

# Modulation of mitochondrial protein phosphorylation by soluble adenylyl cyclase ameliorates cytochrome oxidase defects

Rebeca Acin-Perez<sup>1</sup>, Eric Salazar<sup>2,3</sup>, Sonja Brose<sup>4</sup>, Hua Yang<sup>4</sup>, Eric A. Schon<sup>4</sup>, Giovanni Manfredi<sup>1\*</sup>

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Phosphorylation of respiratory chain components has emerged as a mode of regulation of mitochondrial energy metabolism, but its mechanisms are still largely unexplored. A recently discovered intramitochondrial signalling pathway links CO<sub>2</sub> generated by the Krebs cycle with the respiratory chain, through the action of a mitochondrial soluble adenylyl cyclase (mt-sAC). Cytochrome oxidase (COX), whose deficiency causes a number of fatal metabolic disorders, is a key mitochondrial enzyme activated by mt-sAC. We have now discovered that the mt-sAC pathway modulates mitochondrial biogenesis in a reactive oxygen species dependent manner, in cultured cells and in animals with COX deficiency. We show that upregulation of mt-sAC normalizes ROS production and mitochondrial biogenesis, thereby restoring mitochondrial function. This is the first example of manipulation of a mitochondrial signalling pathway to achieve a direct positive modulation of COX, with clear implications for the development of novel approaches to treat mitochondrial diseases.

## INTRODUCTION

Reversible protein phosphorylation is a mechanism for short-term regulation of oxidative phosphorylation (OXPHOS) in mammalian mitochondria. A well-known example is the pyruvate dehydrogenase kinase (PDK)-dependent phosphorylation of subunit E1 $\alpha$  of pyruvate dehydrogenase (Hansford, 1991; Linn et al, 1969). Amino acid phosphorylation has been identified in several other OXPHOS components, including complex I (Chen et al, 2004; De Rasmio et al, 2008; Papa et al, 1996; Piccoli et al, 2006; Sardanelli et al, 1995; Scacco et al, 2000; Signorile et al, 2002) and complex IV (cytochrome oxidase, COX) of the respiratory chain, where it modulates

enzyme kinetics (Bender & Kadenbach, 2000; Helling et al, 2008; Kadenbach, 2003; Lee et al, 2005; Miyazaki et al, 2003).

Mitochondria contain all the components for OXPHOS protein phosphorylation, including different families of kinases and phosphatases (Horbinski & Chu, 2005; Lu et al, 2007; Pagliarini & Dixon, 2006). The presence of both protein kinase A (PKA) (Pagliarini & Dixon, 2006; Thomson, 2002) and A kinase-anchoring proteins (AKAPs) (Felicciello et al, 2005; Lewitt et al, 2001) has been demonstrated in mammalian mitochondria. In particular, the presence of PKA in the mitochondrial matrix has been shown with different approaches (Livigni et al, 2006; Prabu et al, 2006; Ryu et al, 2005; Schwoch et al, 1990). However, an important missing piece of the puzzle, which is currently being investigated, is the identity of the mitochondrial phosphatases needed to dephosphorylate PKA targets.

We have previously discovered that a soluble adenylyl cyclase (Acin-Perez et al, 2009; Chen et al, 2000; Zippin et al, 2003, 2001) (sAC) is the intra-mitochondrial source of cAMP that regulates PKA, and that stimulation of the mitochondrial sAC-cAMP-PKA (mt-sAC) signalling pathway increases mitochondrial respiration and ATP synthesis, while its inhibition decreases OXPHOS activity (Acin-Perez et al, 2009). This intramitochondrial signalling is necessary to stimulate COX

(1) Department of Neurology and Neuroscience, Weill Medical College of Cornell University, New York, NY, USA.

(2) Tri-Institutional MD-PhD Program, Weill Medical College of Cornell University, New York, NY, USA.

(3) Department of Pharmacology, Weill Medical College of Cornell University, New York, NY, USA.

(4) Department of Neurology, Columbia University, New York, NY, USA.

\*Corresponding author: Tel: +1 212 746 4605; Fax: +1 212 746 8276; E-mail: gim2004@mail.med.cornell.edu

**Table 1. Mitochondrial functional parameters in COX deficient cells**

Cell line	Mutation	Whole cell respiration (fmol O <sub>2</sub> /min/cell)	TMPD/ascorbate respiration (fmol O <sub>2</sub> /min/cell)	COX activity (IU/mg protein)	CS activity (IU/mg protein)	ATP synthesis (nmol ATP/min/mg)
WT	0	4.51 ± 0.319 (22)	27.20 ± 3.67 (7)	0.052 ± 0.016 (6)	0.143 ± 0.025 (9)	33.375 ± 7.558 (3)
CA75	COXI (75%)	2.535 ± 0.300*** (9)	12.29 ± 1.04*** (7)	0.010 ± 0.002*** (6)	0.174 ± 0.005* (9)	8.479 ± 0.607*** (3)
fWTHtert	0	5.625 ± 0.332 (5)	n.d	0.042 ± 0.007 (6)	n.d	23.988 ± 0.146 (3)
fSCO <sub>2</sub> Htert	SCO <sub>2</sub> MT	3.681 ± 0.131** (5)	n.d	0.029 ± 0.005 ** (6)	n.d	10.038 ± 0.238*** (3)

Whole cell respiration, TMPD/ascorbate-dependent respiration, COX enzymatic activity, citrate synthase (CS) activity and ATP synthesis with pyruvate and malate as substrates measured in WT cybrids, heteroplasmic COXI mutant cybrids (CA75, 75% mutant mtDNA), WT human fibroblasts (fWT Htert) and Sco2 mutant human fibroblasts (fSCO<sub>2</sub> Htert). The number in brackets indicates the *n* for each experiment. n.d., not determined.

\**p* < 0.01.

\*\**p* < 0.001.

\*\*\**p* < 0.0001.

activity, since extramitochondrial cAMP is ineffective (Acin-Perez et al, 2009). In addition, we have identified phosphodiesterase activity inside mitochondria, although the specific mitochondrial phosphodiesterase has not yet been identified.

Despite increasing evidence that OXPHOS proteins are subject to reversible phosphorylation, the physiopathological significance of these modifications and the underlying signalling pathways are still elusive. Virtually no information is available on the link between OXPHOS function, reactive oxygen species (ROS) production and mitochondrial cAMP signalling, in mitochondrial disorders, where the components of the respiratory chain are mutated or missing (Dimauro & Davidzon, 2005). OXPHOS protein phosphorylation may be one of the mechanisms whereby cells attempt to adapt to bioenergetic defects by optimizing enzyme activities and improving the balance between energy production and ROS generation (Kadenbach et al, 2009).

Here, we have investigated OXPHOS regulation by the mt-sAC pathway in genetic models of COX deficiency, which is one of the most common causes of inherited mitochondrial disorders (Shoubridge, 2001). We demonstrate that PKA and mt-sAC modulation induce ROS-mediated changes in the regulation of OXPHOS biogenesis that are different in COX deficient and wild type (WT) cells. Strikingly, upregulating the expression of mitochondrial sAC (mt-sAC) in COX defective cells results in a dramatic amelioration of the biochemical defect, with improved COX activity, mitochondrial respiration and ability to grow under strictly aerobic conditions, and at the same time it restores normal regulation of OXPHOS biogenesis.

## RESULTS

### Differential modulation of OXPHOS by PKA in WT and respiratory chain mutant cells

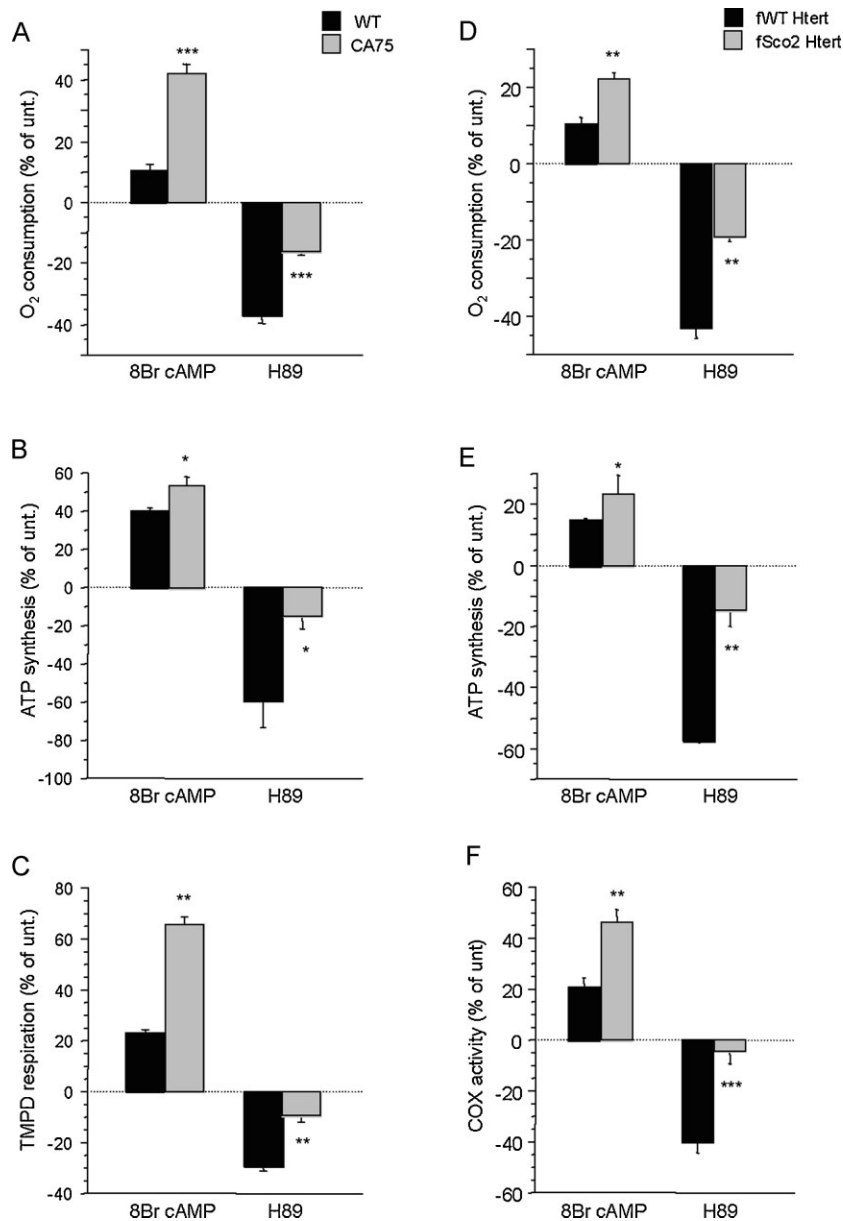
We investigated the PKA-dependent modulation of OXPHOS in mtDNA mutant cybrid cells derived from the fusion of mtDNA-less human osteosarcoma cells with cytoplasts from a patient with a mitochondrial encephalopathy, who harboured a G6930A stop-codon mutation in the COXI gene (Bruno et al, 1999). This mutation in the homoplasmic state results in a complete loss of COX activity, whereas in the heteroplasmic state (*i.e.* a mixture

