

# Protein Phosphorylation and Prevention of Cytochrome Oxidase Inhibition by ATP: Coupled Mechanisms of Energy Metabolism Regulation

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## SUMMARY

Rapid regulation of oxidative phosphorylation is crucial for mitochondrial adaptation to swift changes in fuels availability and energy demands. An intramitochondrial signaling pathway regulates cytochrome oxidase (COX), the terminal enzyme of the respiratory chain, through reversible phosphorylation. We find that PKA-mediated phosphorylation of a COX subunit dictates mammalian mitochondrial energy fluxes and identify the specific residue (S58) of COX subunit IV-1 (COXIV-1) that is involved in this mechanism of metabolic regulation. Using protein mutagenesis, molecular dynamics simulations, and induced fit docking, we show that mitochondrial energy metabolism regulation by phosphorylation of COXIV-1 is coupled with prevention of COX allosteric inhibition by ATP. This regulatory mechanism is essential for efficient oxidative metabolism and cell survival. We propose that S58 COXIV-1 phosphorylation has evolved as a metabolic switch that allows mammalian mitochondria to rapidly toggle between energy utilization and energy storage.

## INTRODUCTION

To maintain cellular energy homeostasis, mitochondria adapt to changes in substrate availability and metabolic demands by modulating oxidative phosphorylation (OXPHOS). Reversible phosphorylation of mitochondrial proteins is an important player in OXPHOS modulation (Hopper et al., 2006; Pagliarini and Dixon, 2006), but only recently have the molecular mechanisms started to emerge. We described a metabolic-sensing signaling pathway in the mitochondrial matrix, involving mitochondrial soluble adenylyl cyclase (sAC), which generates cAMP and activates PKA in response to mitochondrial CO<sub>2</sub> generated in the Krebs cycle. This pathway modulates cytochrome oxidase (COX), the terminal enzyme of the electron transfer chain (ETC), as one of its targets (Acin-Perez et al., 2009a, 2009b).

COX translocates protons across the inner mitochondrial membrane, contributing to the proton electrochemical gradient

used by the ATP synthase to generate ATP. In living cells, COX is a pacemaker of ETC function (D'Aurelio et al., 2001; Dalmonte et al., 2009; Villani et al., 1998), and its activity is modulated by phosphorylation of its subunits (Helling et al., 2008; Lee et al., 2005; Miyazaki et al., 2003; Samavati et al., 2008; Steenaert and Shore, 1997) and by allosteric ATP inhibition (Bender and Kadenbach, 2000; Kadenbach, 2003). However, the molecular mechanisms of COX regulation in response to changes of substrate availability have been elusive.

The nuclear-encoded subunits of COX have tissue specific isoforms (Lomax and Grossman, 1989) regulated during development (Bonne et al., 1993). Subunit IV (COXIV) has two isoforms, COXIV-1 and COXIV-2, which evolutionarily diverged about 320 million years ago. COXIV-1 is expressed ubiquitously, while COXIV-2 is highly expressed in adult lung and only at low levels in brain and heart (Hüttemann et al., 2001).

Here, we identify phosphorylation of amino acid residue S58 in the matrix loop of COXIV-1 as a mechanism for PKA-dependent regulation of COX. We show, by computational means, that S58 is involved in binding ATP at the allosteric site and that its phosphorylation prevents this interaction. S58 phosphorylation modulates the kinetics of the enzyme by controlling ATP allosteric inhibition. We propose that this is a regulatory mechanism of oxidative phosphorylation, because it allows cells to match mitochondrial ETC activity with the availability of metabolic substrates and energy consumption requirements.

## RESULTS

### Serine 58 Is a Candidate Target for Intramitochondrial PKA Phosphorylation of COXIV-1

We had identified COXIV as a putative PKA target (Acin-Perez et al., 2009b). Since most mammalian tissues express COXIV-1 and not COXIV-2, we narrowed our study to the former. Serines and threonines of murine COXIV-1 (NP\_034071.1) were analyzed for probability of phosphorylation. Six candidates were identified (Table 1), four of which were predicted to be PKA targets. Three were excluded: S16 is in the cleaved signaling peptide, S157 is in the COXII/IV interface in the inner membrane, and T142 is in the intermembrane space. The fourth, S58, is localized on the matrix side of COXIV-1, has a high PKA prediction score, and is predicted to be a PKA target by KinasePhos 2.0 and pka prediction site (data not shown).

**Table 1. Prediction of S/T Phosphorylation in Mouse COXIV-1**

NetPhos 2.0 Prediction			PhosphoMotif Finder	
Position	Sequence	Score	Kinase	Localization
S16	KRAISTSVC	0.930	PKA/PKC/Calmodulin K	signal peptide
S58	MLSASQKAL	0.992	PKA/PKC	matrix
S74	WSSLSRDEK	0.966	CK II	matrix
S157	IQGFSAKWD	0.830	PKA/PKC	subunit IV/II interface
T17	RAISTSVCL	0.721	CK II	signal peptide
T142	VAIETKRML	0.949	PKA/PKC	IMS

NetPhos 2.0 predicts six putative phosphoresidues with different probability scores. The amino acid positions refer to the precursor protein, including the signal peptide. Putative kinases for S/T residues were identified by PhosphoMotif Finder. PKA, protein kinase A; PKC, protein kinase C; CK II, casein kinase II. The putative function of the protein domain containing phosphorylated amino acids is indicated (NCBI Reference Sequence features).

