

Complementary action of the PGC-1 coactivators in mitochondrial biogenesis and brown fat differentiation

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Summary

Mitochondria play an essential role in the ability of brown fat to generate heat, and the PGC-1 coactivators control several aspects of mitochondrial biogenesis. To investigate their specific roles in brown fat cells, we generated immortal preadipocyte lines from the brown adipose tissue of mice lacking PGC-1 α . We could then efficiently knockdown PGC-1 β expression by shRNA expression. Loss of PGC-1 α did not alter brown fat differentiation but severely reduced the induction of thermogenic genes. Cells deficient in either PGC-1 α or PGC-1 β coactivators showed a small decrease in the differentiation-dependant program of mitochondrial biogenesis and respiration; however, this increase in mitochondrial number and function was totally abolished during brown fat differentiation when both PGC-1 α and PGC-1 β were deficient. These data show that PGC-1 α is essential for brown fat thermogenesis but not brown fat differentiation, and the PGC-1 coactivators play an absolutely essential but complementary function in differentiation-induced mitochondrial biogenesis.

Introduction

White adipose tissue (WAT) and brown adipose tissue (BAT) are two functionally distinct tissues characterized by two types of fat cells with very different metabolic properties. The principal role of WAT is the storage and release of triglycerides in response to changing systemic energy levels. By contrast, BAT is specialized for thermogenesis, a process that dissipates chemical energy to produce heat (Cannon and Nedergaard, 2004; Lowell and Spiegelman, 2000). Thermogenesis is induced in this tissue in response to cold exposure or diet and occurs through the activity of uncoupling protein 1 (UCP1), expressed selectively in brown adipocytes (Bouillaud et al., 1985). UCP1 is an inner mitochondrial membrane protein that uncouples the mitochondrial proton gradient, so that oxygen consumption is no longer linked to ATP synthesis (Klingenberg and Huang, 1999). Brown fat differentiation is also accompanied by a great increase in mitochondrial biogenesis (Butow and Bahassi, 1999); indeed, brown fat cells have among the highest mitochondrial density of any cell type in mammals (Lindberg et al., 1967).

The sequence of events that regulates formation of BAT during development is poorly understood. Although BAT and WAT are functionally distinct, they share certain key transcriptional regulators that mediate their differentiation (Rosen and Spiegelman, 2000). Early in the differentiation program, C/EBP β and C/EBP δ are transiently expressed and contribute to the expression of PPAR γ and C/EBP α . PPAR γ is the direct driver of fat cell differentiation, while C/EBP α is required to maintain PPAR γ expression and also functions synergistically with PPAR γ to promote the expression of genes found in both brown and white adipocytes (Rosen et al., 2002, 1999; Wu et al., 1999b). The acquisition of the specific morphological and molecular equipment required for brown fat function during differentiation is under the control of PPAR γ -coactivator 1 α (PGC-1 α) (Puigserver et al., 1998). PGC-1 α is induced early in brown fat differentiation

and is preferentially expressed in mature brown adipocytes compared to white adipocytes. Furthermore, ectopic expression of PGC-1 α is sufficient to promote several aspects of differentiation toward the brown fat lineage, including the induction of UCP1 gene expression. PGC-1 α is also rapidly and highly induced by cold exposure and turns on several key components of the adaptative thermogenic program in brown fat, including fuel intake, fatty-acid oxidation, mitochondrial biogenesis, and increased oxygen consumption through coactivation of transcription factors such as peroxisome proliferator-activated receptors (PPARs) and nuclear respiratory factor 1 (Wu et al., 1999a).

Notably, mice deficient in PGC-1 α are cold sensitive with poor expression of UCP1 (Lin et al., 2004). In addition, the brown fat tissues appear morphologically abnormal, with abundant accumulation of large lipid droplets, reminiscent of white adipose tissue. The PGC-1 α null mice also show a complex neurological disorder, with hyperactivity associated with neurodegeneration, especially in the striatal region (Lin et al., 2004; Leone et al., 2005). Brown adipose tissue is abundantly innervated by the sympathetic nervous system (SNS); SNS nerve endings in BAT release noradrenaline, which activates β -adrenergic receptors and a cascade of events leading to BAT cell proliferation, mitochondriogenesis, and increased expression and activation of UCP1 (Himms-Hagen, 1990). Lesions associated with PGC-1 α deficiency in the brain could therefore contribute to defective brown fat function in PGC-1 α ^{-/-} mice.