of mutant and WT mtDNA) the severity of the defect depends on the proportion of mutant mtDNA (D'Aurelio et al, 2001). Heteroplasmic cybrids containing 75% mutant mtDNA (named CA75) have partially reduced COX activity (by 80%), cell respiration (by 45%) and ATP synthesis (by 65%) (Table 1). CA75 cells recapitulate the genetic and biochemical features of mtDNA diseases in patients, where mutations are often heteroplasmic and result in partial OXPHOS defects.

8Br-cAMP, a membrane permeant PKA agonist, stimulated intact cell respiration (Fig 1A), ATP synthesis (Fig 1B), COX-dependent respiration with TMPD-ascorbate as substrates (Fig 1C) and COX enzymatic activity measured spectrophotometrically (data not shown). This stimulation was significantly stronger in CA75 than in WT cells. Conversely, the PKA inhibitor H89 (Chijiwa et al, 1990) decreased respiration (Fig 1A), ATP synthesis (Fig 1B) and COX activity (Fig 1C) significantly less in CA75 than in WT cells.

We then studied OXPHOS modulation by PKA in immortalized fibroblasts derived from a patient with mutant Sco2, a nuclear-encoded copper chaperone protein necessary for COX function (Papadopoulou et al, 1999; Salvati et al, 2002). Sco2 mutant fibroblasts (fSCO<sub>2</sub> Htert) had a partial deficiency in COX activity (by 25%), cell respiration (by 35%) and ATP synthesis (by 55%) (Table 1). fSCO<sub>2</sub> Htert cells showed an enhanced stimulation and blunted inhibition of cell respiration (Fig 1D), ATP synthesis (Fig 1E) and COX activity (Fig 1F) in response to 8Br-cAMP and H89, respectively, as compared to WT fibroblasts (fWT Htert).

To determine whether the different modulating effect of PKA was specific to COX mutants we studied three additional mtDNA mutations. The first (SUA63) contained a homoplasmic C3256T mutation in the tRNA<sup>Leu<sup>UUR</sup></sup> gene (Hao & Moraes, 1996), and had a severe decrease of both complex I and COX assembly (Fig 2A) and decreased respiration (by 50%). SUA63 cells responded to PKA modulation similarly to COXI and Sco2 mutants, with enhanced stimulation and blunted inhibition of OXPHOS by 8Br-cAMP and H89, respectively (Fig 2B), as compared to WT cells (SUB36, 0% mutant mtDNA). The second mutant was a murine cybrid line (E09) containing a homoplasmic missense COXI mutation resulting in a partial COX deficiency (Acin-Perez et al, 2003). Consistent with the results in human cells, E09 cells exhibited an increased COX



**Figure 1. Abnormal PKA modulation of OXPHOS in COX defective cells.** OXPHOS function was assessed in WT and CA75 cybrids incubated in the presence of 8Br-cAMP or H89.

**A.** Mitochondrial respiration in intact cells (WT unt.,  $n = 22$ ; WT 8Br-cAMP,  $n = 9$ ; WT H89,  $n = 9$ ; CA75 unt.,  $n = 9$ ; CA75 8Br-cAMP,  $n = 5$ ; CA75 H89,  $n = 5$ ).  
**B.** ATP synthesis driven by pyruvate plus malate ( $n = 3$  for all samples).  
**C.** TMPD/ascorbate driven respiration ( $n = 7$  for all unt. samples, and  $n = 3$  for all treated samples). OXPHOS function was also assessed in human WT and Sco2 mutant fibroblasts by:  
**D.** Intact cell respiration ( $n = 7$ ),  
**E.** ATP synthesis ( $n = 6$ ) and  
**F.** COX enzymatic activity ( $n = 6$ ). For each cell line, changes induced by the treatments are indicated as a percentage of untreated controls (unt.). \*,  $p < 0.01$ ; \*\*,  $p < 0.001$ ; \*\*\*,  $p < 0.0001$ .

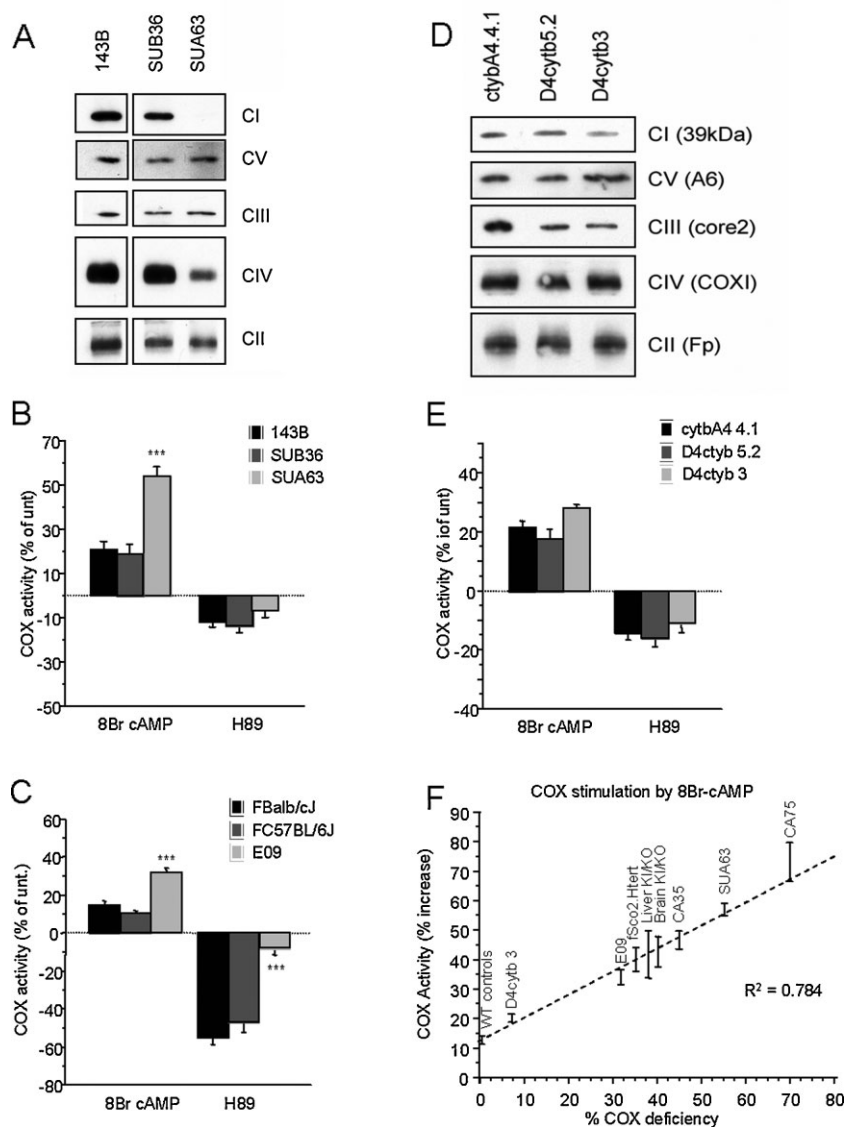
stimulation by 8Br-cAMP and a blunted inhibition by H89, as compared to two WT mouse cybrid lines (Fig 2C). The third mutant was a 4 bp frame-shift deletion in the cytochrome *b* (Cyt *b*) gene (Rana et al, 2000). We tested two heteroplasmic Cyt *b* mutant cybrids, D4cytb 5.2 (75% mutant mtDNA) and D4cytb 3 (90% mutant mtDNA). These mutants, with an isolated defect of Complex III assembly (Fig 2D) and decreased respiration (by 15 and 25%, respectively), showed COX responses to PKA stimulation and inhibition similar to those in WT cybrids (cytbA4.4.1) (Fig 2E).

