 2 mutation,  
15 *FASEB J*, 25 (2011) 1618-1627.

16 [28] R.S. Aiyar, M. Bohnert, S. Duvezin-Caubet, C. Voisset, J. Gagneur, E.S. Fritsch, E.  
17 Couplan, K. von der Malsburg, C. Funaya, F. Soubigou, F. Courtin, S. Suresh, R.  
18 Kucharczyk, J. Evrard, C. Antony, R.P. St Onge, M. Blondel, J.P. di Rago, M. van der Laan,  
19 L.M. Steinmetz, Mitochondrial protein sorting as a therapeutic target for ATP synthase  
20 disorders, *Nat Commun*, 5 (2014) 5585.

21 [29] G. Leman, N. Gueguen, V. Desquiret-Dumas, M.S. Kane, C. Wettervald, S. Chupin, A.  
22 Chevrollier, A.S. Lebre, J.P. Bonnefont, M. Barth, P. Amati-Bonneau, C. Verny, D. Henrion,  
23 D. Bonneau, P. Reynier, V. Procaccio, Assembly defects induce oxidative stress in inherited  
24 mitochondrial complex I deficiency, *Int J Biochem Cell Biol*, 65 (2015) 91-103.

25 [30] O.I. Aruoma, B. Halliwell, B.M. Hoey, J. Butler, The antioxidant action of N-  
26 acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and  
27 hypochlorous acid, *Free Radic Biol Med*, 6 (1989) 593-597.

28 [31] M. Benrahmoune, P. Therond, Z. Abedinzadeh, The reaction of superoxide radical with  
29 N-acetylcysteine, *Free Radic Biol Med*, 29 (2000) 775-782.

30 [32] T. Golias, I. Papandreou, R. Sun, B. Kumar, N.V. Brown, B.J. Swanson, R. Pai, D. Jaitin,  
31 Q.T. Le, T.N. Teknos, N.C. Denko, Hypoxic repression of pyruvate dehydrogenase activity is  
32 necessary for metabolic reprogramming and growth of model tumours, *Sci Rep*, 6 (2016)  
33 31146.



- 1 [33] S. Zhang, M.W. Hulver, R.P. McMillan, M.A. Cline, E.R. Gilbert, The pivotal role of  
2 pyruvate dehydrogenase kinases in metabolic flexibility, *Nutr Metab (Lond)*, 11 (2014) 10.
- 3 [34] K.R. Pryde, J.W. Taanman, A.H. Schapira, A LON-ClpP Proteolytic Axis Degrades  
4 Complex I to Extinguish ROS Production in Depolarized Mitochondria, *Cell Rep*, 17 (2016)  
5 2522-2531.
- 6 [35] D.C. Fuhrmann, I. Wittig, S. Drose, T. Schmid, N. Dehne, B. Brune, Degradation of the  
7 mitochondrial complex I assembly factor TMEM126B under chronic hypoxia, *Cell Mol Life  
8 Sci*, 75 (2018) 3051-3067.
- 9 [36] R. Rafikov, X. Sun, O. Rafikova, M.L. Meadows, A.A. Desai, Z. Khalpey, J.X. Yuan,  
10 J.R. Fineman, S.M. Black, Complex I dysfunction underlies the glycolytic switch in  
11 pulmonary hypertensive smooth muscle cells, *Redox Biol*, 6 (2015) 278-286.
- 12 [37] J. Hirst, M.S. King, K.R. Pryde, The production of reactive oxygen species by complex I,  
13 *Biochem Soc Trans*, 36 (2008) 976-980.
- 14 [38] P.M. Keeney, J. Xie, R.A. Capaldi, J.P. Bennett, Jr., Parkinson's disease brain  
15 mitochondrial complex I has oxidatively damaged subunits and is functionally impaired and  
16 misassembled, *J Neurosci*, 26 (2006) 5256-5264.
- 17 [39] F. Valsecchi, C. Monge, M. Forkink, A.J. de Groof, G. Benard, R. Rossignol, H.G.  
18 Swarts, S.E. van Emst-de Vries, R.J. Rodenburg, M.A. Calvaruso, L.G. Nijtmans, B. Heeman,  
19 P. Roestenberg, B. Wieringa, J.A. Smeitink, W.J. Koopman, P.H. Willems, Metabolic  
20 consequences of NDUFS4 gene deletion in immortalized mouse embryonic fibroblasts,  
21 *Biochim Biophys Acta*, 1817 (2012) 1925-1936.
- 22 [40] J. Hirst, Energy transduction by respiratory complex I--an evaluation of current  
23 knowledge, *Biochem Soc Trans*, 33 (2005) 525-529.
- 24 [41] S.N. Jung, W.K. Yang, J. Kim, H.S. Kim, E.J. Kim, H. Yun, H. Park, S.S. Kim, W.  
25 Choe, I. Kang, J. Ha, Reactive oxygen species stabilize hypoxia-inducible factor-1 alpha  
26 protein and stimulate transcriptional activity via AMP-activated protein kinase in DU145  
27 human prostate cancer cells, *Carcinogenesis*, 29 (2008) 713-721.
- 28 [42] L.B. Sullivan, N.S. Chandel, Mitochondrial reactive oxygen species and cancer, *Cancer  
29 Metab*, 2 (2014) 17.
- 30 [43] E.C. Hinchy, A.V. Gruszczyk, R. Willows, N. Navaratnam, A.R. Hall, G. Bates, T.P.  
31 Bright, T. Krieg, D. Carling, M.P. Murphy, Mitochondria-derived ROS activate AMP-  
32 activated protein kinase (AMPK) indirectly, *J Biol Chem*, (2018).

1 [44] C.A. Wu, Y. Chao, S.G. Shiah, W.W. Lin, Nutrient deprivation induces the Warburg  
2 effect through ROS/AMPK-dependent activation of pyruvate dehydrogenase kinase, *Biochim*  
3 *Biophys Acta*, 1833 (2013) 1147-1156.

4 [45] L. Plecita-Hlavata, J. Tauber, M. Li, H. Zhang, A.R. Flockton, S.S. Pullamsetti, P.  
5 Chelladurai, A. D'Alessandro, K.C. El Kasmi, P. Jezek, K.R. Stenmark, Constitutive  
6 Reprogramming of Fibroblast Mitochondrial Metabolism in Pulmonary Hypertension, *Am J*  
7 *Respir Cell Mol Biol*, 55 (2016) 47-57.