Newly generated polyclonal antibodies against a mouse S58<sub>phospho</sub>-COXIV-1 peptide were used to show that S58<sub>phospho</sub>-COXIV-1 signal in mouse liver mitochondria was increased by 8Br-cAMP (PKA activator) and decreased by H89, PKI 14-22 (PKA inhibitors) and KH7 (sAC inhibitor) (Figure S1 available online), thus confirming S58 as a PKA phosphorylation target.

#### Mutagenesis of S58 of COXIV-1

To prove that S58 phosphorylation is involved in COX regulation we used mouse fibroblasts (C1), where COXIV-1 is silenced (Li et al., 2006), resulting in marked decrease of COXIV-1 expression (Figure S2A), COX activity (Figures S2B and S2C), and cell respiration (Figure S2D).

Transient expressed rat COXIV-1 (NP\_058898.1) that has 92% identity with mouse, but contains one mismatch in the RNAi target region (Figure S3A) that prevents its silencing, partially complemented COX activity in C1 cells (Figures S3B and S3C).

Next, we generated C1 cells stably expressing wild-type (WT), a nonphosphorylatable S58A, or a phosphomimetic S58D COXIV-1. The levels of COXIV-1 in mass cultures (Figure 1A) and six clones (Figure S3D) were recovered to normal levels. Blue-native gel electrophoresis (BNGE) both in first (Figure 1B) and second (Figure S3E) dimensions showed restoration of COX assembly. COX activity was partially rescued but did not reach the values of A9 controls (Figure 1C and Figure S3F), possibly because assembly of rat COXIV-1 in the mouse enzyme results in a mildly defective activity (Dey et al., 2000). We also excluded a compensatory increase in the expression of endogenous COXIV-1 or COXIV-2 (Figures S3G and S3H). Importantly, expression of S58A COXIV-1 resulted in reduced COX activity (Figure 1C and Figure S3F), intact cell respiration (Figure 1D), and COX-driven respiration (Figure 1E), as compared to WT and S58D cells, indicating that loss of COXIV-1 S58 phosphorylation results in COX deficiency.

In WT COXIV-1 cells, PKA stimulation with membrane permeant 8Br-cAMP or its inhibition with H89 resulted in increased and decreased respiration and COX activity, respectively (Figures 1D and 1E). C1 cells displayed a greater OXPHOS increase and a lesser decrease than controls, because the enzyme is constitutively dephosphorylated (Acin-Perez et al., 2009a). However, S58A and S58D cells, which cannot be phosphorylated, had blunted responses to PKA modulation, indicating loss of sensitivity to PKA modulation.