Taken together, these results indicate that PKA-mediated protein phosphorylation regulates COX, and that this regulation is different in WT and COX deficient cells. Basal protein phosphorylation is reduced in COX deficient cells, allowing for greater stimulation by 8Br-cAMP and lesser inhibition by H89.

PKA modulation of OXPHOS is unchanged in respiratory chain defects that do not involve COX.

**Differential modulation of OXPHOS by PKA in WT and COX deficient mice**

The PKA-dependent modulation of OXPHOS was investigated *in vivo*, in a Sco2 KI/KO mouse model, where one allele harboured a E129K mutation, the mouse homologue of the human E140K mutation common to all patients with Sco2 defects (Papadopoulou et al, 1999), and the other a stop codon insertion. The Sco2 KI/KO mice have approximately a 35% reduction in COX activity in brain and liver and a 50% decrease in skeletal muscle (Schon et al, unpublished results). Similar to COX deficient cells, mitochondria from liver and brain of Sco2 KI/KO mice showed increased stimulation and blunted



**Figure 2. PKA modulation of OXPHOS in mtDNA mutant cells.**

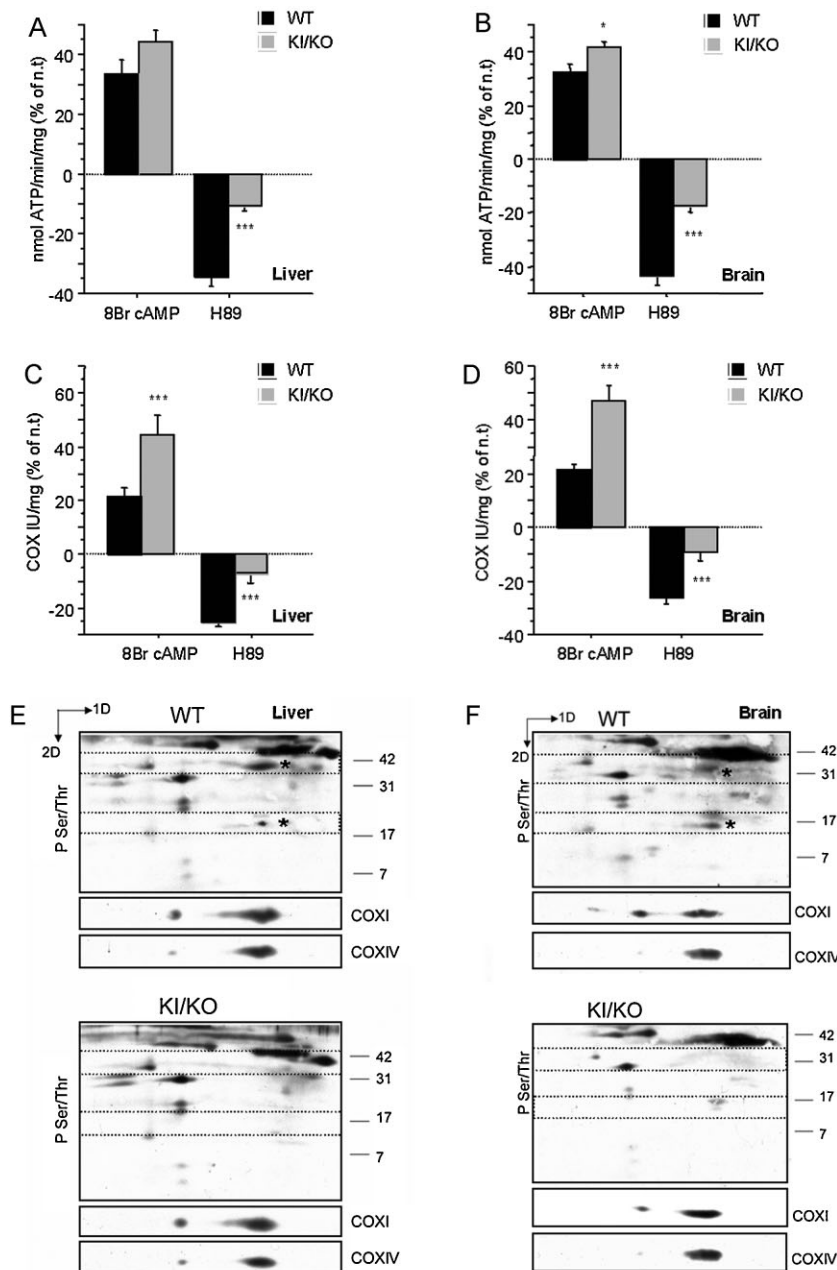
- A. BNGE Western blot of respiratory chain complexes in homoplasmic MELAS cybrids SUA63 and isogenic WT control cybrids SUB36.
- B. 8Br-cAMP and H89 modulation of COX activity in MELAS cybrids and controls ( $n = 7$ ).
- C. 8Br-cAMP and H89 modulation of COX activity in mouse homoplasmic COXI mutant fibroblasts (E09) and WT controls ( $n = 4$ ).
- D. BNGE Western blot of respiratory chain complexes in heteroplasmic Cyt *b* mutant cybrids (D4cytb 5.2 and D4cytb 3) and isogenic WT control cybrids.
- E. 8Br-cAMP and H89 modulation of COX activity in heteroplasmic Cyt *b* mutant cybrids and WT controls ( $n = 8$ ). Values in (B), (C) and (E) are shown as percentage of untreated controls for each cell line.
- F. Correlation plot between the percentage of residual COX activity and the percentage of COX stimulation upon 8Br-cAMP treatment, in the indicated COX deficient models. The percentages of COX stimulation by 8Br-cAMP is relative to untreated and is shown as the average  $\pm$  SD of  $n = 5$  determinations for each indicated COX deficient group. WT controls are averages ( $\pm$  SD) of non-COX deficient cells (143B, fWT Htert, SUB36, FBalb/cJ, FC57BL/6J, cytbA4 4.1, D4cytb 5.2) and tissues (brain WT and liver WT).

$^*$ ,  $p < 0.01$ ;  $^{**}$ ,  $p < 0.001$ ;  $^{***}$ ,  $p < 0.0001$ .

inhibition of ATP synthesis (Fig 3A,B) and COX activity (Fig 3C,D) by 8Br-cAMP and H89, respectively. Similar results were obtained in skeletal muscle (data not shown).

The phosphorylation of COX subunits I and IV, which are targets of mitochondrial PKA (Acin-Perez et al, 2009), was investigated in Sco2 KI/KO mouse mitochondria by 2D blue native gel electrophoresis (2D-BNGE) and Western blot. In both liver and brain from Sco2 KI/KO mice, COXI and COXIV were less phosphorylated than in WT mitochondria (Fig 3E,F). The relative phosphorylation of each subunit was assessed by quantification of the intensities of the immuno-signal obtained with a phospho Ser/Thr antibody over the signal derived from the antibody against the total protein. In liver ( $n = 2$ ), the phospho-COXI/COXI ratios were 0.05 in KI/KO and 0.60 in WT, and the phospho-COXIV/COXIV ratios were 0.01 and 0.3, respectively. In brain ( $n = 2$ ), the phospho-COXI/COXI ratios were 0.01 in KI/KO and 0.35 in WT and the phospho-COXIV/

COXIV ratios were 0.001 and 0.25, respectively. These results indicate that *in vivo* COX deficiency results in decreased basal phosphorylation of COX subunits. COXI hypophosphorylation was also confirmed in the human fibroblast line fSco2 Htert (phospho-COXI/COXI ratio was 0.2 vs. 0.6 in fWT Htert,  $n = 2$ ) and in the mouse fibroblast line E09 (phospho-COXI/COXI ratio: 0.3 vs. 0.6–0.7 in FBalb/cJ or FC57BL/6J, respectively,  $n = 2$ ). Comparing all the systems together, we found that there was an inverse linear correlation ( $R^2$  0.782) between residual COX activity in COX-deficient mitochondria and the proportion of COX activity increased by 8Br-cAMP stimulation (Fig 2F). This correlation did not depend on whether the defect involved loss of COX subunits, such as in CA75, CA35, SUA63 and Sco2 mutants, or a loss of function with normal protein levels, such as in E09 cells. The result of this analysis indicates that COX regulation through PKA phosphorylation depends on COX activity and not on protein levels.



**Figure 3. Abnormal PKA modulation of OXPHOS in COX defective mice.** Liver and brain mitochondria from *Sco2* KI/KO mice and WT control Sv/J mice were treated with 8Br-cAMP or H89. OXPHOS function was assessed by:

**A, B.** ATP synthesis ( $n = 6$ ) and **C, D.** COX enzymatic activity ( $n = 9$ ). For each sample, changes induced by the treatments are indicated as a percentage of untreated controls (unt.). \*,  $p < 0.01$ ; \*\*\*,  $p < 0.0001$ .

**E, F.** Immunodetection of mitochondrial phospho-proteins in 2D-BNGE Western blot probed with anti-phospho Ser/Thr. The areas of the membrane delimited by dashed lines were probed with specific antibodies against COX I and COX IV subunits (shown below each panel) to demonstrate the identity of the phosphorylated proteins and to assess protein loading. The asterisks denote the putative phospho-COX I and phospho-COX IV in WT liver and brain mitochondria, which were undetectable in the KI/KO mitochondria. One representative Western blot of two independent experiments is shown.

