

# Mechanisms Controlling Mitochondrial Biogenesis and Respiration through the Thermogenic Coactivator PGC-1

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## Summary

Mitochondrial number and function are altered in response to external stimuli in eukaryotes. While several transcription/replication factors directly regulate mitochondrial genes, the coordination of these factors into a program responsive to the environment is not understood. We show here that PGC-1, a cold-inducible coactivator of nuclear receptors, stimulates mitochondrial biogenesis and respiration in muscle cells through an induction of uncoupling protein 2 (UCP-2) and through regulation of the nuclear respiratory factors (NRFs). PGC-1 stimulates a powerful induction of NRF-1 and NRF-2 gene expression; in addition, PGC-1 binds to and coactivates the transcriptional function of NRF-1 on the promoter for mitochondrial transcription factor A (mtTFA), a direct regulator of mitochondrial DNA replication/transcription. These data elucidate a pathway that directly links external physiological stimuli to the regulation of mitochondrial biogenesis and function.

## Introduction

Adaptive thermogenesis is the physiological process whereby energy is dissipated in the form of heat in response to external stimuli. The stimuli that have been studied most extensively are exposure to cold and ingestion of high-calorie diets (Rothwell and Stock, 1979).

More recently, there has been a great deal of interest in adaptive thermogenesis as a physiological defense against obesity (Hamann et al., 1996). Numerous experiments, including tissue ablation studies in transgenic mice, clearly show that brown fat-mediated adaptive thermogenesis plays a major role in the prevention of obesity (Himms-Hagen, 1989; Lowell et al., 1993). In humans, there are great differences in how individuals metabolize an intentional caloric overload; some people store most of this energy as fat, while others dissipate much of it through altered energy expenditure, including adaptive thermogenesis (Bouchard et al., 1990; Levine et al., 1999).

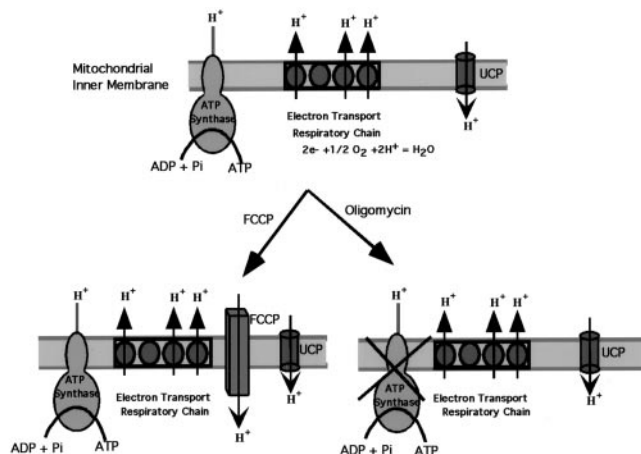
Adaptive thermogenesis occurs primarily in the mitochondria of brown fat and skeletal muscle. Because brown fat is prominent in small mammals like rats and mice, it is believed to play a very important role in these animals (Lowell et al., 1993). Since this tissue is much less obvious in large animals like humans, skeletal muscle is thought to be the site of primary importance for this process. The uncoupling proteins (UCPs) are small, intramembranous mitochondrial proteins that are expressed in a tissue-selective manner and play a key role in thermogenesis (Flier and Lowell, 1997; Ricquier and Bouillaud, 1997). UCP-1 is present exclusively in brown adipose tissue (Jacobsson et al., 1985; Bouillaud et al., 1986), while UCP-3 is expressed in brown fat and skeletal muscle (Boss et al., 1997; Vidal-Puig et al., 1997). UCP-2 is found in most tissues (Fleury et al., 1997; Gimeno et al., 1997). The thermogenic role of UCP-1 has been definitively shown by many gain- and loss-of-function experiments (Lowell et al., 1993; Kopecky et al., 1995; Enerback et al., 1997); the functions of the other UCPs, identified and cloned through their sequence homology with UCP1, are less clear. Both UCP-2 and UCP-3 have been shown to uncouple respiration when expressed ectopically in yeast (Fleury et al., 1997) and mammalian cells (Boss et al., 1998). However, their respective roles in thermogenesis and energy balance of intact animals remain to be determined.

Adaptive thermogenesis is a much more complex process than simply the activation of mitochondrial uncoupling. Other major components are an increase in the number of mitochondria and in the activity of the electron transport system in those mitochondria (Nicholls et al., 1986). The reason for this multilayered program presumably lies in the need to increase the overall rate of fuel oxidation, allowing for the preservation of normal cellular ATP/ADP ratios while “wasting” a significant fraction of metabolic energy in the form of heat.

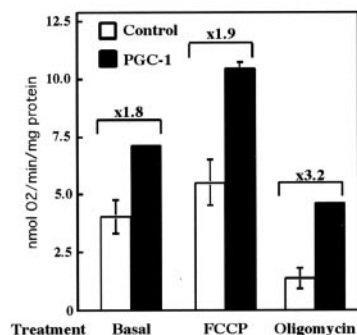
We recently identified a coactivator of nuclear receptors, termed PGC-1, which can activate many aspects of the adaptive thermogenic program. PGC-1, a nuclear protein of 90 kDa, has no overall sequence similarity to any known molecules but contains SR domains and an RNA-binding motif characteristic of certain splicing factors (Puigserver et al., 1998). PGC-1 mRNA is greatly induced in muscle and brown fat upon exposure of mice to cold (4°C), probably due to the activation of the sympathetic nervous system and responding stimulation of

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A



B



$\beta$ -adrenergic receptors (Puigserver et al., 1998). PGC-1 expression is also reduced upon fasting and induced on refeeding, matching alterations in energy expenditure (unpublished data). When coexpressed with PPAR $\gamma$  or the thyroid hormone receptor, PGC-1 greatly increases their transcriptional activity. When ectopically expressed in cultured white fat cells, PGC-1 causes an increase in mRNA for UCP-1, and several genes of the oxidative phosphorylation pathway, including cytochrome c oxidase (COX) subunits II and IV, and ATP synthetase. It also stimulates a doubling of mitochondrial DNA content (Puigserver et al., 1998).