#### Protein Phosphorylation Is Reduced in COXIV-1 S58 Mutants

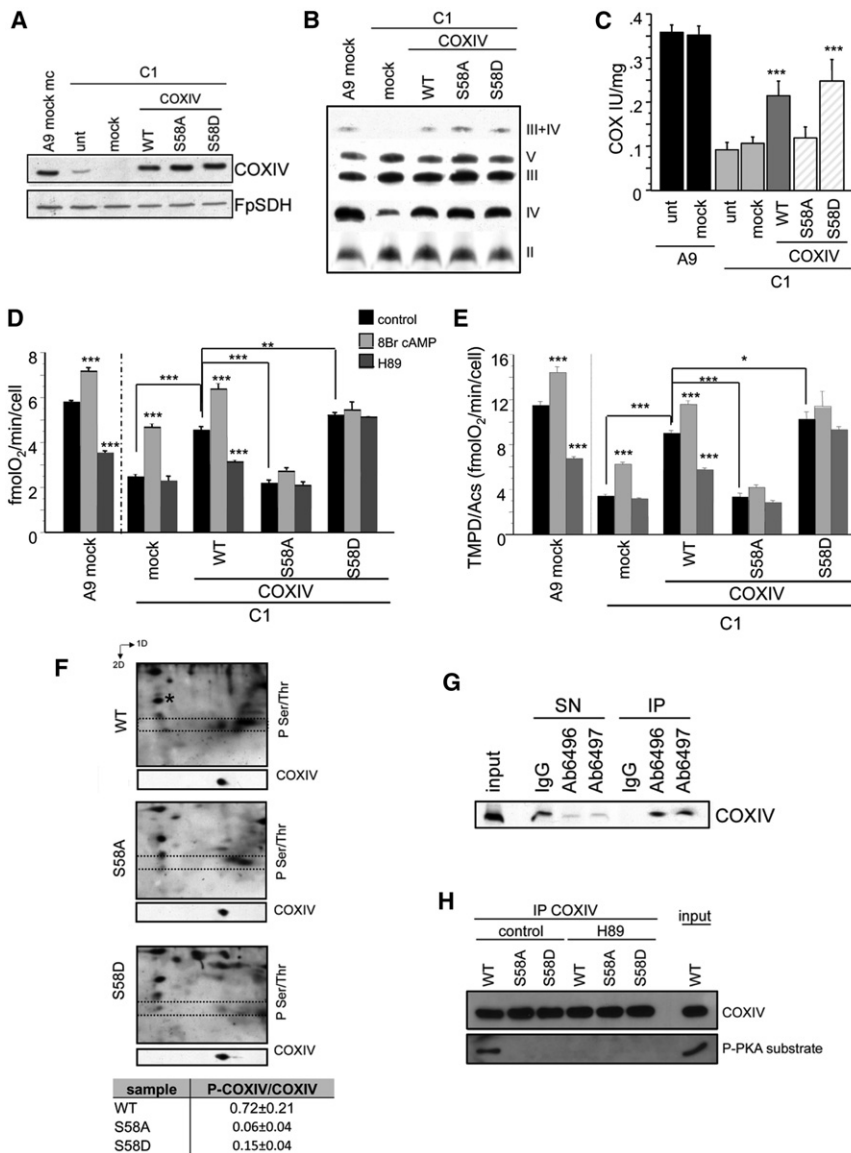
COXIV-1 phosphorylation was investigated by 2D-BNGE in cells treated with 8Br-cAMP. The intensity of phosphorylated COXIV-1 detected with a phospho Ser/Thr antibody was normalized by an unknown phosphoprotein (asterisk in Figure 1F), which was unchanged in all samples. This ratio was then normalized by total COXIV determined with anti-COXIV antibody. S58A and S58D COXIV-1 were less phosphorylated than WT COXIV-1 (Figure 1F), indicating that S58 accounts for most of PKA-dependent COXIV-1 phosphorylation. Most COXIV-1 appeared phosphorylated in A9 control cells, as shown by almost complete immunodepletion from the supernatant after immunoprecipitation with S58<sub>phospho</sub>-COXIV-1 antibodies (Figure 1G). These antibodies did not detect the rat protein, due to sequence differences in the epitope. Nevertheless, by immunoprecipitation with COXIV antibody and detection with an antibody that recognizes PKA phosphorylated peptides, in C1 cells expressing the WT, but not the mutant rat COXIV-1, the protein was phosphorylated, and H89 decreased its phosphorylation (Figure 1H).

#### Molecular Dynamics Simulations of COX Containing WT or Mutant COXIV-1

Dimeric WT (Figure 2A) and mutant COX (COXIV-1 S58A, S58D, and S58<sub>phospho</sub>) were simulated in the membrane by molecular dynamics (MD). The mean backbone (bb) root mean square deviations (rmsd) from the initial structure, and the mean backbone fluctuations (rmsf) around the average structure of each subunit (Table S1) suggest that neither mutation of COXIV-1 S58 to A or D nor its phosphorylation change COX conformation significantly. A superposition of the most prevalent conformations of the matrix domain of WT and mutant COXIV-1 (Figure 2B) did not show major structural changes (bb rmsds between each superimposed structure and the average structure of 0.753, 0.732, 0.702, and 0.629 Å for WT, S58A, S58D, and S58<sub>phospho</sub>, respectively). Thus, the effects of S58A and S58D mutations reflect the functional role played by S58 in COX regulation.

#### ATP Binding Site in the Matrix Domain of COXIV-1

At high ATP:ADP ratios, ATP binds COXIV and other enzyme subunits and reversibly inhibits COX activity (Arnold and Kadenbach, 1997; Bender and Kadenbach, 2000; Napiwotzki et al., 1997). Two ATP binding sites have been proposed in COXIV,



**Figure 1. C1 Cells Expressing WT and Mutant COXIV-1**

(A) Western blot of C1 cells expressing WT, S58A, and S58D COXIV-1 with anti COXIV antibodies. FpSDH is a loading control.

(B) First dimension BNGE to detect COX assembly with anti-COXI antibodies.  $\beta$ ATPase is a loading control.

(C) COX activity measured spectrophotometrically in C1 cells expressing COXIV-1.

(D) Intact cell respiration upon PKA activation or inhibition by 8Br cAMP (1 mM, for 2 hr) or H89 (1  $\mu$ M, for 2 hr), respectively.

(E) Modulation assessed by TMPD/ascorbate-driven respiration. Error bars represent the averages of mass cultures and two clones (in triplicate). Statistical differences are relative to mock C1 cells (transfected with empty vector). A9 mock cells are included as reference. \* $p < 0.01$ ; \*\*\* $p < 0.0001$ . Unt, untransfected cells.

(F) 2D-BNGE and western blot of mitochondria from transfected cells treated with 8Br cAMP (1 mM, for 2 hr), with an anti-phospho Ser/Thr antibody. The dashed box indicates the region of COXIV migration (inset below each panel), when the membrane was reprobbed with anti-COXIV antibody. P-COXIV/COXIV ratios are shown (average of three independent gels  $\pm$  standard deviation [SD]).