**ROS and PGC1 $\alpha$  mediate increased OXPHOS biogenesis in COX I mutant cybrids**

When OXPHOS is impaired, cells need to develop strategies to compensate for the metabolic defect. Adaptive mechanisms include an increase in OXPHOS biogenesis, which is typically observed in muscle fibres harbouring certain mtDNA mutations (ragged red fibres). It has been proposed that ROS can behave as second messengers in the regulation of OXPHOS biogenesis (St-Pierre et al, 2006), and changes in OXPHOS biogenesis mediated by ROS production have been reported in cultured cells with mtDNA mutations (Moreno-Loshuertos et al, 2006). We hypothesized that decreased protein phosphorylation in COX deficient cells is a compensatory mechanism to enhance

ROS production, and thus increase OXPHOS biogenesis. Indeed, ROS were elevated in heteroplasmic COX I mutants (Table 2). Consistently, the activity of citrate synthase (Table 1), a Krebs cycle enzyme and the mtDNA content (Table 2) were also increased. Furthermore, the expression of the cotranslational trans-activating factors PGC1 $\alpha$  and NRF1 that control OXPHOS biogenesis (Scarpulla, 2008) was significantly increased in CA75 heteroplasmic COX I mutant cells (Table 2). Homoplasmic COX I mutants do not have increased ROS production, presumably because the electron flow is abolished, and thus did not upregulate OXPHOS biogenesis (Table 2).

8Br-cAMP decreased ROS production by stimulating COX and improving the efficacy of electron transfer, while H89

**Table 2. ROS production and parameters of OXPHOS biogenesis in COXI mutant cybrids**

Cell line	Mutation load (%)	ROS production (F535 nm/max)	mtDNA/nDNA (% vs. 143B)	PGC1 $\alpha$ /GAPDH (% vs. 143B)	NRF1/GAPDH (% vs. 143B)
143B	0	0.356 $\pm$ 0.078 (34)	100 $\pm$ 9.79 (4)	100 $\pm$ 12.41 (14)	100 $\pm$ 15.2 (12)
ctlB2	0	0.377 $\pm$ 0.041 (8)	n.d.	n.d.	n.d.
CA0	0	0.354 $\pm$ 0.026 (8)	103.9 $\pm$ 7.57 (4)	n.d.	n.d.
CA35	35	0.560 $\pm$ 0.098** (18)	174.33 $\pm$ 26.18** (6)	n.d.	n.d.
CA75	75	0.577 $\pm$ 0.104** (18)	189.13 $\pm$ 13.55** (6)	160.5 $\pm$ 29.52** (6)	167.5 $\pm$ 11.64** (4)
CA100	100	0.383 $\pm$ 0.044 (8)	90.26 $\pm$ 12.73 (4)	n.d.	n.d.

ROS production in untreated cells is expressed as the ratio between the spontaneous DCFDA fluorescence (535 nm emission) and the maximal fluorescence elicited by exposure to H<sub>2</sub>O<sub>2</sub> (300  $\mu$ M). The mtDNA content determined by RT-PCR and normalized by nuclear 28S rRNA gene content is shown as percentage of 143B. PGC1 $\alpha$  and NRF-1 mRNA levels determined by RT-PCR relative to GAPDH mRNA are shown as percentage of 143B. Three independent control cell lines were used: 143B parental osteosarcoma cells, ctlB2 human WT cybrids, CA0 WT cybrids isogenic for the CA COXI mutant cybrids. The number in brackets represents the n for each experiment. n.d., not determined.

\*\* $p < 0.001$ .

increased ROS production in WT cells, but significantly less in heteroplasmic COXI mutants (Fig 4A). This suggests that PKA blockage normally results in increased ROS, but mutant cells that have constitutively lower protein phosphorylation do not up-regulate ROS.

To investigate the role of PKA in OXPHOS biogenesis we used three different luciferase (LUC) reporter constructs. The first one expressed LUC under the control of the PGC1 $\alpha$  promoter (Handschin et al, 2003); the second expressed LUC from the cytochrome *c* full promoter (Cyt *c*-LUC326, Xia et al, 1997), which is under the control of PGC1 $\alpha$  through NRF2 (Scarpulla, 2008); the third had an incomplete Cyt *c* promoter, lacking SP1 and NRF2 binding sites (Cyt *c*-LUC66, Xia et al, 1997). Transfected cells were treated for 48 h with 8Br-cAMP, H89 or the antioxidant *N*-acetyl-cysteine (NAC). PGC1 $\alpha$ -LUC (Fig 4B, upper panel) and Cyt *c*-LUC326 (Fig 4B, lower panel) expression decreased significantly in CA75 cells treated with 8Br-cAMP. Conversely, H89 significantly increased PGC1 $\alpha$ -LUC expression in WT, but not in CA75 cells, confirming that OXPHOS biogenesis in the latter is poorly responsive to PKA inhibition. NAC had similar effects as 8Br-cAMP on the expression of the reporter genes in both WT and CA75 cells and suppressed the increase in LUC activity induced by H89 in WT cells, confirming that the changes in gene expression were mediated by ROS. Cyt *c*-LUC66 did not show any change in expression with any of the compounds (Fig S1 of Supporting Information). Changes in mtDNA content (Fig 4C) and citrate synthase activity (Fig 4D) paralleled those of PGC1 $\alpha$ -LUC and Cyt *c*-LUC326.

In COXI mutant cells, Cyt *c*, which is under the control of PGC1 $\alpha$ /NRF1, was strongly upregulated (Fig 4E). A 48-h treatment with 8Br-cAMP or NAC resulted in a decrease of Cyt *c* (Fig 4F), both in mutant and WT cells, indicating that chronic stimulation of PKA signalling affects OXPHOS biogenesis by decreasing ROS.

A 48 h treatment of WT cells with forskolin/IBMX, which increases cytosolic but not mitochondrial cAMP (Acin-Perez et al, 2009), did not affect ROS production, mtDNA content, cytrate synthase activity and the steady-state levels of Cyt *c* (Fig S2 of Supporting Information), indicating that OXPHOS biogenesis was not affected by cytoplasmic cAMP.

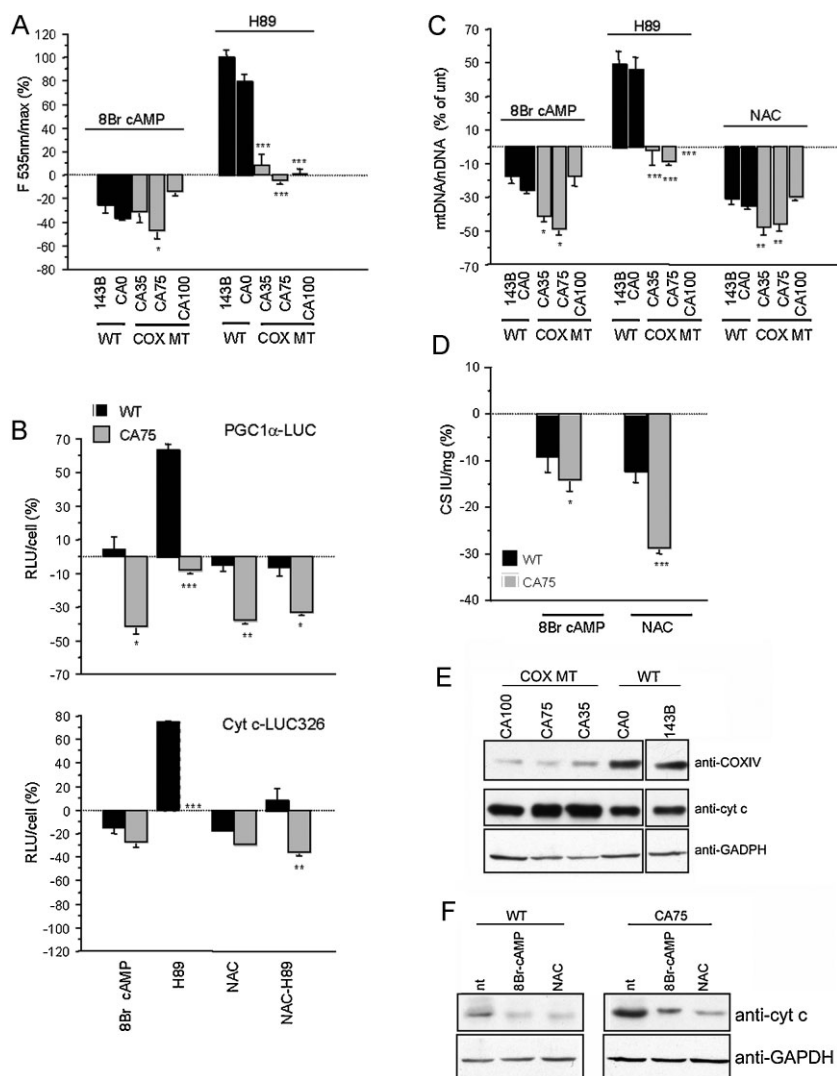
The effects of the chronic administration of 8Br-cAMP were recapitulated using a genetic approach, where an enzymatically active isoform of rat sAC (Hess et al, 2005) was expressed in 293T HEK cells (Fig S3A of Supporting Information), resulting in decreased mtDNA content (Fig S3B of Supporting Information), cytrate synthase activity (Fig S3C of Supporting Information), COXIV and Cyt *c* (Fig S3A of Supporting Information).

To assess the physiological consequences of OXPHOS biogenesis down-regulation we subjected cells to galactose medium, where cells depend on OXPHOS for ATP synthesis (Mattiazzi et al, 2004). In WT cells, the ratio between the doubling time in glucose and in galactose (glu/gal) was decreased by chronic treatment with 8Br-cAMP (Fig S4 of Supporting Information). Furthermore, 8Br-cAMP did not improve the inability of CA75 cells to replicate in galactose medium, because the decrease in OXPHOS biogenesis (Fig 4) offset the stimulation of COX activity.