Two of the major questions to emerge from this work are (1) whether and how PGC-1 can affect a thermogenic program in muscle, where no UCP-1 is expressed; and (2) how can PGC-1, a protein localized to the nucleus, affect mitochondrial DNA replication and transcription. While certain regulators of mitochondrial transcription and replication have been discovered (NRF-1, NRF-2, and mtTFA) (Evans and Scarpulla, 1990; Clayton, 1992; Shadel and Clayton, 1993; Virbasius et al., 1993b), upstream factors that coordinate these to generate complete, functioning mitochondria are virtually unknown. In addition, how this entire program can be linked to environmental stimuli is not well understood. In this report, we investigate how PGC-1 can interact with certain mitochondrial regulatory factors to control biogenesis of this organelle.

Figure 1. Stimulation of Oxygen Consumption by PGC-1

(A) Model illustrating the two predominant modes of respiration in mitochondria: oxidative phosphorylation and uncoupling. Continued oxygen consumption by mitochondria requires that protons reenter the mitochondrial matrix. This can occur via ATP synthase, if ADP is available, or proton leak, such as that mediated by UCPs. These two pathways can be distinguished using either oligomycin, an inhibitor for F<sub>1</sub>/F<sub>0</sub> ATP synthetase, or FCCP, a chemical uncoupler.

(B) PGC-1 increases both oxidative phosphorylation-linked and uncoupling-linked respiration in differentiated myotubes. C2C12 cells infected with virus containing an empty vector or an expression vector for PGC-1 were differentiated into myotubes. At day 5 post-confluence, the cells were gently detached from the dishes and transferred to a chamber for measurements of oxygen consumption. The concentration of FCCP and oligomycin were 2  $\mu$ M and 2.5  $\mu$ g/ml, respectively. Each measurement was performed in triplicate and normalized to the protein content of the cells.

## Results

### Ectopic Expression of PGC-1 in Myotubes Increases Oxygen Consumption

To investigate the role of PGC-1 in regulating adaptive thermogenesis in muscle, we have utilized retroviral vectors to introduce this protein into C2C12 muscle. Because it is very difficult to measure changes in temperature in cultured cells, a more commonly used method is to determine the consumption of oxygen. As illustrated diagrammatically in Figure 1A, both oxidative phosphorylation and uncoupling of mitochondria are linked to respiration; the former generates ATP, while the latter yields heat. As shown in Figure 1B, the basal oxygen consumption of the PGC-1-expressing myotubes (normalized per unit protein) is about 77% higher than that in the control cells. As shown in Figure 1A, FCCP is a chemical uncoupler that can completely uncouple mitochondria and maximize their respiratory capacity; this renders any action of uncoupling proteins irrelevant. The FCCP-stimulated oxygen consumption therefore reflects the number and electron transport activity of mitochondria. Figure 1B shows that the FCCP-stimulated oxygen consumption is increased 1.9-fold in the PGC-1 cells, compared to controls, indicating that these cells have a higher content and/or electron transport activity of mitochondria. Oligomycin, an inhibitor of F<sub>1</sub>/F<sub>0</sub> ATP synthetase, blocks all oxidative phosphorylation-linked

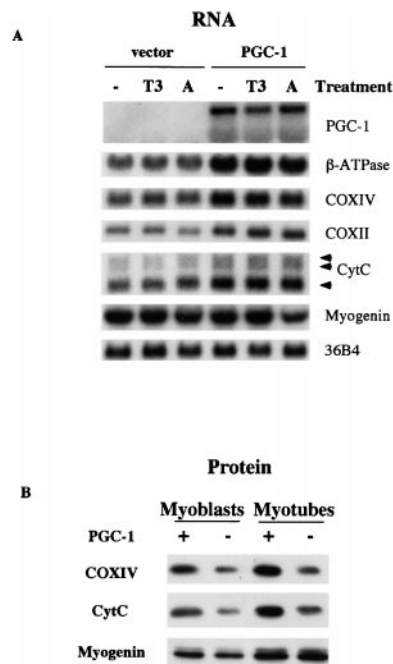


Figure 2. PGC-1 Increases Expression of Genes of the Mitochondrial Respiratory Chain

(A) RNA analysis of PGC-1-expressing cells. Myoblasts expressing PGC-1 and the control cells were induced to differentiation and were then treated with various stimuli, including 100 nM T3 (24 hr) and 1 mM 8-bromo-cAMP (A) (6 hr). Total RNA was extracted and subjected to Northern blot analysis. Probes used for hybridization were PGC-1,  $\beta$ -ATP synthetase, COXII and IV, CytC, and myogenin. A cDNA encoding a ribosomal protein, 36B4, was also used as a control for loading equivalence of RNA.

(B) Protein analysis of PGC-1-expressing cells. Total proteins were extracted from the cells at confluence (myoblasts) or day 5 postconfluence (myotubes) and were subjected to Western blot analysis.

respiration, thus allowing respiration through proton leak (uncoupling) only. In the presence of this drug, the oxygen consumption in the cells expressing PGC-1 is 3.2-fold higher than the controls, indicating that PGC-1 stimulates the uncoupling of mitochondria in these cells. These data indicate that PGC-1 has at least two distinct effects on mitochondrial function in these cells: an increase in total respiration capacity, and an increase in mitochondrial uncoupling.

#### PGC-1 Increases the Expression of Genes Involved in Oxidative Phosphorylation

To investigate how PGC-1 modulates mitochondrial function, we examined the expression of genes involved in respiratory chain function, including those encoded in both the nuclear and mitochondrial genomes. As shown in Figure 2A, endogenous PGC-1 mRNA is barely detectable in the myotubes, while the viral PGC-1 transcript is easily observed. The mRNA for the  $\beta$  subunit of ATP synthetase, encoded in nuclear genome, is increased 2-fold in the PGC-1 cells over the control. Several other components of the respiratory chain, including the nuclearly encoded cytochrome c oxidase subunit IV (COX IV), the mitochondrially encoded cytochrome c oxidase subunit II (COX II), and cytochrome c (CytC),

were also examined. The mRNAs for these three genes are all increased 2- to 3-fold in the cells expressing PGC-1 compared to control cells. Thyroid hormone (T3) and cyclic AMP (cAMP) can be important regulators of thermogenesis in vivo. These PGC-1-mediated gene expression events do not appear to be sensitive to either T3 or 8-bromo-cyclic AMP (A). The expression of myogenin, a regulator of muscle differentiation, is not affected by PGC-1, indicating quantitatively that the differentiation of both cell lines is comparable. In addition to the data shown above for myotubes (Figure 2A), a nearly identical pattern of gene activation by PGC-1 was observed in C2C12 myoblasts (data not shown). We also examined the expression of COXIV and CytC at the protein level by Western blotting. As shown in Figure 2B, both are elevated while myogenin protein remains unaffected.