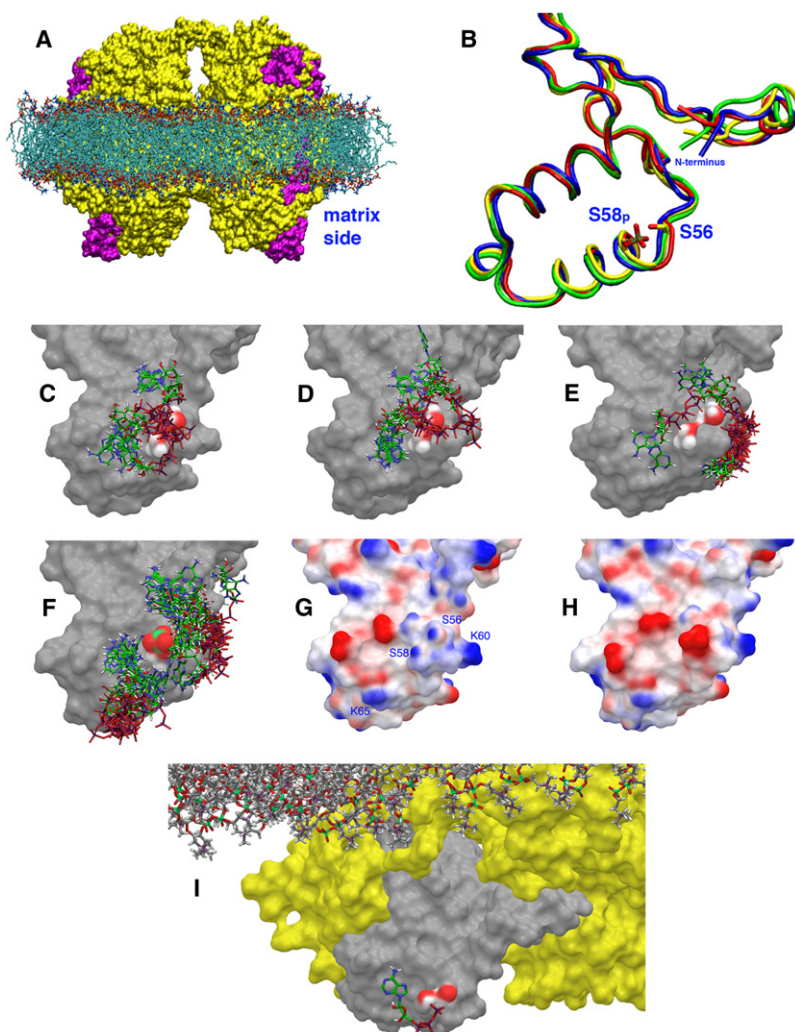
(G) Immunoprecipitation of phosphorylated COXIV-1 in mitochondrial lysates from A9 cells with S58<sub>phospho</sub>-COXIV-1 (Ab6496 or Ab6497) and detection with COXIV antibodies. Rabbit IgG was used as negative control. IP, immunoprecipitation; SN, supernatant.

(H) Immunoprecipitation of COXIV from untreated and H89 treated (1  $\mu$ M, for 2 hr) cells expressing WT, S58A, and S58D COXIV-1.

Error bars indicate standard deviations.

one facing the intermembrane space and regulated by cytosolic ATP (Napiwotzki and Kadenbach, 1998) and another facing the matrix and regulated by intramitochondrial ATP (Arnold and Kadenbach, 1997). We hypothesized that phosphorylation of COXIV-1 S58 regulates allosteric inhibition of COX by matrix ATP. The binding site for ATP in the matrix domain of COXIV-1 was studied with a combination of induced-fit docking (IFD) and QM-polarized ligand docking (QPLD). Five clusters of conformations were identified in the MD simulations of WT and S58<sub>phospho</sub> COXIV-1, and ranked from 1 to 5 based on the number of frames contained (more frames reflect more likely conformations). Charges on the ligand (ATP) and protein were assigned based on a mitochondrial matrix pH of  $8.0 \pm 0.2$  (Metoki and Hommes, 1984). In cluster 1 from WT COXIV-1, there were two groups of ATP binding poses (Figure 2C) characterized by an interaction of the  $\gamma$  and/or  $\beta$  phosphate of ATP with the hydroxyl moieties of the side chains of S56 and S58. In cluster

all frames, Figure 2E). S56 and S58 are situated in the first helix of the matrix domain of COXIV-1 (Figure 2B), and the dipole moment of the helix provides 0.5–0.7 units of positive charge that stabilizes the binding of the  $\gamma$  and  $\beta$  phosphates of ATP (Figure 2G). The phosphate group of S58<sub>phospho</sub> obliterates the positive electrostatic potential (Figure 2H), thereby preventing ATP binding. This is shown by failure of the IFD to identify a pose corresponding to the binding of ATP to the side chains of S56 and S58 of COXIV-1 S58<sub>phospho</sub> (Figure 2F). Low-affinity binding sites for ATP were recognized near the side chains of K60 and K65. The pose with the best IFD score was refined by QPLD (Figure 2I). The phosphate of ATP wraps around the side chain of S58, while the sugar and base are contained in nearby pockets. When COXIV-1 is phosphorylated, the phosphate of S58<sub>phospho</sub> replaces the  $\gamma$  phosphate of ATP, significantly weakening its binding. The pose in Figure 2I was used to calculate by free-energy perturbation (FEP) changes in the binding energy of



**Figure 2. Structural Models of COX and ATP Binding Site on the Matrix Side of COX**

(A) Molecular surface of membrane (teal) embedded dimeric WT COX. The enzyme is in yellow, with the two COXIV subunits in magenta.