#### Mitochondrially targeted sAC (mt-sAC) ameliorates the OXPHOS defect in COXI mutant cells

Treatment of COXI mutant cells with PKA analogues stimulates COX activity and OXPHOS (Fig 1), but it also inhibits mitochondrial biogenesis (Fig 4). Furthermore, chronic treatment with cAMP analogues is likely to have toxic extra-mitochondrial effects. For these reasons we undertook a genetic approach to constitutively increase cAMP selectively in mitochondria. Although a portion of sAC is localized in the mitochondrial matrix (Acin-Perez et al, 2009), it is not known yet which of the multiple splice variants of sAC is targeted to mitochondria and whether any specific sAC isoform is exclusively localized in mitochondria. Thus, to ensure its specific targeting to the mitochondrial matrix we generated a recombinant mitochondrial-sAC (mt-sAC) by adding a N-terminal cleavable presequence from the subunit *c* of human ATPase (Manfredi et al, 2002a) to the rat sAC isoform. We also appended a C-terminal HA-tag to distinguish mt-sAC from endogenous sAC (Fig 5A).

In transiently transfected COS cells, mt-sAC was localized in mitochondria, as evidenced by colocalization with the mitochondrial dye Mitotracker Red, in contrast to sAC that was



**Figure 4. Effects of PKA modulation on ROS production and OXPHOS biogenesis.**

- A.** WT and COX1 mutant cybrids were incubated for 2 h in the presence of 8Br-cAMP or H89 and ROS production was measured by DCFDA fluorescence. Changes in fluorescence induced by the treatments are indicated as a percentage of untreated controls (unt.) ( $n = 9$ ).
- B.** LUC activity in PGC1 $\alpha$ -LUC (upper panel) or Cyt c-LUC 326 (lower panel) transfected WT and CA75 cells, after 48 h treatment with 8Br-cAMP, H89, NAC or H89 plus NAC. Luminescence was normalized by the number of cells (RLU/cell ratio). Values are shown as a percentage of untreated controls for each cell line and represent the mean of three independent transfections, each measured in triplicates.
- C.** MtDNA content after 48 h treatment with 8Br-cAMP, H89 or NAC ( $n = 7$ ).
- D.** Citrate synthase (CS) enzymatic activity after 48 h treatment with 8Br-cAMP or NAC ( $n = 6$ ). In (C) and (D), values are shown as a percentage of untreated controls for each cell line. \*,  $p < 0.01$ ; \*\*,  $p < 0.001$ ; \*\*\*,  $p < 0.0001$ .
- E.** Western blot for COXIV and Cyt c in COX1 mutant cybrids and WT controls ran under the same conditions. Reprobing the blots with GAPDH confirms that same amount of proteins was loaded for all cell lines.
- F.** Western blot for Cyt c in CA75 cybrids and WT controls after 48 h treatment with 8Br-cAMP and NAC. Panels (E) and (F) show representative Western blots from three independent experiments.

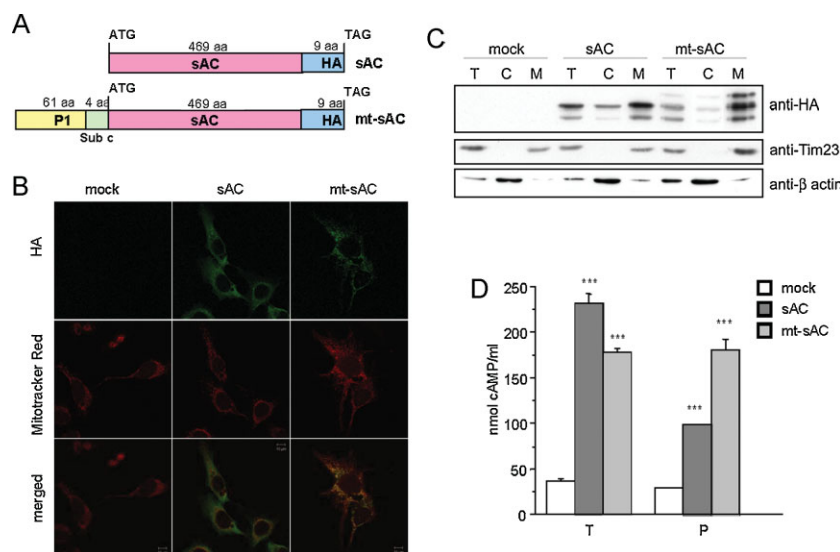
diffusely expressed (Fig 5B). Cell fractionation revealed that mt-sAC was in the mitochondrial fraction, except for a small proportion of unprocessed protein residing in the cytosol, while sAC was both in the cytosol and mitochondria (Fig 5C). After permeabilization of the plasma membrane, cAMP was measured in the pellet fraction (containing organelles) and in the total homogenate from transfected cells. Total cAMP levels were increased in both sAC and mt-sAC expressing cells, but in the latter cAMP was concentrated in the fraction containing mitochondria (Fig 5D).

After determining that the content of total and mitochondrial endogenous sAC was comparable in CA75 and WT cells (data not shown), we stably transfected them with mt-sAC and determined that cAMP was increased in the organellar fraction of mt-sAC expressing cells (Fig S5A of Supporting Information). The COX1 mutation load in clones and mass cultures of transfected CA75 ranged between 62 and 87% (Fig 6A). The expression levels of mt-sAC varied among lines (Fig 6B), and COX activity was increased proportionally to mt-sAC expression (Fig 6C). CA75 lines with higher mt-sAC expression

also had higher COX activity. For example, CA75 mt-sAC clones 1 and 2 had 76 and 72% mutant mtDNA, respectively; but clone 1 with higher mt-sAC expression also had higher COX activity. On the other hand, CA75 mt-sAC clone 3 with high mt-sAC expression had low COX activity due to a very high mutation load (89%).

COX activity in mock transfected CA75 cells was relatively insensitive to the specific sAC inhibitor KH7 (Hess et al, 2005), consistent with down-regulation of COX PKA-dependent phosphorylation. However, KH7 completely abolished the increase in COX activity induced by mt-sAC expression in CA75 cells (Fig S5B of Supporting Information).

Cell respiration in CA75 mass cultures and clone 1 recovered to levels comparable to mock-transfected WT cells (Fig 6D). The functional amelioration of OXPHOS in CA75 cells expressing mt-sAC was further demonstrated by the improvement in the doubling time ratio glu/gal (Fig 6E). Of note, the growth in galactose did not change the mutation load in the mt-sAC CA75 cells (data not shown). At the proteomic level, the amount of phosphorylated COX1 was increased in parallel with the



**Figure 5. mt-sAC is targeted to mitochondria and is enzymatically active.**

- A.** Scheme of the sAC and the mitochondrially targeted sAC (mt-sAC) genetic constructs. P1 is the mitochondrial targeting peptide of the human P1 isoform of ATPase subunit c. Four amino acid of the mature subunit c (sub c) are included in the construct. The rat sAC gene is cloned downstream of the targeting peptide and contains an HA epitope tag.
- B.** COS-1 cells expressing the empty vector (mock), sAC or mt-sAC were stained with Mitotracker (in red) and immuno-stained for HA (in green). In the merged images, colocalization of sAC and mitochondria is indicated by the overlap of the fluorescence (i.e. in yellow).
- C.** sAC and mt-sAC distribution in subcellular fractions of transfected COS-1 cells. Total cell homogenate (T), mitochondrial fraction (M), cytosolic fraction (C). β-Actin and Tim23 are markers of cytosolic and mitochondrial proteins, respectively.
- D.** cAMP levels in sAC and mt-sAC expressing cells measured in total cell homogenates (T) and in the mitochondria containing pellets (P), which result from plasma membrane permeabilization followed by centrifugation. \*\*\*,  $p < 0.0001$ .

improvement of OXPHOS function in mt-sAC expressing CA75 cells (Fig 6F).

The rescue of OXPHOS function induced by mt-sAC expression in CA75 cells correlated with a decrease in ROS production (Fig 7A), PGC1 $\alpha$  (Fig 7B) and NRF1 (Fig 7C) mRNA, mtDNA (Fig 7D) and Cyt *c* content (Fig 7E), all of which reverted to normal levels. mt-sAC expression did not affect the levels of COXI and COXIV, which remained deficient (Fig 7E).

Taken together these results indicate that the constitutive stimulation of cAMP production in mitochondria by expressing sAC targeted to the matrix triggers adaptation mechanisms in COX deficient cells that upregulate OXPHOS function and reduce the need for enhanced ROS-dependent OXPHOS biogenesis.