#### PGC-1 Activates Expression of Uncoupling Proteins in a Cell Type-Selective Manner

The oxygen consumption data shown in Figure 1 indicate that respiration in myotubes expressing PGC-1 is more uncoupled than in controls, suggesting the induction of one or more UCPs. We therefore examined the expression of mRNA for the three known UCPs in myotubes expressing PGC-1 or in control cells, in the presence of various stimuli as indicated. Figure 3A shows that irrespective of PGC-1 expression, UCP-1 mRNA is not detected. UCP-3 mRNA is expressed weakly in these cells but is not affected by PGC-1. In contrast, UCP-2 mRNA is expressed in the control cells and is increased about 3-fold due to PGC-1; again, this induction is independent of the hormone/ligand treatments (T3, 8-bromo-cyclic AMP, and the PPAR $\gamma$  ligand troglitazone) used. This elevation of UCP-2 expression is consistent with the increment of uncoupling-linked oxygen consumption in these cells (3.2-fold; Figure 1B).

Given the very distinct patterns of tissue selectivity of mitochondrial uncoupling in vivo, it is of great interest to know whether this induction of UCP-2 mRNA by PGC-1 is cell type selective. Figure 3B shows that UCP-2 mRNA is not increased by PGC-1 in 3T3-F442A adipocytes. However, UCP-1 mRNA is activated by PGC-1 in these cells (Figure 3B and Puigserver et al., 1998). UCP-3 expression is not detectable in these adipocytes with or without PGC-1. These results indicate that PGC-1 regulates the expression of UCPs, and they suggest that it does so in a cell type-selective manner.

#### PGC-1 Stimulates Mitochondrial Biogenesis

To investigate more directly whether PGC-1 can stimulate mitochondrial proliferation, we first examined the mitochondrial DNA (mtDNA) content in the muscle cells by Southern blot analysis. As shown in Figure 4A, mtDNA content is increased approximately 2-fold by PGC-1. To investigate whether mitochondrial biogenesis per se occurred, we performed transmission electron microscopy of thin sections prepared from these cells. Figure 4B shows that PGC-1-expressing myoblasts have an obvious proliferation of mitochondria when compared with the control cells. Quantitative morphometry performed on more than 100 cells of each group indicates a 57% increase in mitochondrial density (mitochondrial



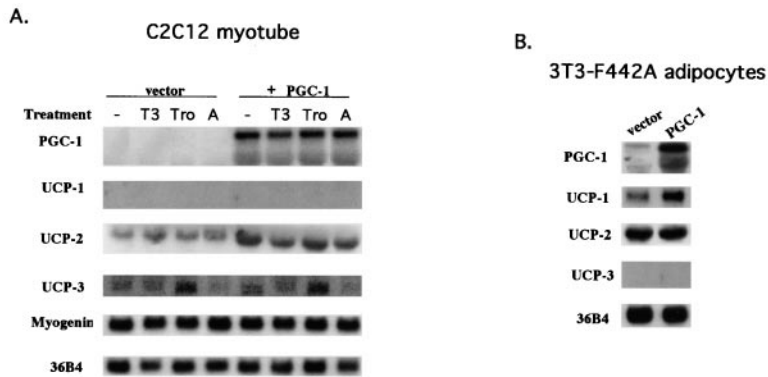


Figure 3. Cell Type-Selective Induction of UCPs through PGC-1

C2C12 and 3T3-F442A cells expressing PGC-1 and their controls were induced to differentiation as described in Experimental Procedures. The C2C12 cells were treated with various stimuli, including 1 mM 8-bromo-cAMP (A, 6 hr), 100 nM T3 (24 hr), or 10  $\mu$ M troglitazone (Tro, 24 hr). 3T3-F442A adipocytes were treated with 100 nM T3 (18 hr) following with 1  $\mu$ M 9-*cis* retinoic acid and 1 mM 8-bromo-cAMP for an additional 6 hr prior to RNA extraction. Total RNA was extracted and subjected to Northern blot analysis using <sup>32</sup>P-labeled cDNAs encoding PGC-1, UCP-1, UCP-2, UCP-3, myogenin, and 36B4.

number/cytoplasmic area) in the cells expressing PGC-1 compared to controls (Figure 4B). No consistent differences were observed in the mitochondrial structure between the two groups, including mitochondrial size or density of cristae.

#### PGC-1 Modulates Regulators of Mitochondrial Transcription/Replication

PGC-1 is localized exclusively to the cell nucleus (Puigserver et al., 1998); this raises the question of how PGC-1

can affect the transcription and replication of the mitochondrial genome. Our attention was drawn to two sets of previously identified transcriptional factors related to mitochondrial function: the nuclear respiratory factors (NRFs) and mitochondrial transcription factor A (mtTFA). The family of NRFs, NRF-1 (Virbasius et al., 1993a) and several NRF-2 isoforms (Virbasius et al., 1993b; Gugneja et al., 1995), has been shown to activate the transcription of a large number of genes involved in respiratory chain function (Scarpulla, 1997). Notably, this includes several genes shown here to be targets of PGC-1 such as  $\beta$ -ATP synthetase, COX IV, and CytC. NRFs also regulate mtTFA (Virbasius and Scarpulla, 1994), a nuclearly encoded factor that translocates to mitochondria where it binds to the D loop of mitochondrial DNA and activates its replication and transcription (Clayton, 1992; Shadel and Clayton, 1993). Targeted disruption of mtTFA in mice caused severe depletion of mtDNA and abolished mitochondrial oxidative phosphorylation/respiration function (Larsson et al., 1998; Wang et al., 1999). Hence, it is conceivable that PGC-1 regulates mitochondrial function, at least in part, through modulating the amounts and/or function of these two key sets of regulators. To test this idea, a luciferase gene driven by the mtTFA promoter was cotransfected into BALB 3T3 cells along with an empty vector or a vector expressing PGC-1. Figure 5A shows that ectopic expression of PGC-1 increases the activity of the mtTFA promoter about 4-fold. Because this promoter has been well characterized, we examined the effects of mutations in the binding sites for several key transcription factors. The activation of the mtTFA promoter is completely abolished by mutating the NRF-1-binding site. In contrast, mutating either the NRF-2- or the Sp1-binding site reduces the activity to a much smaller extent. These data suggest that PGC-1 affects either the expression of NRF-1, augments transcriptional activity, or both. To begin to investigate this, mRNA levels for these mitochondrial regulators were studied. Figure 5B shows that ectopic expression of PGC-1 in either myoblasts or myotube cells causes a huge increase in the expression of NRF-1 and NRF-2 $\alpha$  mRNA. There is also a significant increase in the expression of the crucial NRF target gene, mtTFA.