8

9

1 **FIGURE LEGENDS:**

2 **Figure 1: Different metabolic orientations observed in CI deficient cells.**

3 **A. Cellular metabolic orientation**, combined representation of the use of oxidative ((R-O)/F)  
4 and glycolytic (L/G ratio) capacities for each MT-ND, MT-TRNA and nDNA mutations. The  
5 results are expressed in percent of control cells. \* indicates a significant difference ( $> \pm$   
6 2SD) compared to Ctr. **B. biplot of the PCA-X (dot plots) and loading plot of the**  
7 **explaining contribution of each metabolic parameter (squares) in the model.** CI maximal  
8 functions (CI/CS, st3 MP), maximal respiratory chain phosphorylating capacity (st3 MPS),  
9 maximal cellular oxidative capacity (FCCP: F), oxidative metabolism (Routine respiration:  
10 R), cellular phosphorylating respiration (R-O), relative cellular oxidative metabolism (R/F  
11 and R-O/F), glycolytic metabolism (L/G), parameters measured for each patient cell line were  
12 analyzed by a non-supervised multivariate process using the SIMCA software. The resulting  
13 model shows two components (cumulated R2X=0.722). The B group included most of the  
14 cells with nuclear mutated genes, the two MT-TL1 HL and the MT-TK cells. The A group  
15 encompassed all the MT-ND mutated cells, the MT-TL LL and the MT-TP cells, and the three  
16 nuclear mutated NDUFA9, NDUFS4.1 and NDUFV1.1 cells. **C1. Orthogonal Projections to**  
17 **Latent Structures Discriminant Analysis (OPLS-DA) analysis of the two patient groups**  
18 **A and B**, determined in the PCA analysis. **C2. Corresponding Volcano plot.** In the  
19 “Volcano” plot, the x-axis represents the coefficient of the regression between the X  
20 (analyzed parameters) and Y (response) matrix, while the variable importance for the  
21 projection (VIP) is represented on the y-axis. The VIP summarizes the importance of each  
22 variable in defining both the X matrix and the correlation between X and Y. Variables with a  
23 VIP value  $\geq 1$  are considered as pertinent for the model. **D. Oxidative metabolism** was  
24 analysed on intact cells in glucose medium by measuring the: **D1. Routine respiration,**

1 corresponding to the cellular respiration rate, **D2. Phosphorylating respiration (R-O)**,  
2 determined after ATP synthesis inhibition by oligomycin. **D3. Use of the spare capacity for**  
3 **phosphorylation ((R-O)/F)**, *i.e.* part of the maximal respiratory capacity used to sustain  
4 oxidative phosphorylation (R-O). **E. Glycolytic metabolism. E1. Cellular Glucose**  
5 **consumption and Lactate production**, measured in culture media. **E2. Use of the glycolytic**  
6 **capacity**, determined by the conversion rate of the glucose (G) to lactate (L) (L/G ratio). **F.**  
7 **Cellular ATP/ADP ratio** measured with or without oligomycin (Oligo, 4μg/ml) to inhibit  
8 mitochondrial ATP synthesis. **G. Cellular metabolic orientation**, determined by the ratio  
9 between the glucose to lactate fueling (G to L) and the use of oxidative capacity ((R-O)/F).  
10 **H1. CI activity**, normalized to the CS one. **H2. CI-linked maximal respiration** in coupling  
11 conditions with malate, pyruvate (St3 MP).. **I1. Correlation between the metabolic**  
12 **orientation and the CI /CS ratio. I2. Correlation between the metabolic orientation and**  
13 **the CI-linked maximal respiration (St3 MP). J. Cellular NAD<sup>+</sup>/NADH ratio** determined  
14 on fresh cell lysates from Ctr and patient fibroblasts. **K. Correlation between the metabolic**  
15 **orientation and cellular NAD<sup>+</sup>/NADH ratio.**

16 A, D1, D2, D3, E1, E2, F, G, H1, H2, J: Analyses were performed at least on two biological  
17 replicate for each cell lines of each group. Results are presented as means ± SEM. \* indicates  
18 a significant difference compared with Ctr group and § a significant difference among patient  
19 groups (A vs B). I1, I2 and K: statistical significance of correlations was evaluated by the *F*  
20 test using Sigma Plot Software.

21

22 **Figure 2: Induction of CI disassembly, but not CI deficiency, triggers a metabolic shift**

1 **A. CI assembly analysis. A1. In the first immunoblot, CI holoenzyme level was analyzed**  
2 **by sequential hybridization of** NDUFB6+NDUFS2 and SDHA antibodies. A2. The  
3 assembly intermediates were detected in parallel on the same samples but different  
4 immunoblots by sequential hybridization with anti-NDUFAF4, NDUFAF1, NDUFAF2,  
5 specifically targeting different assembly intermediates (see Supplementary Figure 1), and  
6 SDHA or. Analyses were performed at least in two biological replicates for each cell lines of  
7 each group. **B1. Left panel. CI holoenzyme quantity.** The CI holoenzyme quantity was  
8 normalized to the CII one and is represented as a mean  $\pm$  SEM for each group. **Right panel.**  
9 **Assembly intermediates.** The total Assembly Intermediate (A.I.) quantity was normalized to  
10 the CII one and is represented as a mean  $\pm$  SEM for each group. **B2. Accumulation of**  
11 **assembly intermediates relative to CI holoenzyme quantity. C. Correlation between the**  
12 **metabolic orientation and CI assembly. D. Analysis of : Cellular metabolic orientation**  
13 determined by the ratio between the use of glycolytic capacity (L/G) and the use of oxidative  
14 capacity ((R-O)/F) in the four FA cells and three AB cells (upper left panel) displaying  
15 different **CI assembly profiles** (upper right panel) but comparable CI functions (**maximal CI**  
16 **activity (I/CS) and CI-linked respiration** (St3 MP, lower left panel)) and **maximal**  
17 **respiratory chain capacity**, either in coupled state (permeabilized cell, St3 MPS) or in  
18 uncoupled state (intact cells, FCCP, lower right panel). The blue bars represent the Ctr  
19 values. § indicates a significant difference between FA cells and AB ones.

20 B1, B2: Analyses were performed at least in two biological replicates for each cell lines of  
21 each group. Results are presented as means  $\pm$  SEM. \* indicates a significant difference  
22 compared with Ctr group and § a significant difference among patient groups (FA vs AB). C:  
23 statistical significance of correlations was evaluated by the *F* test. Ctr, n=11; Group A, n=19;  
24 Group B, n=10.

1 **Figure 3: Pharmacologically-induced CI disassembly but not CI deficiency induced**  
2 **metabolic rewiring in Ctr fibroblasts.**

3 **A, B, C. Effect of induced CI deficiency (A) or disassembly (B, C) on Ctr fibroblasts**  
4 using either **A. Piericidin A** (5 nM) vs DMSO (1:5,000), **B. sodium pyriothione** (NaPyr,  
5 1 $\mu$ M) vs DMSO (1:5,000) or **C. transfection with *NDUFAF1* or Scramble siRNA**, for 48H  
6 in each condition. **A1, B1, C1. Quantification of CI holoenzyme and assembly**  
7 **intermediates.** Relative amounts of CI holoenzyme and assembly intermediates from treated  
8 fibroblasts are normalized to CII and expressed relative to the vehicle / scramble conditions.  
9 **A2, B2, C2 Maximal cellular oxidative capacity** (F), and **Oxidative metabolism**, i.e.  
10 Routine respiration (R) and phosphorylating respiration (R-O) determined on Ctr and treated  
11 fibroblasts. **A3, B3, C3. Metabolic compensations**, i.e. use of the maximal capacity to  
12 sustain cellular oxidative phosphorylation ((R-O)/F) or oxidative metabolism (R/F). **A4, B4,**  
13 **C4. Glycolytic metabolism**, determined by the conversion rate of the glucose to lactate (L/G  
14 ratio) measured in culture media. **A5, B5, C5. Cellular metabolic orientation**, represented  
15 the use of oxidative ((R-O)/F) and glycolytic (L/G ratio) capacities of cells. Results are  
16 expressed relative to the vehicle / scramble conditions and represented as a mean  $\pm$  SEM. n=3  
17 independent experiments for each treatment. # indicates a significant difference compared to  
18 vehicle or scramble.