(B) Superposition of the matrix domain of COXIV in WT (blue), S58A (red), S58D (green), and S58<sub>phospho</sub> (yellow), as derived from the most prevalent conformations in the MD.

(C) Binding poses of ATP on the surface of WT COXIV-1 in the most prevalent conformation (cluster 1). S56 and S58 side chains atoms are color coded (gray, carbon; red, oxygen; white, hydrogen).

(D and E) Binding poses of ATP on the surface of WT COXIV-1 in the conformations of cluster 2 and 3, respectively.

(F) Binding poses of ATP on the surface of S58<sub>phospho</sub> COXIV-1 derived from the entire MD simulation superimposed on the conformation of cluster 1. The phosphate moiety of S58<sub>phospho</sub> is color coded (green, phosphorus; gray, carbon; red, oxygen; white, hydrogen).

(G and H) Molecular surfaces of WT COXIV-1 and S58<sub>phospho</sub> COXIV-1 colored according to electrostatic potentials (blue, +1 kT/e; red, -1 kT/e).

(I) Matrix side of COX (yellow surface) embedded in the membrane (shown as sticks) with ATP bound to subunit IV-1 (gray surface).

ATP, when S58 is replaced by A or D or is phosphorylated.  $\Delta\Delta G_{\text{bind}}$  values for ATP with respect to the WT were  $0.82 \pm 2.9$  kcal/mol for S58A,  $7.15 \pm 3.5$  kcal/mol for S58D, and  $8.6 \pm 3.5$  kcal/mol for S58<sub>phospho</sub> (more positive values indicate weaker binding), corresponding respectively to a  $\sim 4$ -fold,  $\sim 175,000$ -fold, and  $\sim 2,100,000$ -fold increase in  $K_d$ .

#### ATP Allosteric Inhibition of COX Is Regulated by COXIV-1 Phosphorylation

Since S58 of COXIV-1 is a PKA target and S58<sub>phospho</sub> prevents ATP binding, we investigated the functional consequences of these molecular interactions. COX activity was measured in permeabilized cells in an ATP-regenerating system with increasing concentrations of reduced cytochrome *c*, in the presence of either ATP or ADP. WT COXIV-1 cells had ATP-induced inhibition at all cytochrome *c* concentrations, while S58A cells were insensitive to ATP, and S58D cells had a lesser degree of ATP inhibition (Figures 3A–3D).

Next, mitochondria were sonicated and treated with pyrophosphatase to hydrolyze ATP and ADP. The removal of ATP from all binding sites eliminated the differences in COX activity

between WT and S58 mutant cells (Figure 3E), indicating that ATP is responsible for the differences.

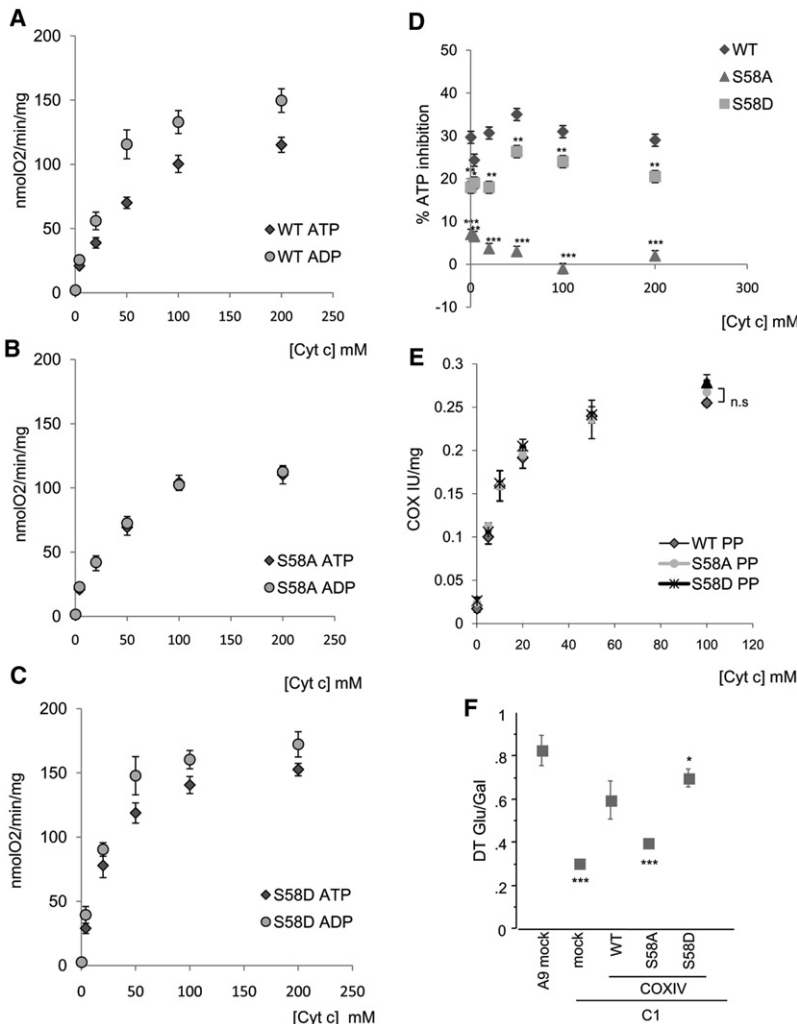
#### COXIV-1 S58 Phosphorylation Is Necessary for Efficient OXPHOS Function