To investigate whether PGC-1 increases the transcriptional activity of ectopically expressed NRF-1 through coactivation of this factor, we cotransfected PGC-1 and NRF-1, along with a luciferase reporter construct containing a minimal promoter linked to four copies of an

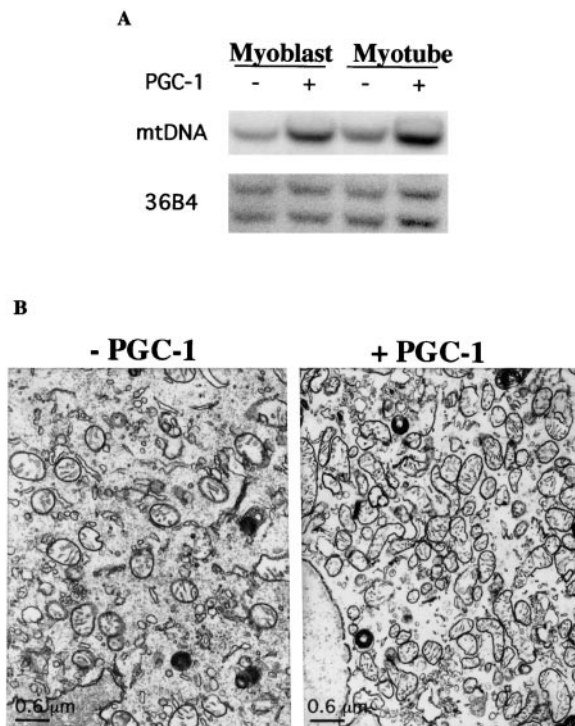


Figure 4. PGC-1 Stimulates Mitochondrial DNA Replication and Biogenesis

(A) Southern blot analysis of mitochondrial and genomic DNA. Total cellular DNA was isolated from C2C12 cells expressing PGC-1 and their controls, in both the myoblast and myotube states. Ten micrograms of DNA was digested with NcoI as a probe and subjected to Southern blot analysis using a cDNA for COX II as a probe for mtDNA. The blot was then stripped and hybridized to a cDNA for 36B4, a nuclearly encoded gene.

(B) Transmission electron microscopy of PGC-1 expressing myoblasts and control cells. The magnification is 8750 $\times$ .

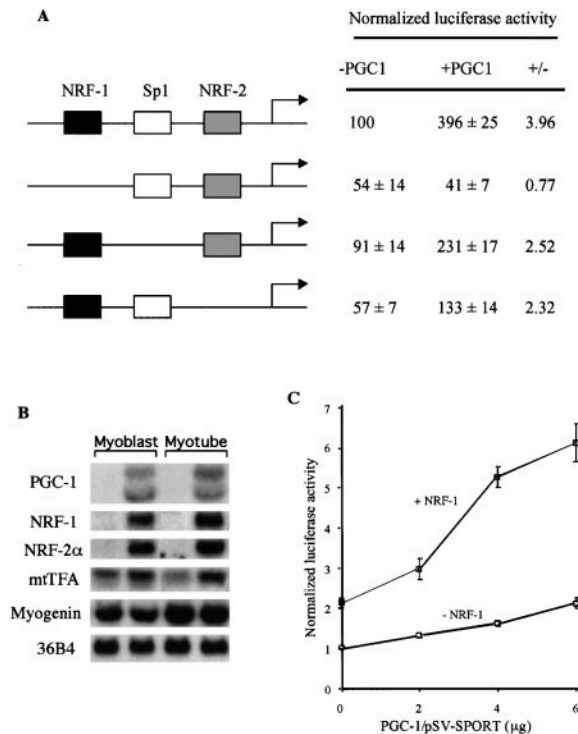


Figure 5. PGC-1 Controls Amount and Activity of Transcriptional Regulators of Mitochondrial Genes

(A) PGC-1 increases the activity of the mtTFA promoter. BALB/c 3T3 cells were transfected with 0.25 μg of CMV/β-gal, 0.5 μg of the indicated mtTFA promoter luciferase reporter construct, and 4 μg of pSV/SPORT or pSV/SPORT-PGC-1. Luciferase activities were normalized to a cotransfected β-gal control. The data represent means of three independent experiments, each performed in duplicate.

(B) PGC-1 induces mRNA for the NRFs and mtTFA. C2C12 cells expressing PGC-1 and the control cells were grown to confluence (day 0) or induced to differentiate for 5 days. Total RNA was extracted and 20 μg of each sample was subjected to Northern blot analysis. Probes used in hybridization were cDNAs for human NRF-1, NRF-2α, and mouse mtTFA. The same blot was also hybridized to cDNA for myogenin and 36B4.

(C) Coactivation of NRF-1 by PGC-1. BALB 3T3 cells were transfected with 1 μg of a luciferase reporter gene containing 4× NRF-1-binding site, 0.5 μg of pGS5- NRF-1 expression plasmid, and the indicated amount of pSV/SPORT-PGC-1. Luciferase activities were normalized to a cotransfected β-gal control. The data represent the means of two independent experiments performed in triplicate.

NRF-1-binding site. Figure 5C shows that the expression of PGC-1 alone in the 3T3 cells does not activate the reporter construct significantly. However, coexpression of PGC-1 and NRF-1 causes a large increase in transcriptional activity, suggesting that PGC-1 can increase the activity of a given quantity of NRF-1.

#### Identification of Domains that Mediate PGC-1/NRF-1 Interactions

A possible physical interaction between PGC-1 and NRF-1 was investigated first by coexpressing NRF-1 with a protein containing full-length PGC-1, fused to the yeast Gal4 DNA-binding domain used as a tag. COS cells were transiently transfected with various combinations of the DNA plasmids as indicated in Figure 6A.

The transfected cells were lysed and subjected to immunoprecipitation and Western blotting using Gal4 and NRF-1 antibodies, respectively. As shown in Figure 6A, NRF-1 is only detected in the immunoprecipitates when PGC-1 is coexpressed, indicating that PGC-1 and NRF-1 stably associate in these cells.