19

20 **Figure 4: ROS overproduction is involved in metabolic reprogramming**

21 **A. Analysis of free N module accumulation** in Ctr and patient cells. After BN-PAGE, free N  
22 module and fully assembled CI (bound N module) were detected using anti-NDUFS1  
23 antibody. **A1. Representative blots** of one of the duplicate experiments. The different groups

1 are highlighted by different colors: the Ctr cells are in blue, FA cells are in red and AB cells  
2 in green. **A2. Quantification of free N module relative to fully assembled CI (bound N  
3 module)** in 10 Ctr, 14 FA and 10 AB cell lines. **B. ROS production** measured by  
4 fluorescence detection of Amplex Red oxidation on permeabilized fibroblasts. Dedicated CI  
5 and CI+CII-linked ROS production was sequentially analysed using substrates of CI and  
6 CI+CII as followed: first, NADH supply to CI was induced by adding pyruvate and malate  
7 (MP) in the presence of saturating ADP concentration. Then, NADH supply was further  
8 increased by addition of glutamate (MPG). Finally, Succinate (MPGS) was added to measure  
9 the ROS production with convergent CI+II electron flow into the Q-junction. ROS production  
10 was normalized to the corresponding oxygen consumption measured in parallel in the same  
11 oxygraphic chambers (n=5 for Ctr, n=9 for FA and n=9 for AB). Results are represented as a  
12 mean  $\pm$  SEM. \* indicates a significant difference with Ctr and § with the FA groups. **C.  
13 Correlation between the CI-linked ROS production and the relative unbound N module  
14 (upper left panel), the cellular metabolic orientation (upper right panel) and the CI  
15 quantity (lower left panel). D. ROS production analysis in NaPyr and vehicle-treated  
16 cells** successively respiring with CI substrates (pyruvate + malate, pyruvate + malate +  
17 glutamate) and CI+CII substrates (pyruvate + malate + glutamate + succinate). # indicates a  
18 significant effect of NaPyr addition. **E. Effect of mt-PQ treatment on cell metabolism and  
19 CI assembly in Ctr and AB cells. E1. ROS production analysis** with CI substrates (malate,  
20 pyruvate, glutamate). ROS overproduction was induced in Ctr fibroblast by mito-Paraquat  
21 (mt-PQ) treatment (5  $\mu$ M, 48H). **E2. Maximal coupled CI-linked respiration** (malate,  
22 pyruvate, glutamate, St3 MPG) after mt-PQ treatment. **E3. Maximal CI activity** normalized  
23 to CS one after mt-PQ treatment. **E4. Routine respiration (R) and phosphorylating  
24 respiration (R-O). E5. Glycolytic metabolism** i.e. the ratio of the lactate production to  
25 glucose consumption measured in culture media (L/G) determined on Ctr and mt-PQ treated

1 fibroblasts. n=3 different Ctr cell lines, analysed in two biological replicates. Results are  
2 represented as a mean  $\pm$  SEM. \* indicates a significant difference with Ctr and # indicates a  
3 significant effect of mt-PQ. **E6. Blue Native PAGE analysis of DMSO and mtPQ treated**  
4 **cells.** Left panel: representative Blue Native PAGE gel; Right panel: Quantification of CI  
5 holoenzyme (CI), assembly intermediates (AI) and respiratory chain complexes (CI, CII, CIII  
6 et CIV) after DMSO or mtPQ incubation in Ctr cells n=3 in duplicate. Results are represented  
7 as a mean  $\pm$  SEM. # indicates a significant effect of mt-PQ ( $p < 0.05$ ). **F. Effect of NAC**  
8 **treatment on cell metabolism and CI assembly in Ctr and AB cells. F1. Oxidative**  
9 **metabolism** in Ctr and AB cells treated with the antioxidant N-Acetyl Cysteine (NAC, 1 mM,  
10 48H) **Left, Routine respiration** (R, left panel) and **Right, Phosphorylating respiration** (R-  
11 O, right panel). **F2. Glycolytic metabolism**, determined by the conversion rate of the glucose  
12 to lactate (L/G ratio) measured in culture media. **F3. CI assembly analysis.** Left panel:  
13 representative Blue Native PAGE gel sequentially hybridized with NDUFS2+NDUFB6 and  
14 SDHA; Right panel: Quantification of CI holoenzyme (CI). Experiments were performed on 4  
15 different Ctr cell lines (3 different Ctr cell lines for BN-PAGE experiments), and 5 different  
16 AB cell lines, using representative cell lines with different assembly defects, i.e. MT-TL1.3,  
17 NDUFV1.2, NDUFS4.3, NDUFB3 and NDUFS7. Results are represented as a mean  $\pm$  SEM. #  
18 indicates a significant effect of the treatment. \* indicates a significant difference with Ctr cells  
19 in the same conditions.

20

21 **Figure 5: ROS/AMPK pathway is involved in limitation of substrate supply to**  
22 **respiratory chain by inhibition of PDH in AB cells.**

23 **A1. AMPK phosphorylation level** analyzed by Western blotting in cell lysates. Left panel:  
24 representative blot, right panel: quantitation of P-AMPK (in green) relative to total AMPK (in



1 red). Each cell line was analyzed in two biological replicates, Ctr: n=10, FA: n=14 and AB:  
2 n=9. Results are represented as a mean  $\pm$  SEM. \* indicates a significant difference with Ctr.  
3 **A2. Effect of ROS overproduction on AMPK phosphorylation.** Left panel: representative  
4 blot of Ctr cells treated with Vehicle (DMSO 1:5000) or mt-PQ, right panel: quantitation of P-  
5 AMPK (in green) relative to total AMPK (in red). Western blots realized on n=3 Ctr in  
6 duplicate, # indicates a significant effect of the treatment. **B1. PDH phosphorylation level**  
7 analyzed by Western blotting in cell lysates. Left panel. Representative blots. Phospho-PDH,  
8 in red and total PDH, in green. Right panel. Quantitation of P-PDH relative to total PDH.  
9 Each cell line was analyzed in two biological replicates, Ctr: n=6, FA: n=10 and AB: n=9.  
10 Results are represented as a mean  $\pm$  SEM. \* indicates a significant difference with Ctr. **B2.**  
11 **Effect of ROS overproduction on PDH phosphorylation.** representative blot of P-PDH (in  
12 red) and total PDH (in green) in Ctr cells treated with Vehicle (DMSO 1:5000) or mt-PQ and  
13 quantitation of P-PDH relative to total PDH expressed in percent of Veh treated cells.  
14 Western blots realized on n=3 Ctr in duplicate, # indicates a significant effect of the  
15 treatment. **C. Effect of inhibition of ROS production and of AMPK on PDH**  
16 **phosphorylation.** Left panel: Representative blots of PDH phosphorylation level analyzed by  
17 Western blotting in cell lysates. Phospho-PDH, in red and total PDH, in green. Right panel:  
18 quantitation of P-PDH relative to total PDH. Experiments were performed on 4 different Ctr  
19 cell lines and 5 different AB cell lines, using representative cell lines with different assembly  
20 defects, i.e. MT-TL1.3, NDUFV1.2, NDUFS4.3, NDUFB3 and NDUFS7. Results are  
21 represented as a mean  $\pm$  SEM. # indicates a significant effect of the treatment. **D. Analysis of**  
22 **pyruvate metabolism and TCA cycle functioning.** Oxidation of pyruvate and malate was  
23 followed on permeabilized fibroblasts from Ctr (n=4), FA (n=7), and AB (n=7) groups.  
24 Malate, the oxaloacetate precursor, was added with pyruvate to support full TCA cycling and  
25 respiration was induced by sub-saturating ADP concentration (0.5mM, 60% of Vmax) to

1 mimic the phosphorylating respiration observed in intact cells. NAD<sup>+</sup> was omitted to preserve  
2 an eventual allosteric regulation of PDH and/or TCA cycle.