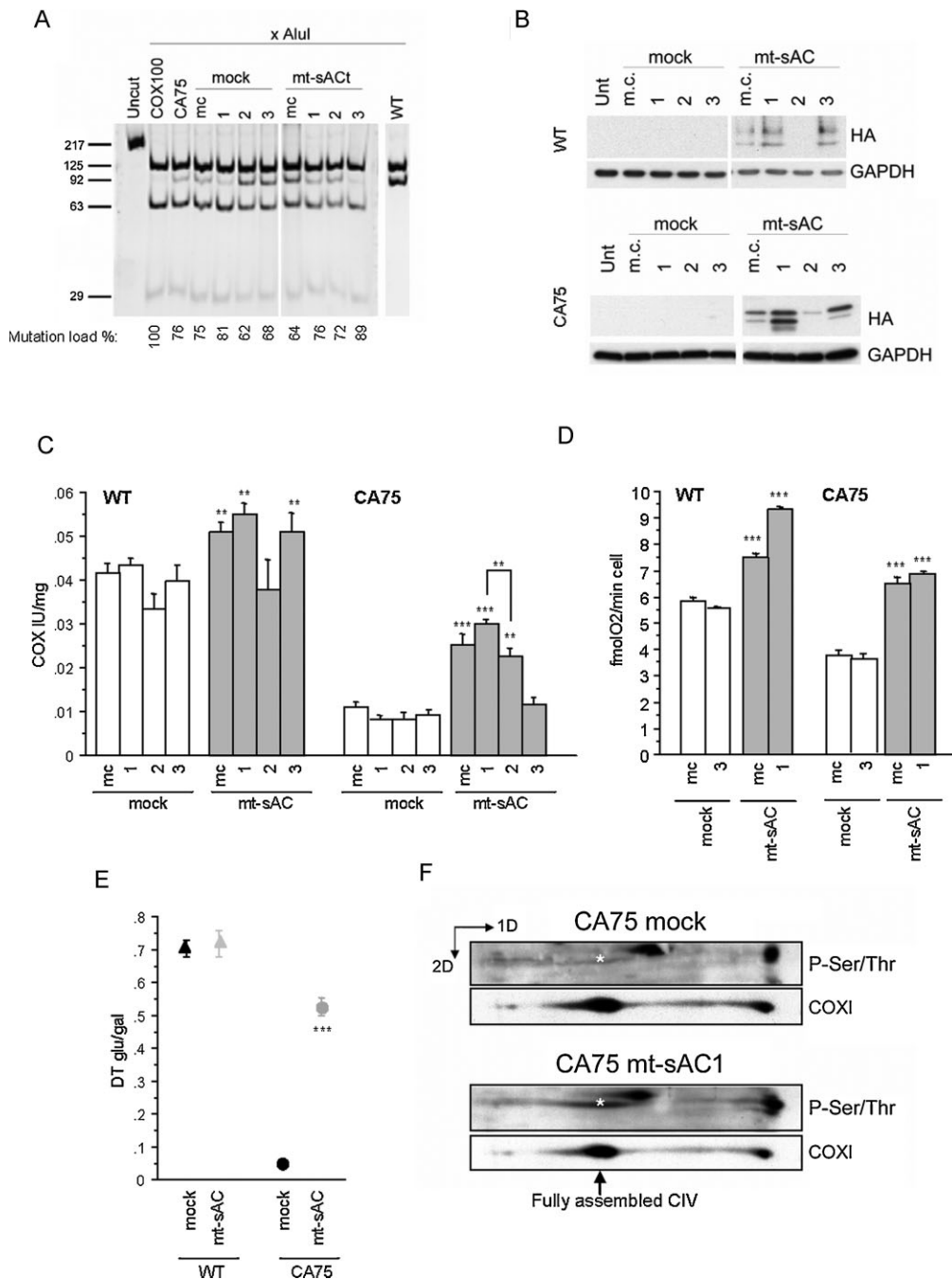
## DISCUSSION

We have demonstrated that OXPHOS regulation by mitochondrial PKA is different in normal and COX defective cells and tissues, in humans and mice. OXPHOS activity in COX deficient systems is strongly stimulated and poorly inhibited by PKA agonists and antagonists, respectively, because there is a constitutive down-regulation of PKA-dependent protein phosphorylation. COX defective cells suppress phosphorylation of COX subunits and residual enzymatic activity, thereby increasing OXPHOS biogenesis via a ROS-mediated PGC1 $\alpha$ /NRF1 pathway (Irrcher et al, 2009; St-Pierre et al, 2006). However, the ROS-mediated compensatory mechanism may come at a high price, because it enhances oxidative stress with potentially detrimental long-term effects. Interestingly, the up-regulation of ROS and OXPHOS biogenesis is only found in heteroplasmic, but not in homoplasmic, COXI mutants. The latter have a complete loss of COX activity, resulting in retrograde blockage of the pathways feeding reducing equivalents to the respiratory chain (Vives-Bauza et al, 2006), whereas a partial COX defect facilitates electron escape from the respiratory chain (Zuckerbraun et al, 2007). Undoubtedly,

metabolic factors other than ROS may also contribute to the regulation of mitochondrial biogenesis in COX deficient cells. For example, changes in the AMP/ATP ratio could signal to PGC1 $\alpha$  through AMP kinase (Bronner et al, 2004; Jager et al, 2007). Nevertheless, the observation that homoplasmic COXI mutant cells, which do not overproduce ROS, fail to upregulate mitochondrial biogenesis suggests that ROS may play an important role. The mechanism whereby COX deficient cells suppress COX phosphorylation, despite having all the components of the mt-sAC pathway in place, remains to be fully elucidated, but it is likely to result from reduced levels of CO<sub>2</sub> produced by the Krebs cycle, which in mutant cells is slowed down by decreased utilization of NADH in the respiratory chain (Acin-Perez et al, 2009).

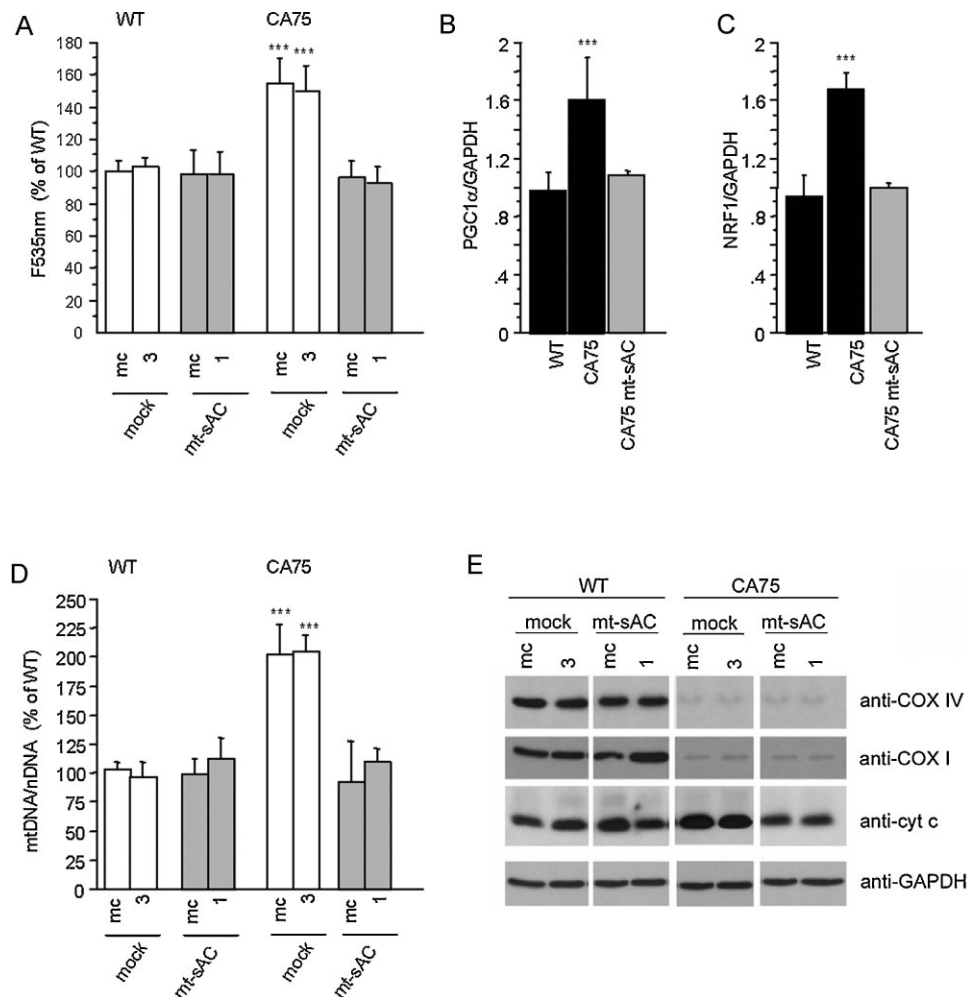
In COX deficient cells, OXPHOS can be ameliorated by stimulation of the PGC1 $\alpha$  pathway of mitochondrial biogenesis. Increased expression of PGC1 $\alpha$  by transgenic or pharmacological means resulted in a remarkable improvement of OXPHOS function and clinical phenotype in a mouse model of muscle COX deficiency (Wenz et al, 2008). The mt-sAC pathway opens a new window of opportunity to ameliorate mitochondrial defects. By genetic manipulation we stably upregulated mt-sAC and induced a long-term metabolic adaptation. mt-sAC expression dramatically improved mitochondrial function in COX deficient cells, which adapted to the sustained rise in mitochondrial cAMP by increasing OXPHOS activity. Therefore, activation of the mt-sAC pathway is an alternative approach to improve OXPHOS in COX deficient cells, without enhancing ROS





**Figure 6. mt-sAC expression in COXI mutant cell lines.**

- A.** COXI mutation load measured by last hot cycle PCR followed by *AluI* digestion in mass culture (m.c.) and individual clones of CA75 stably expressing mt-sAC. The level of heteroplasmy indicated as a percentage of mutant mtDNA is indicated below each lane, and represents the mean value of two independent determinations. WT lane originates from a different gel run in parallel with the CA75 samples.
- B.** mt-sAC expression in total cell homogenates of WT and CA75 m.c. and clones ran under the same conditions determined by immunoblot with HA antibody. Reprobing the blots with GAPDH confirms that same amount of proteins was loaded for all cell lines.
- C.** COX enzymatic activity in cell lysates of WT (on the left) or CA75 (on the right) expressing mt-sAC ( $n = 6$ ).
- D.** Intact cell respiration in WT (on the left) or CA75 (on the right) cells expressing mt-sAC ( $n = 6$ ).
- E.** The ratio of the doubling time in glucose medium and in galactose medium (DT glu/gal) in WT or CA75 cells expressing mt-sAC ( $n = 4$ ). In panels, C–E, \*,  $p < 0.01$ ; \*\*,  $p < 0.001$ ; \*\*\*,  $p < 0.0001$ .
- F.** 2D-BNGE of isolated mitochondria from mt-sAC expressing or mock transfected CA75 cells followed by successive immunodetection for phosphorylated proteins and COXI. The position of the fully assembled, monomeric, COX (CIV) is indicated by the arrow. The asterisk indicates the phosphorylated COXI, which is significantly lower in mock transfected than in mt-sAC expressing cells. The blots shown are representative of two independent experiments.