To identify the domains of PGC-1 responsible for this interaction, two GST-PGC-1 fusion proteins were generated containing amino acids 1–180 and 1–403, respectively. These fusion proteins were immobilized on glutathione agarose beads and incubated with in vitro-translated, <sup>35</sup>S-methionine-labeled NRF-1. As shown in Figure 6B, 35% of the input NRF-1 is bound to amino acids 1–403 of PGC-1, while almost none is bound to the GST. Deletion of amino acids 181–403 essentially eliminates all binding above that seen with the GST control protein. For comparison and to assure the functionality of these proteins, we also examined the interaction of these two GST-PGC-1 fusions with PPARγ. As shown previously, amino acids 180–403 contain a major domain of interaction with PPARγ; however, considerable binding is still observed to amino acids 1–180 of PGC-1. Thus, these data indicate that PGC-1 interacts with NRF-1 through amino acids 180–403. This is also in the region of a major interaction domain with PPARγ (Puigserver et al., 1998).

To identify the domains of NRF-1 that interact with PGC-1, the PGC-1-GST fusion proteins were used in combination with reticulocyte translations of various portions of NRF-1. Figure 6C shows that truncation of the C terminus of NRF-1 at amino acid 380, which deletes the major transactivation domain, does not reduce the binding of this protein to PGC-1 compared to the full-length NRF-1 (1–503). Deletion of the N terminus (amino acids 1–107) of NRF-1, which contains important serine phosphorylation sites and the nuclear localization signal (NLS), also does not ablate the interaction between NRF-1 and PGC-1. In contrast, deletion of amino acids 1–142, which contains part of the DNA-binding domain, completely abolishes this interaction. An internal fragment of NRF-1 from amino acids 108–304, containing the entire DNA-binding domain, can bind to PGC-1 as well as the full-length NRF-1. This data localized the region of NRF-1 required for interaction with PGC-1 to amino acids 108–143, which overlaps part of the region required for DNA binding. The major domains of PGC-1 and NRF-1 are shown schematically in Figure 6D. It is worth noting that PGC-1 has a distinct region (amino acids 180–403) that is utilized to interact with both PPARγ and NRF-1, transcription factors that belong to two completely different families. PGC-1 also contains a second domain overlapping with the transcriptional activation domain that allows it to interact with PPARγ and perhaps other nuclear receptors.

Finally, to test the notion that the mitochondrial biogenesis mediated by PGC-1 requires the PGC-1/NRF-1 interaction, we have suppressed NRF-1 activity through the use of a dominant-negative allele of NRF-1. Since NRF-1 functions as a homodimer, we expressed an allele that lacks the transactivation domain and therefore suppresses wild-type NRF-1 activity (U. A. and R. S., unpublished data). C2C12 cells were transiently transfected with a plasmid that coexpresses both green fluorescence protein (GFP) and PGC-1, along with either empty

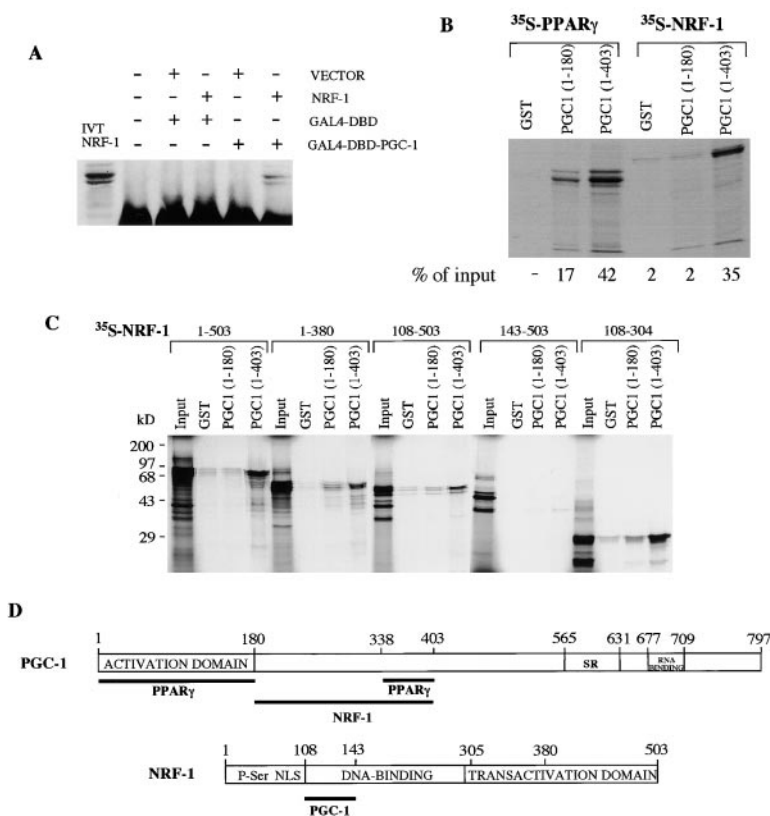


Figure 6. Physical Interaction of PGC-1 with NRF-1

(A) Interaction between PGC-1 and NRF-1 in cells. Vectors expressing the GAL4-DBD, GAL4-DBD-PGC-1, and NRF-1 were transfected in COS cells. Cells were harvested 48 hr after transfection and subjected to immunoprecipitation and Western blot analysis.

(B) Mapping of the domain of PGC-1 interacting with NRF-1 in vitro. Portions of PGC-1 fused to GST were bound onto beads and incubated with in vitro translated [<sup>35</sup>S]-PPAR $\gamma$  or [<sup>35</sup>S]-NRF-1. The beads were then washed, and the bound proteins were eluted and analyzed by autoradiography after SDS-PAGE. The retained PPAR $\gamma$  and NRF-1 were quantified using a PhosphorImager, and the results shown were expressed as percentages of the inputs.

(C) Mapping of the domain of NRF-1 interacting with PGC-1 in vitro. PGC-1 deletions fused to GST were bound to beads and incubated with different [<sup>35</sup>S]-NRF-1 fragments. The bound proteins were washed, eluted, and analyzed by autoradiography after SDS-PAGE.