To study the physiological significance of S58 phosphorylation, we assessed cell growth in WT and S58 mutant cells in medium containing galactose as the main carbon source, where cells are forced to utilize OXPHOS to generate ATP (Acin-Perez et al., 2009a). C1 cells had a severe growth defect in galactose (relative to glucose, Figure 3F). Cells expressing WT or S58D COXIV-1, but not S58A COXIV-1, had a significant improvement of growth in galactose, suggesting that loss of S58 COXIV-1 phosphorylation results in COX inhibition and a failure to maintain ATP synthesis, when cells need to upregulate oxidative metabolism.

#### DISCUSSION

The discovery of a signaling system through sAC and PKA (Acin-Perez et al., 2009b) contributed to our understanding of the regulation of mitochondrial metabolism. However, the molecular mechanisms underlying this regulation had not been defined.

Many proteins involved in OXPHOS function are targets of phosphorylation (Balaban, 2010; Deng et al., 2011; Hopper et al., 2006). In this study, we focused on COX, but it is likely that other OXPHOS components may be involved in PKA-mediated metabolic regulation (Papa et al., 2008). COX contains 13 subunits, ten of which are phosphorylated at various sites



**Figure 3. COX Allosteric Inhibition by ATP in Cells Expressing COXIV-1 and Its Role in OXPHOS.**

(A–C) Oxygen consumption using increasing concentrations of cytochrome c in the presence of ATP (5 mM) or ADP (5 mM) measured in WT (A), S58A (B), and S58D (C) permeabilized COXIV-1-expressing cells (n = 3). (D) Percentage of ATP inhibition at different cytochrome c concentrations from values from (A)–(C) (n = 3). (E) COX activity measured spectrophotometrically in sonicated mitochondria after pyrophosphatase (PP) treatment (n = 6 for each point). (F) Doubling time ratio (n = 3) of cells grown in glucose versus galactose medium (DT Glu/Gal). Statistically significant differences are relative to WT COXIV-1 cells. \*p < 0.01; \*\*p < 0.001; \*\*\*p < 0.0001. Error bars indicate standard deviations.

are inhibited and fuel utilization diminishes, resulting in low intramitochondrial CO<sub>2</sub> levels, low sAC activity and cAMP, and low PKA activity. COXIV-1 is dephosphorylated and COX activity inhibited by ATP, shunting substrates toward fat and glycogen accumulation. On the other hand, when cellular ATP consumption is high, large amounts of substrates are oxidized, CO<sub>2</sub> stimulates COXIV-1 phosphorylation, transiently preventing ATP inhibition and allowing for maximal electron flux through COX and high ATP production. Since the phosphomimetic S58D results in a small increase in COX activity and the S58<sub>phospho</sub>-COXIV-1 antibodies immunocapture most COXIV-1, we inferred that normally the majority of COXIV-1 is phosphorylated. Studies in more complex organisms will assess whether this applies to organs and tissues in vivo.

The S58 residue is conserved among mammals (Table S2), but not in nonmammalian species, suggesting that S58 has coevolved with mammals. Furthermore, two different isoforms of COXIV are found in many species. Similarly to other nuclear-encoded COX subunits, COXIV plays a regulatory role, in part through differential temporal and spatial expression of its isoforms. COXIV-2 does not have a PKA phosphorylation site corresponding to S58. Instead, it has three glutamic acids, carrying negative charges that may impede ATP binding. COXIV-2 confers higher activity to COX than COXIV-1, thereby allowing for a fast electron flux and possibly reducing the risk of forming reactive oxygen species in highly oxygenated tissues (Hüttemann et al., 2007). Under oxygen deprivation, COXIV-2 is induced in brain, where it abolishes sensitivity to ATP inhibition (Horvat et al., 2006). Furthermore, an increase of COXIV-2 expression regulated by hypoxia-inducible factor 1 occurs under hypoxia, in association with COXIV-1 degradation (Fukuda et al., 2007). These responses could be part of a hypoxia-preconditioning program to prevent the wave of free radical production, after reperfusion.