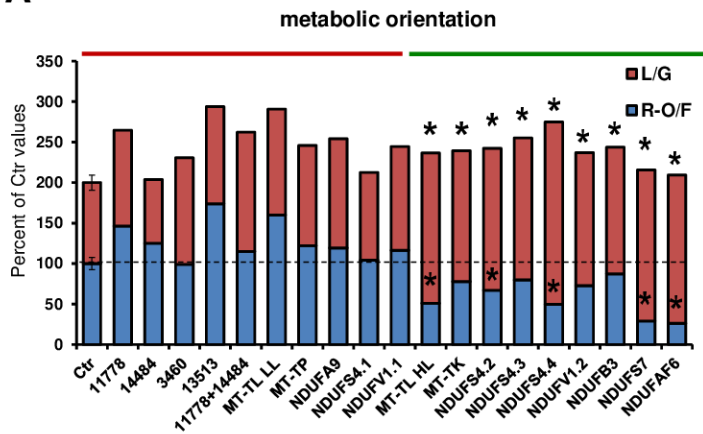
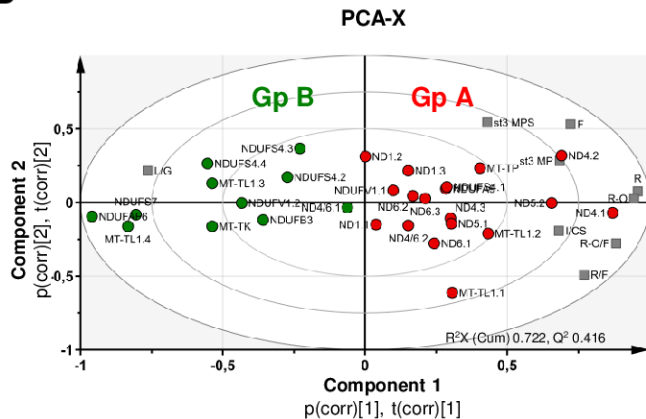
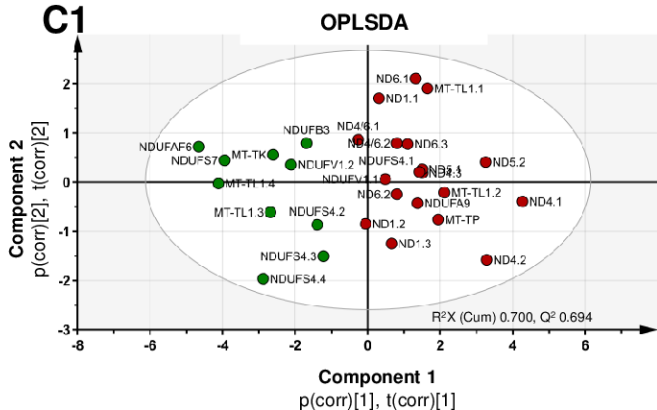
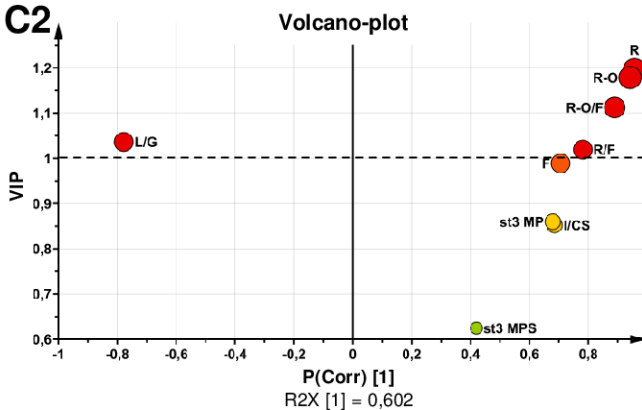
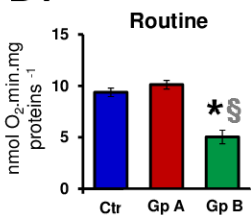
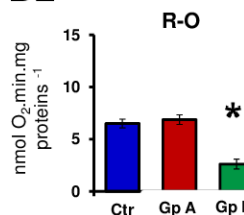
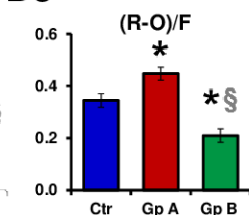
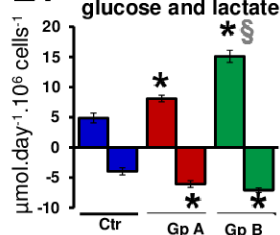
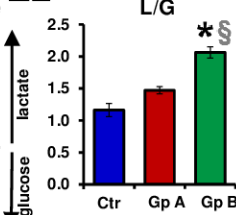
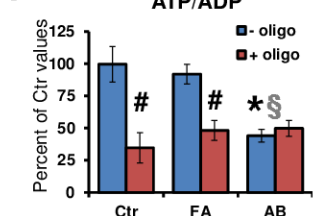
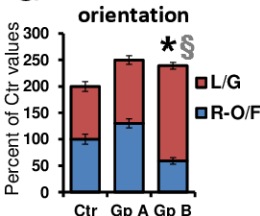
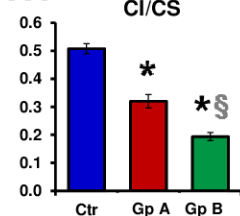
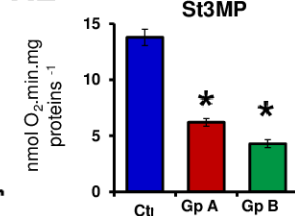
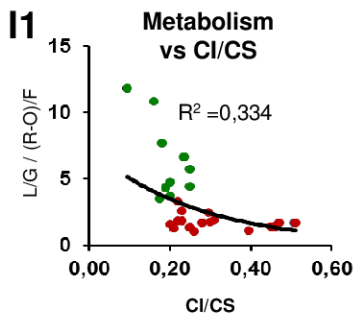
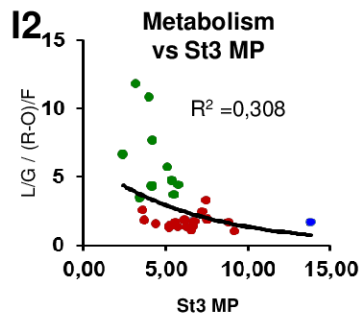
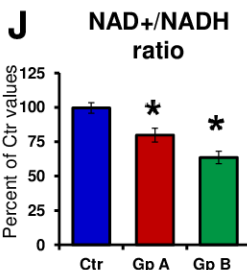
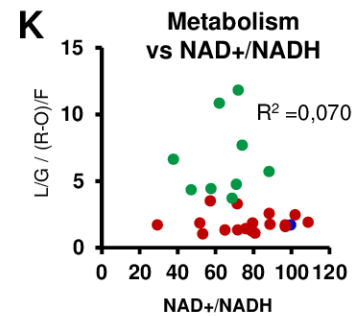
3

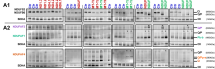
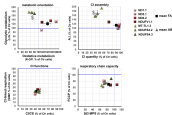
4 **Figure 6: Sum up of the discriminative parameters of metabolic adaptations induced by**  
5 **CI deficiency**