(D) Schematic representation of key interaction domains on PGC-1 and NRF-1. Note that there is overlap in the domains of PGC-1 that interact with NRF-1 and PPAR $\gamma$ .

vector or the NRF-1 dominant-negative allele, NRF-1/DN. The expression of GFP allowed us to select the transfected cells using flow cytometry. To quantitate mitochondrial number, we have utilized a specific dye (MitoTracker Red CMXRos) that yields a fluorescence signal proportional to the density of mitochondria. As shown in Figure 7, expression of PGC-1 caused an increase of MitoTracker fluorescence signal over the control cells detected by the FACS analysis, consistent with the stimulation of mitochondrial biogenesis. Choosing a fluorescence intensity of  $10^2$  as an arbitrary reference point, 48% of the vector-transfected cells have a signal below this threshold (Figure 7A), while only 11% of the PGC-1-transfected cells have a signal below this value (Figure 7B). Cotransfection with the dominant-negative NRF-1 almost completely inhibited this effect of PGC-1 (Figure 7C), restoring 39% of the cells to a value below the  $10^2$  threshold. In addition, we measured the mtDNA content of the same number of these three groups of cells by Southern blot analysis. Consistent with the data in Figure 7, PGC-1 increased the content of mitochondrial DNA to 80% over the control, while this increase was reduced to only 10% over the control when NRF-1/DN was coexpressed with PGC-1 (data not shown). These data indicate that the biogenesis of mitochondria stimulated by PGC-1 requires the function of NRF-1.

## Discussion

The ability to alter mitochondrial number and function is an important adaptive response in all eukaryotes. In mammals, regulation of mitochondrial number and activity is affected by the amounts and types of food ingested

(Brooks et al., 1980), the levels of various hormones (Nelson, 1990), alterations in environmental temperatures (Himms-Hagen, 1990), and by aging, hypoxia, and various environmental stresses (Wallace, 1999). The control of mitochondria biogenesis in response to cold, fasting, and overfeeding is actually part of a broader program of adaptive thermogenesis that is largely centered on muscle and brown adipose tissue (BAT). This program also involves the uncoupling of oxidative phosphorylation through expression of one or more UCPs. The study of the promoters of known mitochondrial genes has led to the identification of two sets of transcription factors that directly regulate mitochondrial gene expression. NRF-1 is not related to any known gene families; however, it has homology in the DNA-binding domain to two developmental regulatory factors, sea urchin P3A2 and erect wing (EWG) protein of *Drosophila* (Virbasius et al., 1993a). NRF-2 belongs to the ETS family; these factors regulate a large number of mitochondrial genes encoded in the cell nucleus, including mtTFA (Scarpulla, 1997). mtTFA, a member of the HMG gene family, is of special interest in that it translocates into mitochondria and initiates the transcription and replication of the mitochondrial genome (Shadel and Clayton, 1993). Genetic and biochemical studies strongly suggest that these factors, acting together, can bring about mitochondrial biogenesis and increase oxidative phosphorylation (Poyton and McEwen, 1996). What is much less clear is how these factors can be coordinated into a program that is tissue selective (in the case of mammals) and can be responsive to the demands imposed by the environment.

A model emerging from the data presented here and



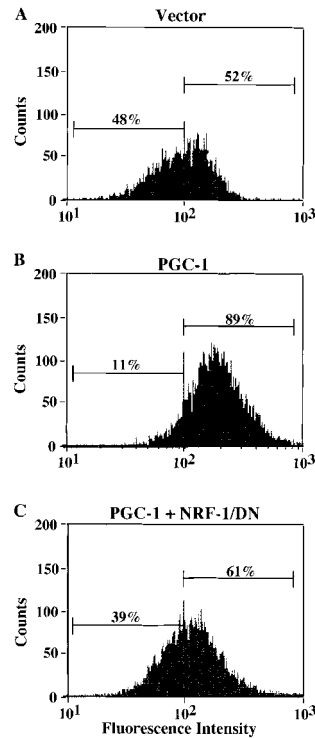


Figure 7. A Dominant-Negative Allele of NRF-1 Blocks the Effect of PGC-1 on Mitochondrial Biogenesis

C2C12 myoblasts were transfected with an IRSE bicistronic expression vector encoding both PGC-1 and GFP (PGC1-IRSE2-EGFP) along with either empty vector (pSG5) (B) or the dominant-negative NRF-1, pSG5-NRF-1/DN (C). The control cells (A) were transfected with pSG5 and the GFP vector (pIRSE2-EGFP). Cells were stained with MitoTracker Red CMXRos 60 hr posttransfection. The GFP-positive cells were sorted, and the MitoTracker fluorescence of these cells was analyzed by flow cytometry. The mean of the MitoTracker fluorescence was 115.6 in (A), 219.2 in (B), and 138.0 in (C).

in Puigserver et al. (1998) illustrates how PGC-1 can link the external environment directly to mitochondrial biogenesis and gene expression (Figure 8). Certain environmental states, such as cold temperatures or high-calorie diets, are known to increase the activity of the sympathetic nervous system, leading to the release of norepinephrine. This neurotransmitter triggers activation of the  $\beta$ -adrenergic receptors, resulting in the elevation of intracellular cAMP and stimulation of adaptive thermogenesis in skeletal muscle and brown fat (Himms-Hagen, 1989). One of the main effectors of this response appears to be PGC-1. Previous studies demonstrated a dramatic induction of PGC-1 by low temperatures in the muscle and brown fat in mice. In addition, PGC-1 is reduced with fasting and elevated upon refeeding (data not shown). That these environmental responses are ultimately relayed by cAMP was further suggested by the fact that PGC-1 expression could be induced in culture brown fat cells by the  $\beta$ -adrenergic agonist isoproterenol (Puigserver et al., 1998). As shown here, PGC-1 powerfully induces mRNA for NRF-1 and NRF-2 $\alpha$ , regulators of multiple target genes such as COX IV,  $\beta$ -ATP synthetase, and mtTFA. In addition, PGC-1 binds to and coactivates NRF-1, increases its transcriptional activity on target genes, including mtTFA. It is important