The presence of S58 in COXIV-1 of mammalian species could be related to a specific need for metabolic regulation in animals exposed to variations in ambient temperature and

(Thomson, 2002). With a combined proteomic (Acin-Perez et al., 2009b) and informatics strategy, we determined that S58 in COXIV-1 fulfilled the criteria for being accessible to matrix PKA and part of a PKA target motif. Furthermore, phosphorylation of this residue had been shown earlier in bovine heart mitochondria (Hellung et al., 2008). We pinpointed S58 of COXIV-1, as a player in COX regulation by reversible phosphorylation and identified S58 as part of the allosteric binding site for ATP on the matrix side of COXIV-1, showing that phosphorylation of S58 dramatically weakens the interaction with ATP. Furthermore, we have shown that lack of COXIV-1 phosphorylation in S58A mutant cells results in reduced COX activity and defective growth under oxidative conditions. These results suggest a link between activation of the intramitochondrial CO<sub>2</sub>-cAMP-PKA pathway and phosphorylation of COXIV-1 S58, which regulates COX allosteric inhibition by matrix ATP. This interpretation does not exclude that extramitochondrial ATP may bind other sites in COX and regulate its activity differently.

Our findings suggest that mitochondria can switch from a “storage mode” to a “consumption mode.” When ATP builds up in mitochondria, because of reduced requirements, enzymes

food availability. In modern days, metabolic regulation through reversible phosphorylation of respiratory chain subunits may represent a trigger for metabolic diseases, such as obesity and diabetes, or a potential target for therapy with approaches to modulate consumption and storage of metabolic supplies.

## EXPERIMENTAL PROCEDURES

### Cell Lines and Treatments

The A9 cells and COXIV-1 silenced cells (C1) were reported elsewhere (Li et al., 2006). Cell replication in glucose and galactose medium was measured as described (Acin-Perez et al., 2009a). 8Br-cAMP 1 mM (Sigma-Aldrich) and H89 1  $\mu$ M (Calbiochem) for 2 hr were used to stimulate or inhibit PKA, respectively.

### Mitochondrial Isolations

Mitochondria-enriched fractions were obtained from  $20 \times 10^6$  cells, as described elsewhere (Birch-Machin and Turnbull, 2001). Mitochondria isolation from mouse liver and pharmacological treatments were performed as described (Acin-Perez et al., 2009b).

### Cloning and Transfection of rat COXIV-1

Rat COXIV-1 complementary DNA (cDNA) was obtained by reverse transcription (Superscript reverse transcriptase, Invitrogen) of rat PC12 cells total RNA using appropriate primers (Supplemental Experimental Procedures) and cloned in pcDNA3.1 (Invitrogen). S58A and S58D mutants of COXIV-1 were generated by in situ mutagenesis with the QuickChange Site-Directed Mutagenesis kit (Stratagene) with appropriate primers (Supplemental Experimental Procedures). Stable cell lines expressing WT, S58A, and S58D COXIV-1 were generated by transfection with FuGene6 (Roche), followed by selection in 250  $\mu$ g/ml G418 for 3 weeks.

### Oxygen Consumption and Enzymatic Assays

Oxygen consumption determinations in intact cells were carried out as described (Hofhaus et al., 1996). COX activity was measured spectrophotometrically on mitochondrial fractions (2–5  $\mu$ g protein) or in cell lysates (30–50  $\mu$ g protein) as described (Birch-Machin and Turnbull, 2001). COX allosteric inhibitory effects of ATP were measured as described (Bender and Kadenbach, 2000) in the presence of ATP or ADP (5 mM). Cell respiration was measured in 50  $\mu$ g Tween-permeabilized cells with increasing concentrations of reduced cytochrome c (0.25–200  $\mu$ M).

For pyrophosphatase treatments, mitochondria were sonicated as described (Acin-Perez et al., 2009b), centrifuged at 20,000 g for 5 min at 4°C, and resuspended in 10 mM Tris-HCl (pH 7.4), 25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM  $K_2HPO_4$ , 0.05 mM EDTA, 5 mM  $MgCl_2$ , 1 mg/ml BSA. Pyrophosphatase (2 Units, Sigma-Aldrich) was added for 10 min at 25°C. COX activity was measured (Birch-Machin and Turnbull, 2001) with increasing concentrations of reduced cytochrome c (0.1–100  $\mu$ M).

### mRNA Content Measurements by RT-PCR

mRNA levels of mouse COXIV-1 and 2 and rat COXIV-1 and  $\beta$ -actin were measured with a LightCycler RNA Master SYBR Green I kit (Roche), in a LightCycler capillary RT-PCR instrument (Roche) with appropriate primers (Supplemental Experimental Procedures).

### Immunoprecipitation of COXIV

Mitochondrial lysates (dodecyl maltoside; 1.6 g/g protein) (Schägger and von Jagow, 1991) were incubated overnight with 1–3  $\mu$ g antibody (COXIV-1 or Ab6496 or Ab6497) or control rabbit IgG bound to protein G Sepharose beads (Invitrogen). Beads were pulled down and washed twice with PBS containing 0.5% dodecyl maltoside. Proteins were eluted in 50–100  $\mu$ l of 0.5M Tris-HCl (pH 6.8), 5% glycerol, 2% SDS, and 100 mM DTT and used for western blot analyses.