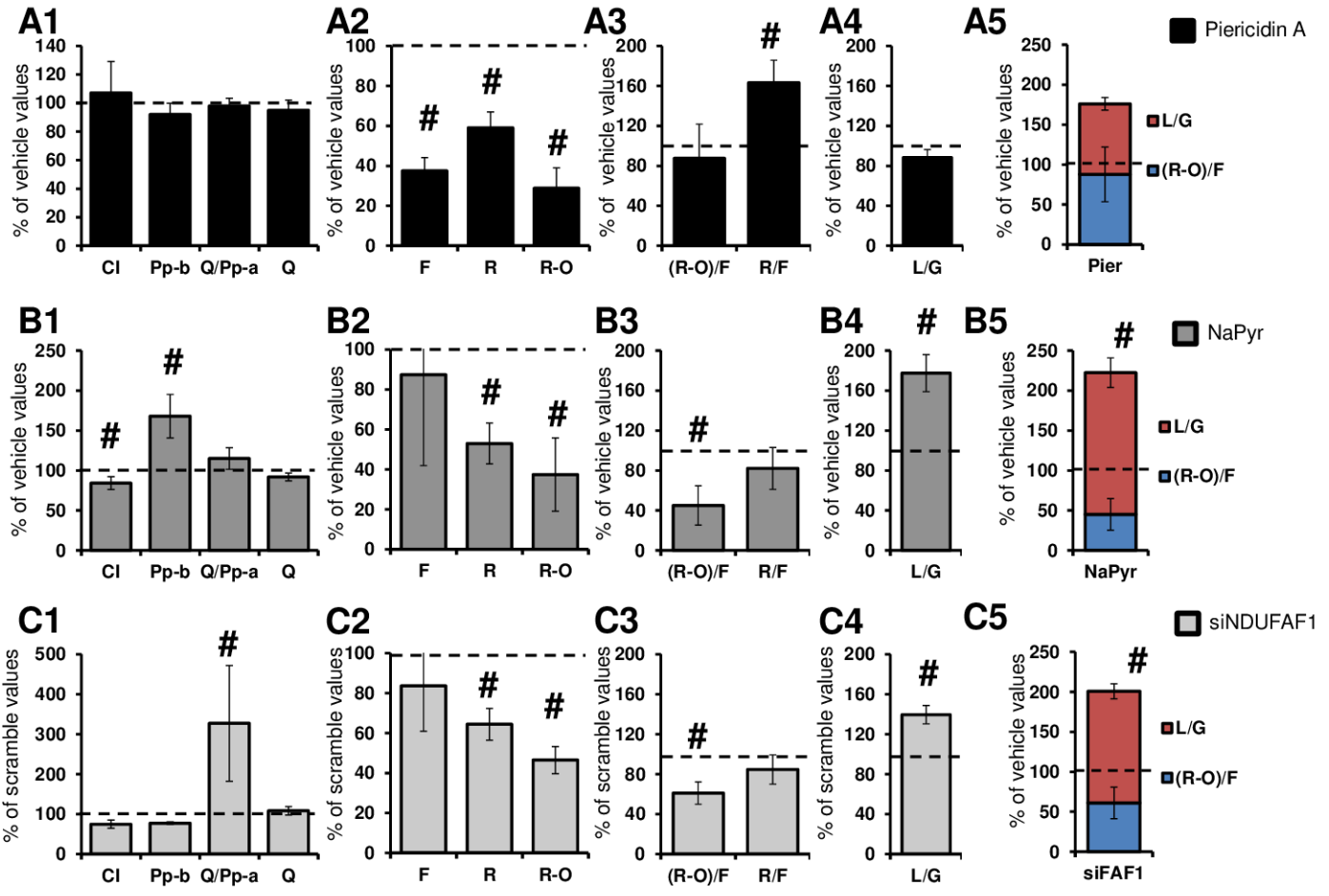
6 **Principal Component Analysis (PCA-X) of the cohort of CI deficient patients. A1: biplot**  
7 **of the PCA-X (dot plots) and loading plot of the explaining contribution of each**  
8 **metabolic parameter (squares) in the model.** CI maximal activity (CI/CS, st3 MP),  
9 maximal respiratory chain phosphorylating capacity (st3 MPS), maximal cellular oxidative  
10 capacity (FCCP: F), oxidative metabolism (Routine respiration: R), cellular phosphorylating  
11 respiration (R-O), relative cellular oxidative metabolism (R/F and R-O/F), glycolytic  
12 metabolism (L/G), AMPK phosphorylation, ADP/ATP ratio, NAD<sup>+</sup>/NADH ratio and CI  
13 assembly (CI holoenzyme quantity: CI and Assembly Intermediates, A.I.) parameters were  
14 measured for each control (Ctr) and patient cell line and analyzed by a non-supervised  
15 multivariate process using the SIMCA software ( $R^2X(\text{Cum})=0.661$  and  $Q^2=0.423$ ). **A2.**  
16 **Volcano plot of the orthogonal Projections to Latent Structures Discriminant Analysis**  
17 **(OPLS-DA, ( $R^2X(\text{Cum})=0.637$  and  $Q^2=0.86$ ) analysis of patient cell lines.** In the “Volcano”  
18 plot, the x-axis represents the coefficient of the regression between the X (analyzed  
19 parameters) and Y (response) matrix, while the variable importance for the projection (VIP) is  
20 represented on the y-axis. The VIP summarizes the importance of each variable in defining  
21 both the X matrix and the correlation between X and Y. Variables with a VIP value  $\geq 1$  are  
22 considered as pertinent for the model. **B. Correlation matrix of the PCA model** obtained  
23 from the cell cohort (patient cells, n=29) for all biochemical parameters. I/CS: maximal CI  
24 activity (NADH Ubiquinone Reductase) reported to that of citrate synthase (CS). St3 MP:

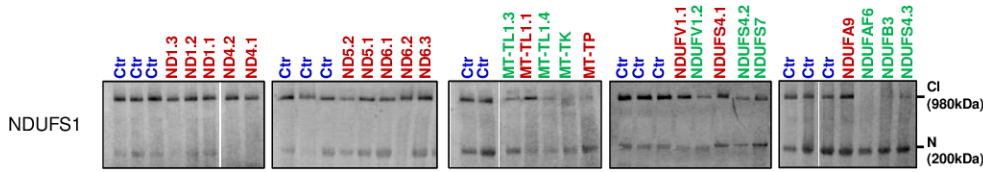
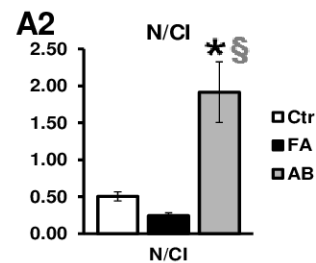
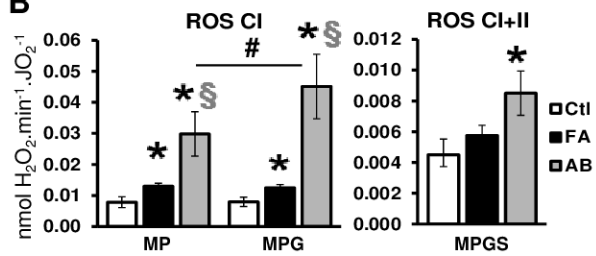
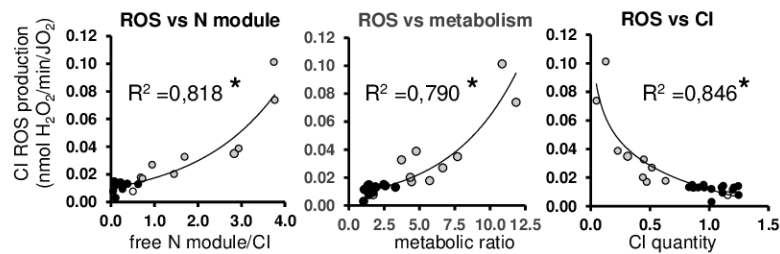
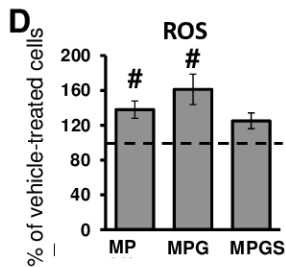
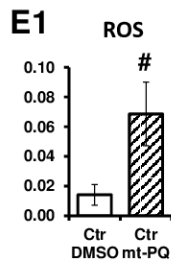
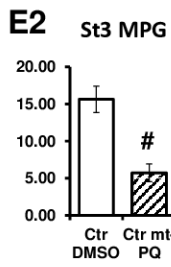
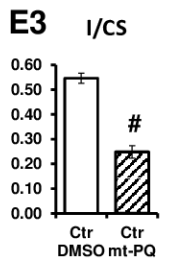
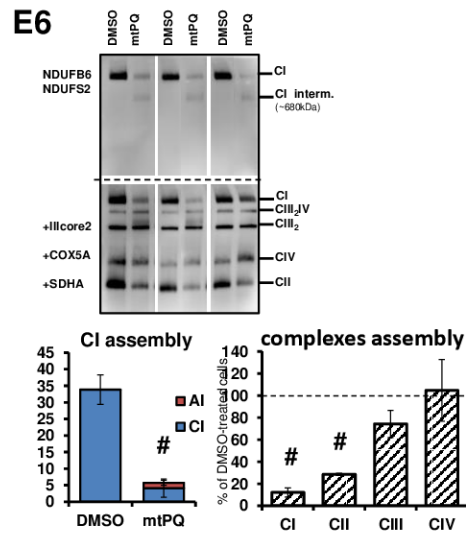
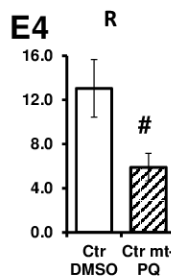
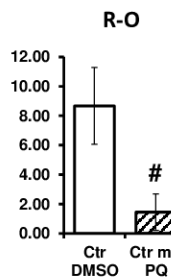
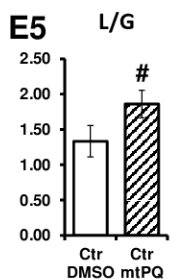
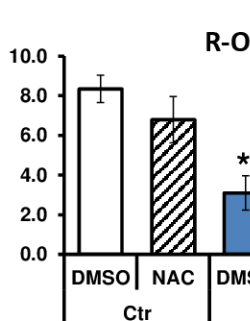
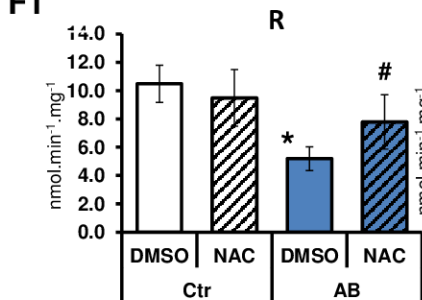
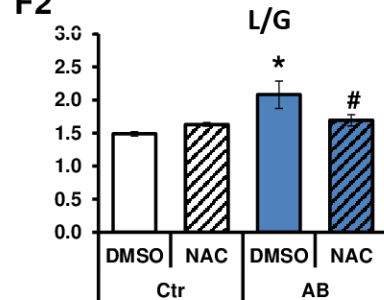
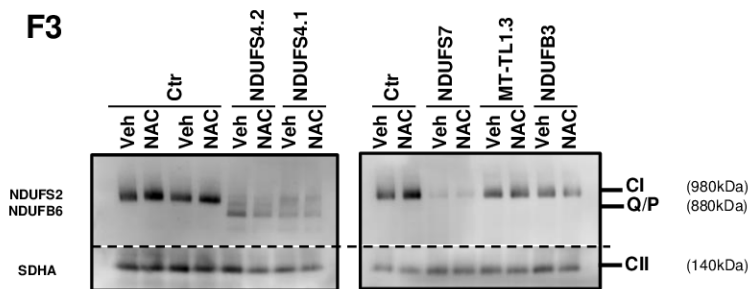
1 maximal CI-linked phosphorylating respiration measured on permeabilized cells. St3 MPS:  
2 maximal CI+CII-linked phosphorylating respiration measured on permeabilized cells, i.e.  
3 maximal OXPHOS capacity. F: maximal uncoupled respiration measured on intact cell  
4 respiring in glucose medium, i.e. maximal cellular oxidative capacity. R: Routine respiration  
5 measured on intact cell, i.e. oxidative metabolism, R-O: Phosphorylating respiration measured  
6 on intact cell as the oligomycin-sensitive respiration, R-O/F: part of the spare respiratory  
7 chain capacity used for ATP synthesis, R/F: part of the spare respiratory chain capacity to  
8 sustain respiration, L/G: glycolytic metabolism estimated by the Lactate/Glucose ratio, P-  
9 PDH : phosphorylation status of the PDH, CI: quantity of CI holoenzyme, A.I.: quantity of CI  
10 assembly intermediates, N/CI: quantification of the accumulation of free N module compared  
11 with CI holoenzyme, ROS: ROS production per oxygen consumed in presence of CI  
12 substrates, NAD: cellular NAD<sup>+</sup>/NADH ratio, ATP/ADP: cellular ATP/ADP ratio, P-AMPK  
13 : AMPK phosphorylation status.

14

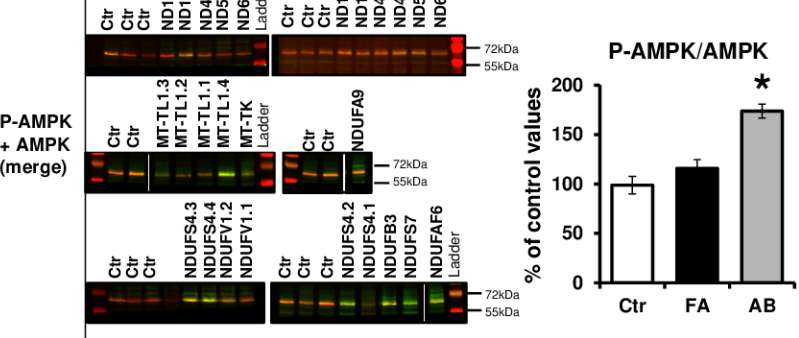
**A****B****C1****C2****D1****D2****D3****E1****E2****F****G****H1****H2****I1****I2****J****K**