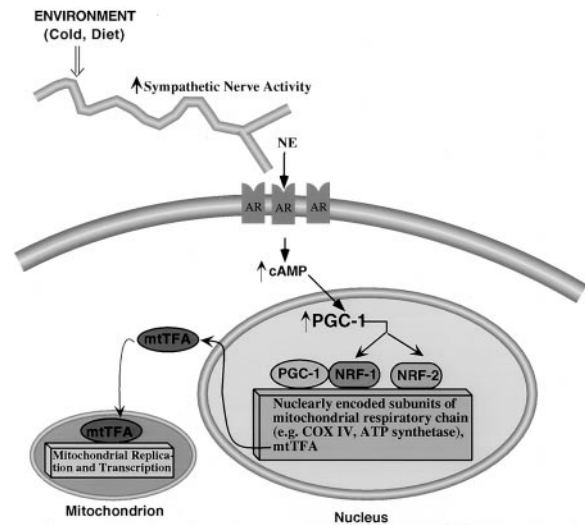


Figure 8. Pathway of Mitochondrial Biogenesis through PGC-1

Cold and high-calorie diet stimulate the sympathetic nervous system leading to the release of norepinephrine. NE triggers the activation of the  $\beta$ -adrenergic receptors (AR) resulting in the elevation of intracellular cAMP and inducing the expression of PGC-1. PGC-1 activates the expression of the subunits of respiratory chain and mtTFA through the induction of the expression of NRFs and the coactivation of NRF-1-mediated transcription. mtTFA subsequently translocates into mitochondrion and directly activates the transcription and replication of mtDNA.

to note that this model, implicating NRF-1 as an important target of PGC-1, is based upon both gain-of-function (Figure 5) and loss-of-function experiments (Figure 7).

The uncoupling of oxidative phosphorylation through the UCPs is an important feature of mammals. This process allows for tolerance of cold, resuscitation from hibernation, and (of more interest to human residents of the industrial world!) prevention of obesity (Flier and Lowell, 1997). Our previous work illustrated that PGC-1 could increase the expression of UCP-1 in cultured brown fat cells (Puigserver et al., 1998). Since thermogenesis in muscle is believed to be much more quantitatively important in humans, the molecular mechanisms of uncoupling in this tissue are of particular interest. As shown in Figure 1, PGC-1 expression in the cultured muscle cells showed a powerful effect on oxygen consumption, increasing it by 77%. To put this into a physiological context, prolonged exposure of animals at 4°C or direct injection with  $\beta$ -adrenergic receptor agonists rarely increases oxygen consumption by more than 100% (Holloway et al., 1991). Hence, PGC-1 can alter this parameter by as much as it is typically changed, even in extreme experimental settings.

Transcriptional coactivators, including PGC-1, increase transcription by directly binding to transcription factors and increasing their activity. The transcription factor(s) utilized by PGC-1 in the regulation of the endogenous UCP-2, NRF-1, and NRF-2 genes is not known. Since UCP-2 is known to be regulated by PPAR $\gamma$  and PPAR $\gamma$  ligands in fat cells (Sears et al., 1996; Camirand et al., 1998), it is possible that PGC-1 is binding to this factor on the UCP-2 promoter in the muscle cells. Because PGC-1 binds to and coactivates PPAR $\gamma$  in a ligand-independent manner (Puigserver et al., 1998), the

lack of dependence on troglitazone for the PGC-1-mediated induction would still be consistent with a role for PPAR $\gamma$ . The NRF-1 and NRF-2 promoters have not yet been well characterized, so whether PGC-1 is coactivating a nuclear receptor, NRF-1, or some as yet unidentified transcription factor binding to this promoter is unclear.

A striking and potentially important feature of coactivation by PGC-1 is that it is promoter selective. While UCP-2 and UCP-3 are both expressed in the muscle cells, and both have been reported to be activated by PPAR $\gamma$  (Kelly et al., 1998; Shimokawa et al., 1998), only UCP-2 mRNA is induced by PGC-1 in the muscle cells. Similarly, our previous work showed that some but not all PPAR $\gamma$  target genes in fat cells were regulated by PGC-1 (Puigserver et al., 1998). It will thus be crucial to determine how PGC-1 can discriminate between particular promoters, even when working through an identical transcription factor. It may be notable that for both transcription factors where PGC-1-binding domains have been mapped (PPAR $\gamma$  and NRF-1), this binding occurs in the identified DNA-binding regions. Hence, the simplest explanation for the promoter selectivity of PGC-1 would be if coactivator docking alters the DNA binding properties of these transcription factors in a way that allows them to function through a subset or a different set of DNA-binding sites.

PGC-1 represents one of the first examples of execution of a particular biological program at the level of the transcriptional coactivator. Prior to this, Roeder's lab had shown that OCA-B, a coactivator of Oct factors that is expressed specifically in B cells, was essential for proper B cell development (Luo et al., 1992). Since there are innumerable examples of biological programs regulated at the level of the transcription factor, it is interesting to speculate why adaptive thermogenesis and mitochondrial biogenesis should be controlled through regulated expression of a particular coactivator. The answer could lie in the fact that these programs require coordination of transcriptional functions which are *themselves* complex networks. Adaptive thermogenesis requires inputs from the  $\beta$ -adrenergic, thyroid hormone, and PPAR $\gamma$  system, expression of various uncoupling proteins in a cell-selective manner, and a whole cascade of mitochondrial transcription factors. This process must be regulated tightly in both space and time upon certain environment cues. Working through a coactivator that can interact with multiple transcription factor families, as many coactivators including PGC-1 can clearly do, would allow coordination of many otherwise disparate transcriptional regulators into a program of whole-body physiology.

Although PGC-1 can clearly coordinate this complex program of adaptive thermogenesis, it is of considerable future interest to know whether PGC-1 can regulate the biogenesis and electron transport systems of mitochondria, without uncoupling oxidative phosphorylation. This question is crucial because PGC-1 is expressed (though not cold-inducible) in certain tissues not ordinarily associated with adaptive thermogenesis, such as brain and heart (Puigserver et al., 1998). Indeed, it is difficult to imagine circumstances whereby the uncoupling of oxidative phosphorylation in the heart would not be highly

deleterious to the organism. There are two obvious solutions to this dilemma. First, as shown in Figure 4, expression of UCPs is cell type selective, and hence, PGC-1 may control mitochondrial number in some tissues without stimulating uncoupling. Another possibility is that mechanisms may exist, through alternative splicing or protein modification, to "subdivide" the program of gene expression controlled by PGC-1, allowing for oxidative phosphorylation but not uncoupling. Further experiments in cardiac and neuronal cells will be required to investigate this.