### Western Blot Analyses

For denaturing SDS-PAGE proteins (10–25  $\mu$ g from cell lysates and 25  $\mu$ l from the immunoprecipitated) were electrophoresed in a 12.5% acrylamide dena-

turing gel and electroblotted onto PVDF (BioRad) filters. For 1D-BNGE and 2D-BNGE, 50–75  $\mu$ g mitochondrial protein was applied on a 5%–13% gradient BN gel followed by separation in a 12.5% denaturing gel (Schägger and von Jagow, 1991). After electrophoresis, proteins were electroblotted onto PVDF filters. For protein detection, the following antibodies were used: FpSDH, Core2, COXIV, and  $\beta$ ATPase (all from Invitrogen); cyt c (BD Pharmingen); GAPDH (Abcam); phospho Ser/Thr (BD Biosciences); and PKA-P-substrate (Cell Signaling). Rabbit polyclonal antibodies against the mouse COXIV-1 S58<sub>phospho</sub>-peptide VAHVMTLSApSQKALKEKE (Ab6496 and Ab6497) were generated (Phosphosolutions).

### Informatics Analyses

Phosphorylation prediction was by NetPhos 2.0 software (<http://www.cbs.dtu.dk/services/NetPhos/>) with a threshold score >0.5. Kinase prediction was with PhosphoMotif Finder ([http://www.hprd.org/PhosphoMotif\\_finder/](http://www.hprd.org/PhosphoMotif_finder/)). PKA-dependent phosphorylation prediction was by Kinase Phos 2.0 (<http://kinasephos2.mbc.nctu.edu.tw/>). Potential PKA phosphorylation sites were predicted by PKA Prediction Sites (pkaPS) (<http://mendel.imp.ac.at/sat/pkaPS/>). COXIV-1 cDNA sequence alignment was by Nucleotide BLAST (<http://blast.ncbi.nlm.nih.gov/>). Protein sequence alignments were by ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>).

### Molecular Dynamics Simulations

Models of membrane embedded COX were built from the X-ray structure of dimeric bovine COX (PDB entry 1V55) with Desmond (D.E. Shaw Research) (Bowers et al., 2006), by addition of an explicit bilayer of 1-palmitoyl-2-oleoyl-phosphatidylcholine around COX and solvation of the enzyme and both sides of the membrane with SPC water (Berendsen et al., 1981); a minimum distance of 12 Å was left between any protein atom and the edge of the simulation box. The ensemble was energy minimized under periodic boundaries condition using the 2005 OPLS-AA force-field (Jorgensen et al., 1996). MD simulations (1.2 ns) were carried out with Desmond in the NPT ensemble at 298.15K (25°C), via the Nose-Hoover thermostat method (Evans and Holian, 1985) and the Martyna-Tobias-Klein barostat method (Martyna et al., 1994). SHAKE constraints (Ryckaert et al., 1977) were imposed on all the heavy-atom-hydrogen covalent bonds. Coordinates were saved every 4.8 ps.

### Identification of the Binding Site for ATP in the Matrix Domain of COXIV

Identification of the ATP binding site in the matrix domain of COXIV-1 was by IFD (Sherman et al., 2006). The QPLD protocol (Cho et al., 2005) was used to refine selected poses to improve the partial charges on the ligand atoms in a docking run, by replacing them with charges derived from quantum mechanical calculations on the ligand in the field of the receptor. IFD and QPLD were implemented in the Schrödinger Suite 2010 (Schrödinger LLC.). FEP (Zwanzig, 1954) were calculated with Desmond in the NPT ensemble at 298.15K in the protein:ATP complex+solvent and the protein alone+solvent environments. Simulations ran for 0.6 ns for each  $\lambda$  window (12 windows for each perturbation). Results were collected and analyzed by the Bennett method (Bennett, 1976; Shirts et al., 2003).  $\Delta\Delta G_{\text{bind}}$  values were calculated by subtraction of the protein+solvent  $\Delta G_{\text{FEP}}$  from the complex+solvent  $\Delta G_{\text{FEP}}$  value.  $K_d$  changes for ATP binding to COXIV-1 were calculated from the relationship

$$\Delta\Delta G_{\text{bind}} = -RT \ln (K_{d,\text{WT}}/K_{d,\text{Mutant}}),$$

where R is the gas constant and T is the absolute temperature.

Electrostatic potentials at the molecular surface were determined with the Poisson-Boltzmann solver of QSite (Schrödinger LLC), using a continuum model with a solvent dielectric constant  $\epsilon_s = 78$ , and a protein dielectric constant  $\epsilon_p = 2$ .

### Statistical Analyses

Comparisons among groups were made with one-way ANOVA. Pair-wise comparisons were made by post hoc Fisher PLSD test with StatView (Adept Scientific). Differences were considered statistically significant at  $p < 0.05$ . In all graphs, error bars indicate the standard deviations.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at doi:10.1016/j.cmet.2011.03.024.

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