**Figure 7. mt-sAC expression normalizes ROS-dependent OXPHOS biogenesis in COX deficient cells.**

- A.** ROS production measured by DCFDA fluorescence in WT and CA75 mt-sAC expressing cells and mock-transfected controls. Values represent the percentage of DCFDA fluorescence relative to WT non-transfected cells ( $n = 15$ ). PGC1 $\alpha$
- B, C.** PGC1 $\alpha$  and NRF-1 mRNA levels measured by RT-PCR and normalized by GAPDH mRNA levels, in non-transfected WT and CA75 cells and in CA75 mt-sAC expressing cells ( $n = 6$ ).
- D.** mtDNA content in WT and CA75 mt-sAC expressing cells and mock-transfected controls. Values represent the percentage of mtDNA/nuclear DNA ratio relative to WT non-transfected cells ( $n = 9$ ).
- E.** Western blot of total cell homogenates from WT and CA75 mt-sAC expressing cells and mock-transfected controls ran under the same conditions immunoprobed for Cyt c and for COX subunits I and IV. Reprobing the blots with GAPDH confirms that same amount of proteins was loaded for all cell lines. The blots shown are representative of two independent experiments.

production and OXPHOS biogenesis. In a therapeutic perspective, the two approaches, PGC1 $\alpha$  stimulation and mt-sAC upregulation, could be complementary.

In line with our previous observation that COXI and COXIV are putative targets for the mt-sAC pathway (Acin-Perez et al, 2009), we find that these two subunits are hypophosphorylated in COX deficient mitochondria, suggesting that PKA-mediated phosphorylation of these two proteins is critical for enzymatic activity. Phosphorylation of various COX subunits has been recognized for over a decade (Bender & Kadenbach, 2000; Helling et al, 2008; Lee et al, 2005; Miyazaki et al, 2003; Samavati et al, 2008; Steenaart & Shore, 1997), although the physiological

meaning of these phosphorylation events is still not completely understood. Cumulatively, COX subunits have more than 50 potential PKA target residues (Helling et al, 2008), but only a handful of targets have been identified so far. It is likely that *in vivo* multiple targets are simultaneously phosphorylated or dephosphorylated based on the needs for short-term reversible regulation of OXPHOS. So far, no specific phosphatases for any subunit of the respiratory chain have been described. Therefore, it is hard to predict the metabolic consequences of PKA modulation by analysing the effects of phosphorylation of individual amino acids *in vitro*. Extensive proteomic studies will be needed to determine under which conditions and at which

residues COX is phosphorylated *in vivo*. Nevertheless, our results indicate that, in living cells, stimulation of COX phosphorylation by mitochondrial PKA results in increased enzymatic activity, which can be maintained over a prolonged period of time.

In summary, we have identified a novel pathway of OXPHOS regulation in healthy and COX deficient cells. We show that genetic manipulation of the mt-sAC pathway results in a resetting and optimization of the balance between energy metabolism, ROS production and OXPHOS biogenesis in COX deficient cells (summarized in the scheme of Fig 8). These findings provide new clues on the ways that mitochondria cope with metabolic defects and on potential pharmacologic interventions that target the mt-sAC pathway to improve mitochondrial function. It will be important to extend these observations to other mitochondrial disorders arising from genetic mutations of the mitochondrial or nuclear genome that affect the respiratory chain, as well as acquired mitochondrial defects that develop in the course of diverse neurodegenerative diseases (Kwong et al, 2006).

## METHODS

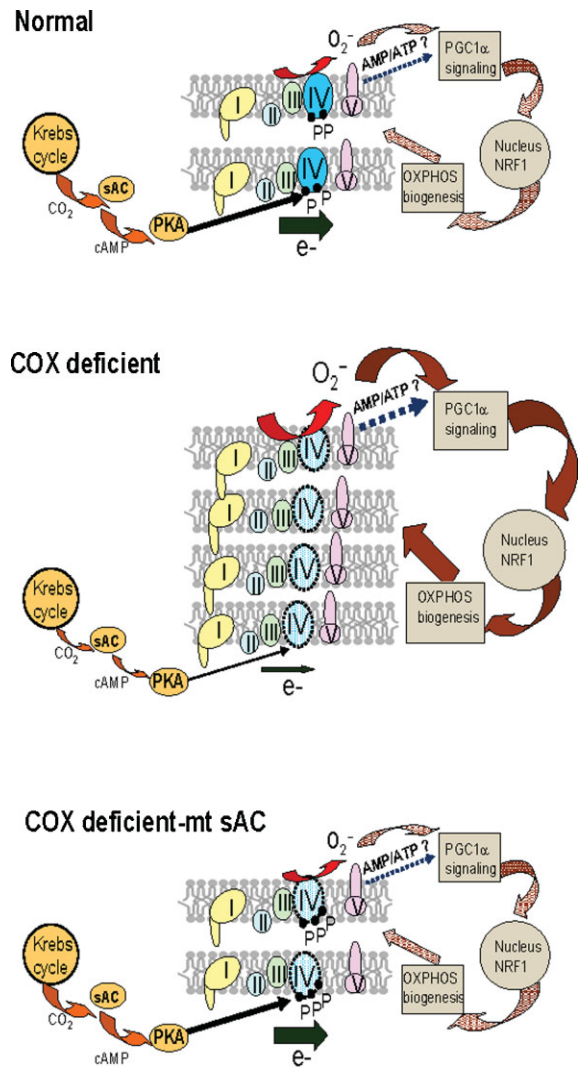
### Cell culture and pharmacological treatments

The previously described cybrids cell lines were derived from fusion of 143B osteosarcoma  $\rho^0$  cells with enucleated cells from patients harbouring mtDNA mutations (King & Attardi, 1989). Cybrids were grown in Dulbecco modified Eagle's medium (DMEM, Invitrogen) containing 4.5 g/l glucose and 1 mM pyruvate, supplemented with 10% foetal bovine serum (FBS, Invitrogen) and 50  $\mu$ g/ml uridine (Sigma-Aldrich). COS-1 and 293T HEK cells were grown in the same conditions with the exclusion of uridine. Human fibroblasts were immortalized by expression of human telomerase as described (Lochmuller et al, 1999), and grown in the same conditions. For growth in galactose, cells were placed in medium without glucose and containing 4.5 g/l galactose (Sigma-Aldrich) plus 1 mM pyruvate and 10% dialyzed FBS. Cell replication rates in glucose and galactose medium were measured as described previously (Moreno-Loshuertos et al, 2006).

The concentrations of the compounds used on cultured cells for this study were as follows: 8Br-cAMP 1 mM (Sigma-Aldrich); H89 1  $\mu$ M (Calbiochem); NAC 2.5 mM (Sigma-Aldrich); forskolin 10  $\mu$ M (Sigma-Aldrich); 3-isobutyl-1-methylxanthine 50  $\mu$ M (IBMX, Sigma-Aldrich), KH7 50  $\mu$ M.

### Generation of *Sco2* KI/KO mice

To clone the murine *Sco2* gene a genomic fragment (65d3) containing the *Sco2* gene was isolated from a 129 SvJ mouse BAC library (Genome System, Inc.). The isolated 5 kb fragment containing the entire *Sco2* gene was subcloned into the *KpnI* site of pBluescript II KS+ and used to generate the targeting vector. To generate *Sco2* knock out (KO) mice a 2 kb fragment composed of the gene encoding the neomycin phosphotransferase (NEO cassette) flanked by two 34-mer identical loxP sites (loxP-NEO-loxP) was inserted in the *PacI* site (converted from a *HindIII*), 30 bp downstream of the start codon of *Sco2*. To generate E129K mutant *Sco2* knock in (KI) mice a single base pair mutation, which converted nucleotide 385 in the mouse *Sco2* sequence from G to A, was introduced by *in situ* mutagenesis. The NEO cassette was inserted in the *PacI* site (converted from a *SmaI* site), 643 bp downstream of the stop codon of



**Figure 8. Schematic model of the proposed mitochondrial mt-sAC pathway of OXPHOS regulation in normal and COX deficient conditions.** In normal mitochondria, the pace of electron ( $e^-$ ) flow through the respiratory chain complexes (I–IV) is finely regulated by the activity of complex IV (COX), which in turn is modulated by phosphorylation of its subunits (P, COX phospho-residues) by mitochondrial PKA. This regulation responds to the  $\text{CO}_2$  produced by the Krebs cycle through mitochondrial sAC. Electron flow is linked to ROS production (e.g. superoxide,  $\text{O}_2^-$ ) and ATP synthesis, which in turn may affect the cellular AMP/ATP ratio. These, and other putative factors (indicated by ?), have signalling functions on the regulation of nuclear genes involved in mitochondrial biogenesis, such as PGC1 $\alpha$  and NRF1. In COX deficient mitochondria with decreased electron flow, Krebs cycle activity is dampened and  $\text{CO}_2$  production reduced, resulting in hypophosphorylation of COX, increased ROS production and increased signalling for compensatory mitochondrial biogenesis through the PGC1 $\alpha$ –NRF1 axis. In COX deficient cells increased COX phosphorylation by the sAC–cAMP–PKA pathway, thereby increasing electron flow and normalizing the signalling for OXPHOS biogenesis.