**A****B1****A.1****B2****C****D**

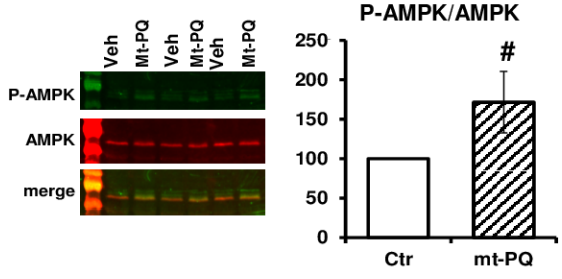


**A1****A2****B****C****D****E1****E2****E3****E6****E4****R-O****E5****F1****F2****F3**

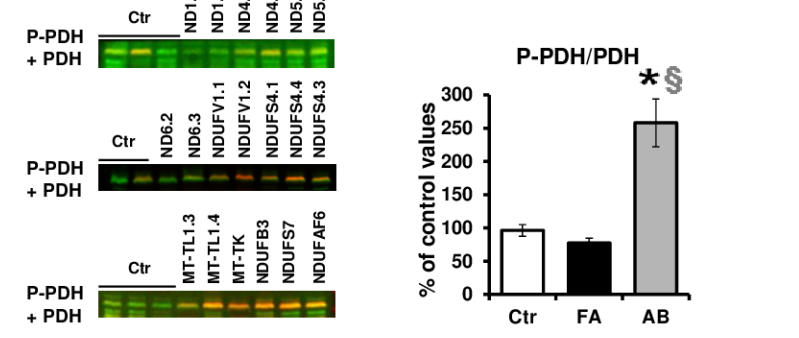
**A1**



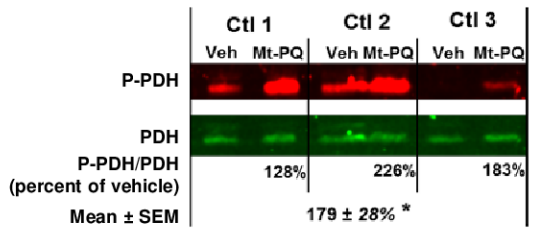
**A2**



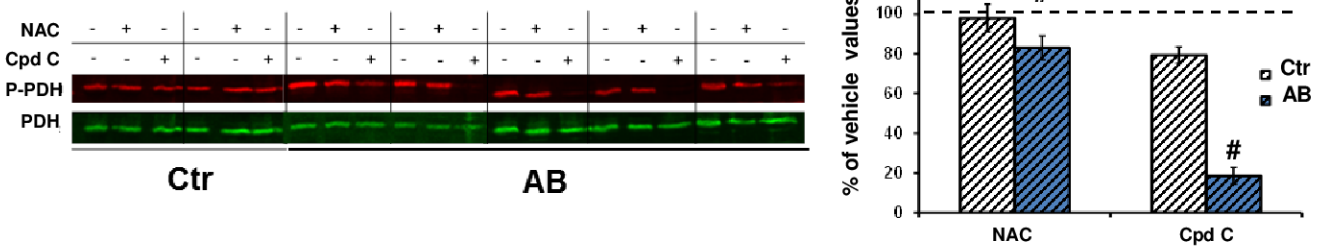
**B1**



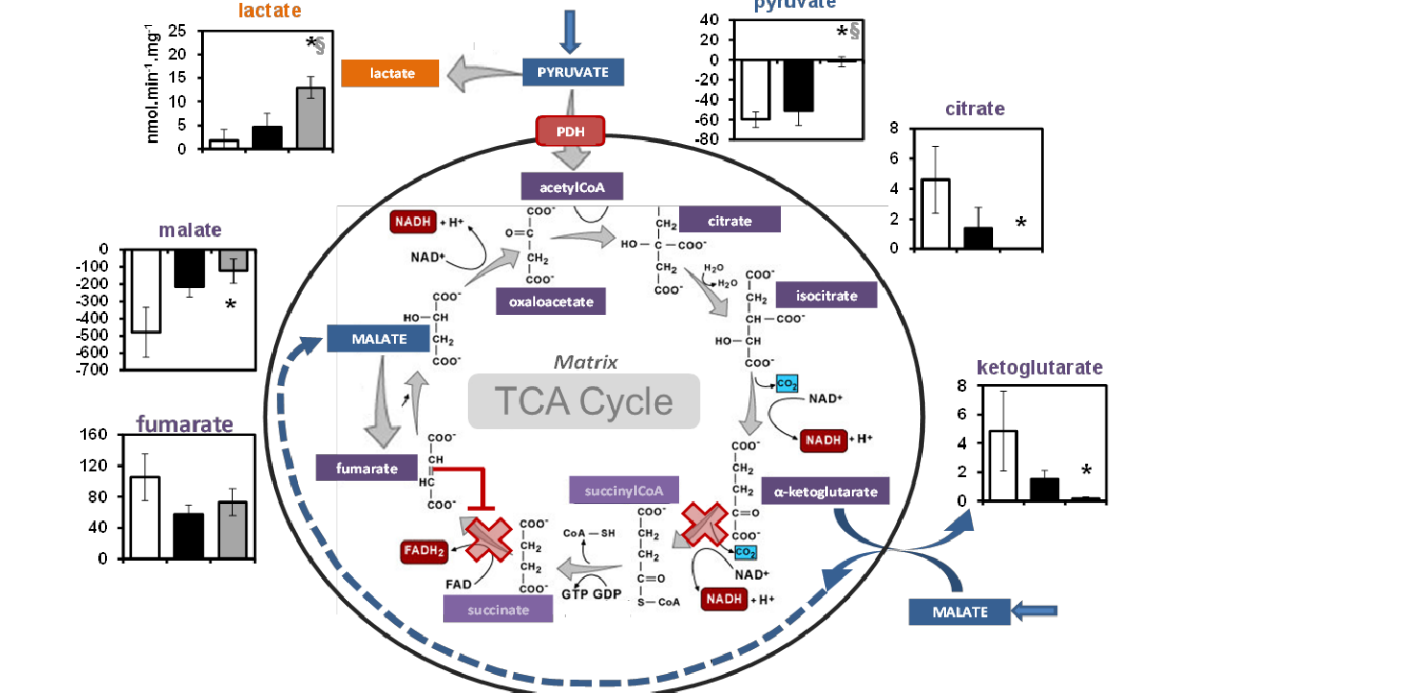
**B2**



**C**

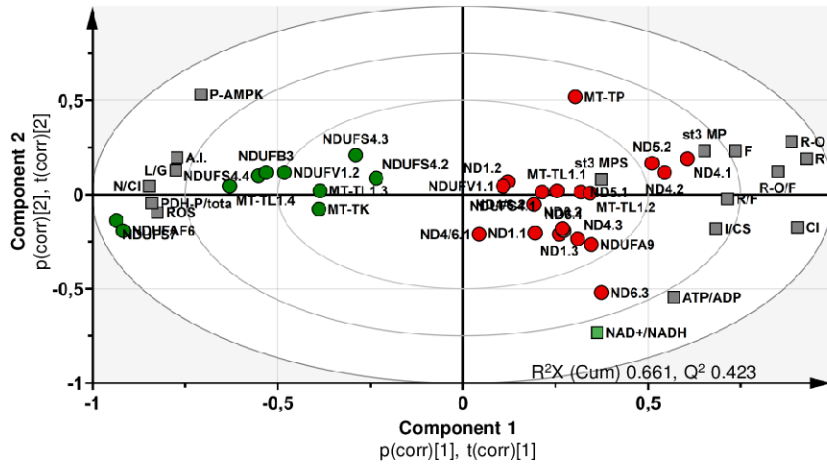


**D**

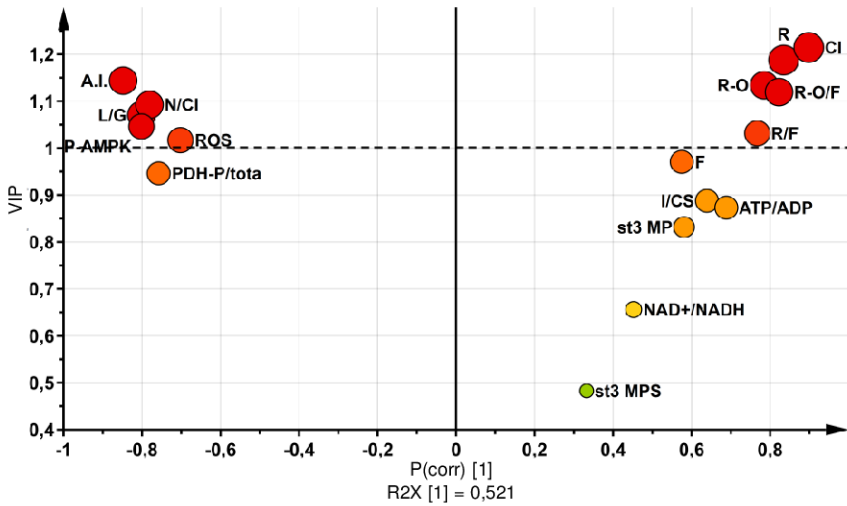




# A1



# A2



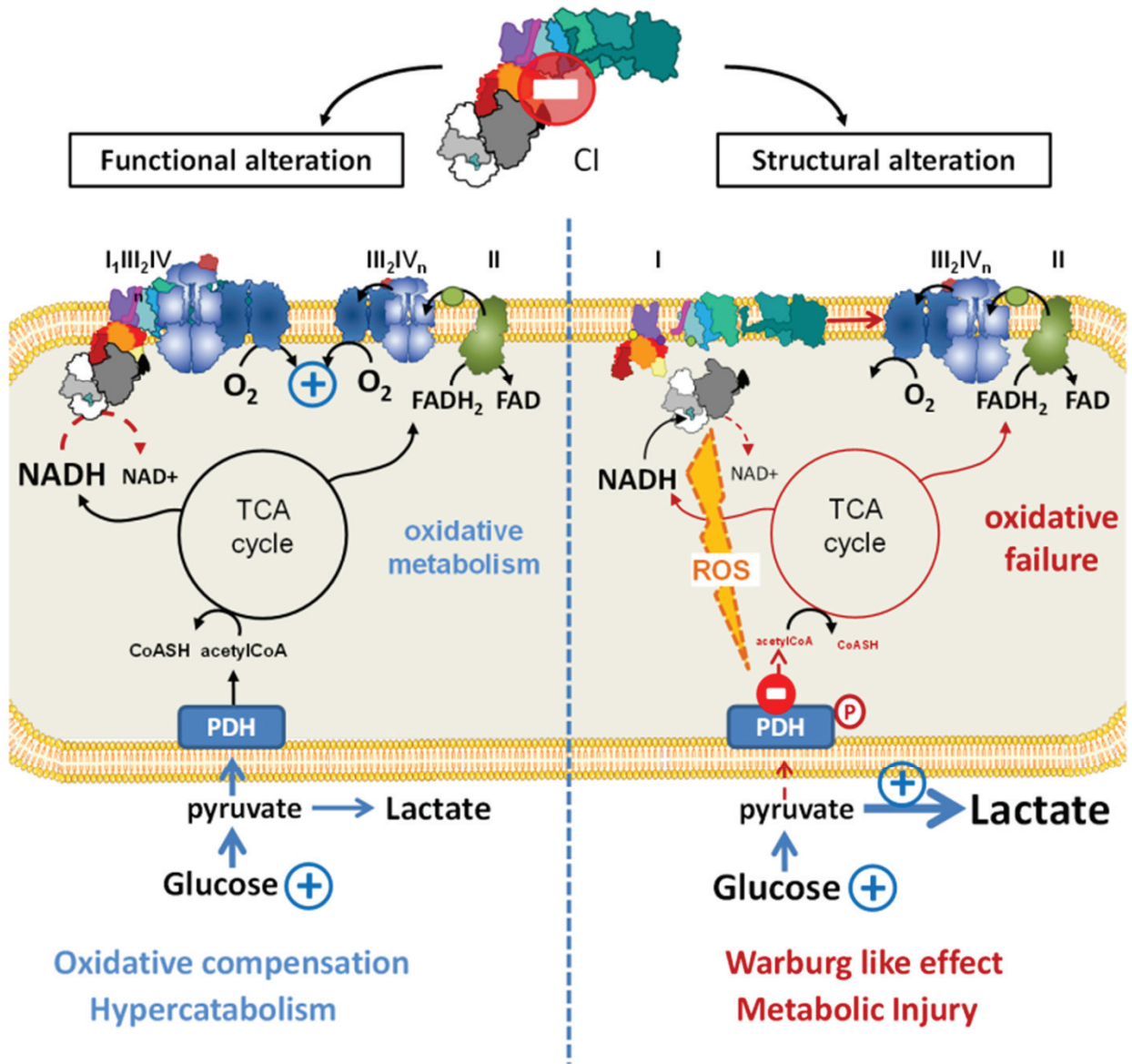
# B

	I/CS	st3 MP	st3 MPS	F	R	R-O	R-O/F	R/F	L/G	NADH	CI	A.I.	ROS	N/CI	ATP/ADP	P-PDH	P-AMPK
I/CS	1.000	0.408	0.075	0.483	0.589	0.569	0.495	0.466	-0.607	0.395	0.567	-0.586	-0.536	-0.556	0.180	-0.501	-0.490
st3 MP		1.000	0.280	0.579	0.616	0.574	0.460	0.371	-0.603	0.095	0.424	-0.511	-0.512	-0.451	0.436	-0.572	-0.521
st3 MPS			1.000	0.353	0.410	0.405	0.321	0.250	-0.224	0.246	0.272	-0.222	-0.216	-0.145	0.392	-0.410	-0.059