#### Experimental Procedures

##### Cell Culture, Retroviral Infection, and Differentiation

C2C12 cells were purchased from ATCC and cultured in DMEM containing 10% cosmic calf serum (CCS, Hyclone). These myoblasts were infected with retrovirus containing pBabe-puromycin or pBabe-PGC-1 using the viral packaging system as described previously (Puigserver et al., 1998). Following puromycin selection, virally infected cell lines were grown to confluence in DMEM with 10% CCS. They were then cultured in DMEM containing 2% heat-inactivated bovine calf serum (Hyclone) to induce differentiation into myotubes. 3T3-F442A stable cell lines were generated and induced to differentiation as described (Puigserver et al., 1998).

##### Protein Binding Assays

The GST-PGC-1 fusion proteins were constructed by cloning the cDNA fragments encoding either amino acids 1–180 or 1–403 into the EcoRI site of pGEX 5X-3 or the BamHI-XhoI sites of pGEX 5X-2, respectively. The PPAR $\gamma$  expression vector, pSV/SPORT-PPAR $\gamma$ , and the NRF-1 deletions were generated as described (Virbasius et al., 1993a; Tontonoz et al., 1994; Gugneja et al., 1996). These fusion proteins were expressed and purified, and in vitro binding reactions were performed as in Puigserver et al. (1998). In vitro proteins labeled with  $^{35}$ S-methionine were prepared using the TNT coupled transcription-translation system (Promega). Labeled proteins were incubated with equal amounts of either the GST protein or with the GST-PGC-1 fusion proteins bound to beads for 1 hr at room temperature. The beads were washed, and the bound proteins were eluted and analyzed by autoradiography after SDS-PAGE.

##### Transcriptional Activation Assays

BALB 3T3 cells were transiently transfected using the calcium phosphate method. Forty hours posttransfection, cells were harvested for luciferase and  $\beta$ -gal assays. The human mtTFA luciferase promoter construct was made by inserting a 0.95 kb MluI/BglII fragment, which included the 122 bp minimal essential mtTFA promoter, from the previously described mtTFA-RC4/CAT plasmid (Virbasius and Scarpulla, 1994) into the pGL3 basic vector (Promega). The different mutants of the mtTFA-RC4/CAT plasmid were transferred to pGL3 basic in the same manner. The luciferase construct, 4xNRF1/Luc, and pSG5/NRF-1 were generated as described (Virbasius et al., 1993a; Gugneja et al., 1996).

##### Northern and Southern Blot Analysis

Total RNA was isolated from cultured cells by Trizol (GIBCO) extraction. RNA samples were analyzed by electrophoresis, transferred, and hybridized as previously described (Tontonoz et al., 1994). For Southern blots, total DNA (including genomic and mitochondrial DNA) was isolated from cells and processed as described (Maniatis et al., 1989).

##### Western Blot Analysis

Cells were lysed in PBS buffer containing 1% Triton X-100, 0.1% deoxycholate, 1 mM PMSF, and protease inhibitors. Fifty micrograms of these total protein extracts from each sample was submitted to SDS-PAGE and transferred to PVDF membrane (Millipore). Western blot analysis was performed using an ECL Western blot kit (Amersham) according to the manufacturer's instructions. The antibody against CytC was purchased from PharMingen and used at



a 1:200 dilution. The COX IV antibody was obtained from Molecular Probes and used at a concentration of 10 ng/ml. The myogenin antibody was used at a 1:25 dilution.

#### Immunoprecipitations

To study protein-protein interactions in cells, various expression plasmids were transfected into COS cells using Superfect (Qiagen). Total cell extracts were prepared by incubating the cells on ice for 30 min in the lysis buffer (10 mM Tris [pH 8.5], 250 mM NaCl, 1% NP40, 1 mM EDTA, 0.1 mM PMSF) supplemented with freshly prepared protease inhibitors (Boehringer). Approximately 700 µg of whole-cell extract protein was incubated with a polyclonal anti-GAL4-DBD antibody (Santa Cruz) for 2 hr at 4°C, followed by an incubation overnight with a mixture of protein A/G Sepharose beads. Immunoprecipitates were washed three times with a washing buffer (50 mM Tris HCl, 150 mM NaCl, 5 mM EDTA, 0.5% NP40, 0.1 mM PMSF) supplemented with freshly prepared protease inhibitors. Immunocomplexes were resolved by SDS-PAGE and transferred to a PVDF membrane (Millipore). Western blots were performed using an anti-NRF-1 as described previously (Gugneja and Scarpulla, 1997) and developed using the ECL method (Amersham).

#### Measurement of Respiration in Whole Cells

Cells were washed with PBS and gently detached from the plates with a cell lifter. They were then resuspended in PBS and transferred to a 1 ml Clark type oxygen electrode chamber that was connected to a circulating water bath at 37°C. After recording the basal respiration, the maximum respiration of cells induced with 2 µM FCCP was measured. The uncoupled respiration was determined in the presence of the ATP synthase inhibitor, oligomycin (2.5 µg/ml). After finishing all the measurements, cells were collected from the chamber, and their protein contents were determined using the BCA kit (Pierce).

#### Electron Microscopy

Cell lines containing a PGC-1 expression vector or control vector were grown to about 70% confluence and gently detached from the plates with a cell lifter. They were washed with PBS and centrifuged at 3000 rpm for 5 min to form a pellet. The cell pellet was fixed with 2% glutaraldehyde in PBS for 2 hr at 4°C and then postfixed in 1% osmium tetroxide in phosphate buffer 0.1 M (pH 7.4), dehydrated in ethanol, and embedded in an Epon-Araldite mixture. Thin sections were obtained with a MT-X ultramicrotome (RMC, Tucson, Arizona), stained with lead citrate and examined with a transmission electron microscopy Philips CM10 (Eindhoven, The Netherlands). For each condition, 100 cells were examined at a final magnification of 8750×. The cytoplasmic area was obtained with the aid of an image analysis system (KS 100, Kontron, Germany).

#### FACS Analysis

C2C12 myoblasts were transiently transfected with 6 µg of PGC1-IRSE2-EGFP with 4 µg of either pSG5 or PSG5-NRF1/DN that lacks amino acids 305–503 (Virbasius et al., 1993b). The control cells were transfected with 6 µg of pIRSE2-EGFP (Clontech) and 4 µg of pSG5. Sixty hours posttransfection, cells were incubated in the medium containing 50 nM of MitoTracker Red CMXRos (Molecular Probes) for 30 min at 37°C. They were then trypsinized and resuspended in PBS. The GFP-positive cells were sorted, and the MitoTracker fluorescence of these positive cells was analyzed on a MoFlo flow cytometry (Cytomation).

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