## The paper explained

### PROBLEM:

Mitochondrial disorders are common genetic diseases of energy metabolism that affect one in every 5000 people. Impaired mitochondrial metabolism is also associated with aging and many neurodegenerative disorders. Deficiency of COX, a key mitochondrial respiratory chain enzyme, is a biochemical hallmark of mitochondrial disorders, either genetically determined or acquired in course of neurodegeneration and aging. No effective therapy is currently available for mitochondrial diseases.

### RESULTS:

The data here presented demonstrate that a recently discovered intra-mitochondrial signalling pathway, the mt-sAC pathway, is differentially regulated in healthy and COX-deficient

mitochondria from humans and mice. COX deficient cells utilize the mt-sAC pathway for compensatory up-regulation of mitochondrial biogenesis through a free radical-mediated signalling to the nucleus. Importantly, genetic activation of the mt-sAC pathway stimulates COX phosphorylation, thereby enhancing its activity and correcting mitochondrial dysfunction in COX deficient cells.

### IMPACT:

This study defines the mt-sAC signalling pathway as a mechanism for metabolic adaptation to mitochondrial dysfunction and a novel potential target for therapeutic interventions to treat mitochondrial diseases.

*Sco2*. To generate *Sco2* KI/KO compound heterozygous mice the *Sco2* homozygous KI mice were crossed with *Sco2* heterozygous KO mice.

### Animal care

All experiments were performed according to the Institutional Animal Care and Use Committee-approved protocol at the Columbia University Medical Center (CUMC protocol number AAAA9997), which is consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### Mitochondrial isolation from mouse tissue

Mouse liver and brain mitochondria were isolated as described (Fernandez-Vizarrá et al, 2002). PKA activity was modulated by 8Br-cAMP (1 mM) and H89 (1  $\mu$ M) in mitochondria and skeletal muscle homogenates as described elsewhere (Acin-Perez et al, 2009).

### Cloning of sAC and mt-sAC

A C-terminal HA tag was added to the cDNA sequence from the rat sAC gene (a gift from Dr Levin and Dr Buck, Department of Pharmacology, Weill Medical College of Cornell University) by PCR amplification using appropriate primers (Methods of Supporting Information). The PCR product was digested with *AgeI* and *NotI* and cloned in the eukaryotic expression vector pTurbo-G418 (Evrogen-Axxora). To direct sAC to mitochondria (mt-sAC) the targeting signal of subunit c of ATPase (P1) (Manfredi et al, 2002b) plus four amino acids of the mature protein were cloned in frame at the N terminus of sAC using *NheI* and *AgeI* sites.

### Cell transfection

Reporter constructs expressing LUC under the control of the complete Cyt *c* promoter (Cyt *c*-LUC 326) or an incomplete promoter (lacking NRF-1 and CREB binding sites, Cyt *c*-LUC 66) (Xia et al, 1997) and under the control of the PGC1 $\alpha$  promoter (PGC1 $\alpha$ -LUC) (Handschin et al, 2003) were used to transfect cells with FuGene6 (Roche). LUC

activity was measured on lysates from  $3 \times 10^5$  transfected cells using the Luciferase Assay System (Promega) in a luminometer (Optocomp I-MGM Electronics).

Stable mt-sAC cell lines were generated by transfection of 143B and CA75 cell lines followed by selection in 300  $\mu$ g/ml G418 (Geneticin, Invitrogen) for 3 weeks.

Measurements of the mutation loads in COXI mutant clones was performed by last-hot cycle PCR followed by *Afl* digestion as described elsewhere (D'Aurelio et al, 2006).

### sAC and mt-sAC localization

COS-1 cells transiently transfected with sAC or mt-sAC were plated on cover slips and stained with Mito-tracker Red (Invitrogen) followed by fixation in 4% paraformaldehyde for immunocytochemistry using HA antibodies (Abcam). Transfected cells were analysed using a Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY) equipped with a 63 $\times$ /1.25 NA oil lens. A series of z-sections were taken spanning the thickness of the cell with intervals between sections set at 0.7  $\mu$ m. Z-stack images were projected onto a single plane using the LSM Image Browser software (Carl Zeiss Microimaging, Inc.). Digital magnification was 2 $\times$  (total magnification was 126 $\times$ ).

For cell fractionation and intracellular localization of sAC or mt-sAC,  $4 \times 10^6$  cells were transfected and mitochondria were isolated as described elsewhere (Birch-Machin & Turnbull, 2001). The total cell homogenate, the cytosolic fraction and the mitochondrial fraction were analysed by Western blot using HA antibodies.

### Oxygen consumption and enzymatic assays

O<sub>2</sub> consumption determinations in intact cells were carried out in an oxygraph equipped with a Clark electrode as previously described (Hofhaus et al, 1996). TMPD (1 mM) dependent respiration was assayed in intact cells after blocking the respiratory chain upstream of COX by addition of antimycin A (20 nM).

COX and cytrate synthase enzymatic activities were measured spectrophotometrically on isolated mitochondria (2–5 µg of protein) or in cell lysates (30–50 µg of protein) as described (Birch-Machin & Turnbull, 2001).

ATP synthesis in isolated mitochondria (15–25 µg of protein) or in cells permeabilized with digitonin ( $2 \times 10^6$  cells) was measured using a kinetic luminescence assay, as described (Vives-Bauza et al, 2007).

#### ROS production in intact cells

Cells were grown in glucose medium for 48 h in a 96-well plate and then incubated with the different compounds for 2 h. ROS were measured by 2',7'-dichlorofluorescein diacetate (H<sub>2</sub>-DCFDA; Invitrogen) fluorescence as previously described (Acin-Perez et al, 2009).

#### MtDNA content and mRNA levels by real time PCR

MtDNA content and mRNA levels of PGC1α, NRF-1 and GAPDH were measured with LightCycler-FastStart DNA Master SYBR Green I kit (Roche) or LightCycler<sup>®</sup> RNA Master SYBR Green I kit (Roche), respectively in a LightCycler capillary RT-PCR instrument (Roche) using appropriate primers (Methods of Supporting Information).

#### Western blot analyses

For denaturing SDS-PAGE 10–25 µg of protein from cell lysates, cytosolic or mitochondrial fractions was electrophoresed in a 12.5% acrylamide/bisacrylamide gel and electroblotted onto PVDF (BioRad) filters.

For 2D-BNGE, 50–75 µg of mitochondrial protein was applied on a 5–13% gradient BN gel followed by separation in a 12.5% denaturing gel (Schagger & von Jagow, 1991). After electrophoresis, proteins were electroblotted onto PVDF filters and sequentially probed with specific antibodies.

For protein detection the following antibodies were used: anti-NDUFA9 (Complex I), anti-Fp (Complex II), anti-core 2 (Complex III), anti-COXI and anti-COXIV (Invitrogen); anti-Cyt c (BD Pharmingen); anti-GAPDH and anti-HA (Abcam); anti-β-actin (Sigma-Aldrich); anti-Tim 23 and phospho Ser/Thr residues (BD Biosciences); anti-A6 (Complex V) (Rabbit polyclonal, Eric Schon, unpublished).

#### cAMP measurements

cAMP measurements were performed in transient or stable cell lines after subcellular fractionation. Fractions containing organelles (mitochondria) were collected after the cells were permeabilized with digitonin (50 µg/million cells) for 1 min and centrifuged at 2500 × g for 1 min. cAMP measurements were performed as described (Acin-Perez et al, 2009).

#### Statistical analyses

Comparisons between groups were made using one-way ANOVA. Pair wise comparisons were made by *post-hoc* Fisher PLSD test. Differences were considered statistically significant at  $p < 0.05$ . Data analyses were performed using the statistical program StatView. In all experiments, error bars indicate standard deviations (Adept Scientific, UK).

#### Author contributions

Rebeca Acin-Perez has designed the experiments together with Giovanni Manfredi, performed most of the work, and

wrote the manuscript. Eric Salazar has performed the cAMP assays. Sonja Brosel has generated the immortalized human Sco2 fibroblasts. Hua Yang has generated the Sco2 KI/KO mice. Eric A. Schon is the principal investigator on the Sco2 work conducted in his laboratory and has made substantial intellectual contributions to this manuscript. Giovanni Manfredi is the laboratory head and has conceived and directed the project as well as written the manuscript together with Rebeca Acin-Perez.

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Supporting information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

#### For more information

United Mitochondrial Disease Foundation

<http://www.umdf.org>

Mitomap, a human mitochondrial genome database

<http://www.mitomap.org/>

#### Author's website

Giovanni Manfredi:

<http://www.med.cornell.edu/research/gmanfredi/index.html>

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