F				1.000	0.816	0.743	0.456	0.197	-0.411	0.277	0.676	-0.476	-0.688	-0.645	0.230	-0.647	-0.409
R					1.000	0.956	0.827	0.701	-0.602	0.243	0.783	-0.694	-0.724	-0.712	0.406	-0.736	-0.558
R-O						1.000	0.853	0.699	-0.616	0.117	0.721	-0.647	-0.670	-0.681	0.440	-0.686	-0.467
R-O/F							1.000	0.872	-0.640	0.174	0.693	-0.639	-0.671	-0.653	0.486	-0.721	-0.482
R/F								1.000	-0.581	0.181	0.559	-0.663	-0.422	-0.443	0.404	-0.511	-0.479
L/G									1.000	-0.236	-0.701	0.610	0.592	0.600	-0.592	0.569	0.553
NAD+/NADH										1.000	0.380	-0.392	-0.216	-0.217	0.331	-0.321	-0.479
CI											1.000	-0.709	-0.802	-0.880	0.552	-0.832	-0.761
A.I.												1.000	0.402	0.533	-0.494	0.482	0.586
ROS													1.000	0.885	-0.366	0.937	0.491
N/CI														1.000	-0.493	0.823	0.695
ATP/ADP															1.000	-0.543	-0.658
PDH-P/total																1.000	0.600
P-AMPK																	1.000

positive correlation

negative correlation

# Graphical abstract



**Metabolic reprogramming in CI deficient cells**