It is thus clear that PGC-1 α is required for proper brown fat function. However, it is not clear whether the altered morphology and function of this tissue is due to an altered state of cell differentiation—a differentiation to a more “white fat” phenotype—or to a defective brown fat thermogenic program. It is also unclear whether the defect in BAT in the PGC-1 α knockout animals is autonomous to brown adipose cells or is a result of defective SNS signaling. To investigate these questions, we

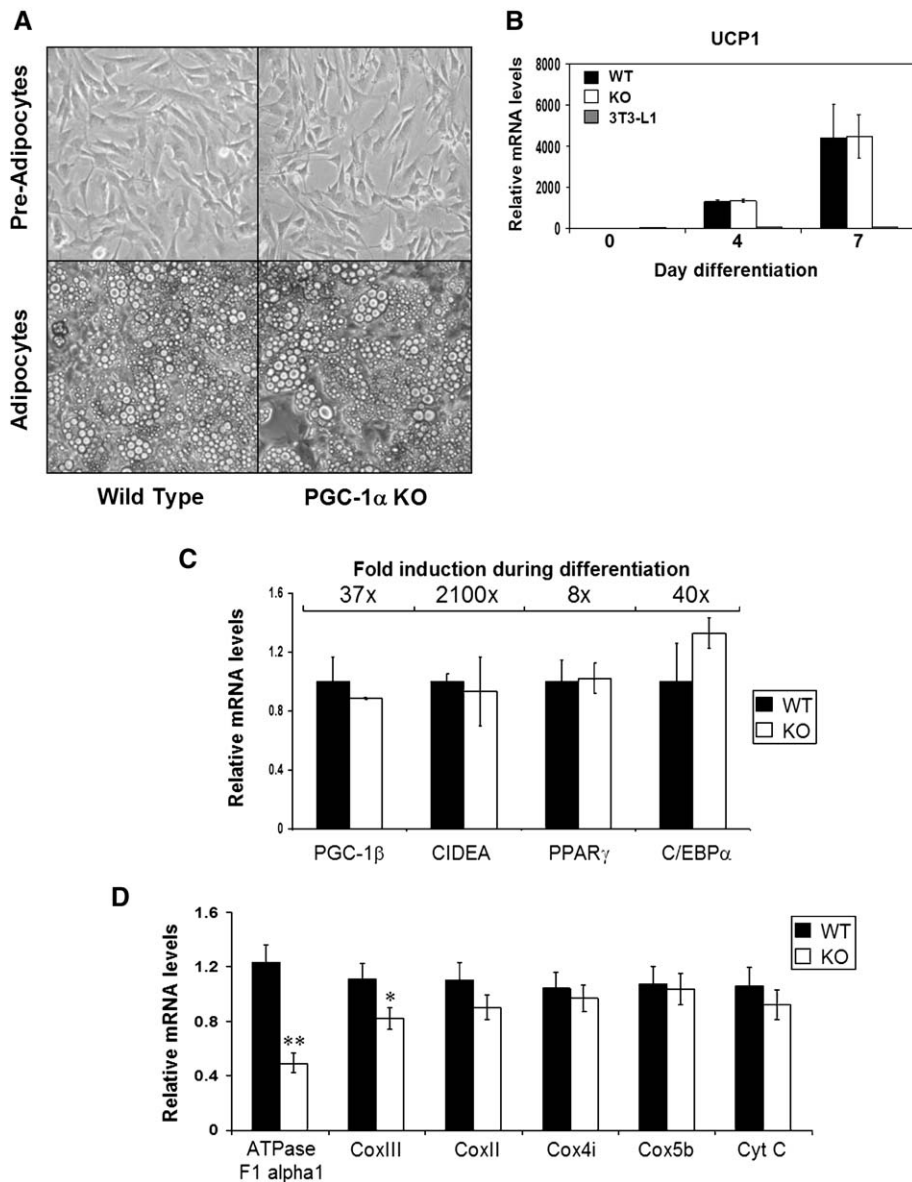


Figure 1. Lipid accumulation and expression of brown fat-selective genes during differentiation in absence of PGC-1 α .

A) Brown preadipocytes and mature brown adipocyte morphology is shown for wild-type and PGC-1 α null cells.

B) Expression of UCP1 mRNA, as assessed by real-time PCR at days 0, 4, and 7 of the differentiation protocol. Note the selective induction of UCP1 in brown fat cells compared to the 3T3-L1 white fat cells.

C) mRNA expression of genes selective for brown fat cells (CIDEA, PGC-1 β) or those common to all fat cells (PPAR γ , C/EBP α) was examined by real-time PCR in mature wt and PGC-1 α KO adipocytes. For expression of each gene, the fold induction of mRNA expression in mature adipocytes compared to preadipocytes is indicated.

D) Real-time PCR analysis of mitochondrial gene mRNA levels in mature wt and PGC-1 α KO brown adipocytes. * $p < 0.05$. ** $p < 0.01$. Data in (B), (C), and (D) represent mean \pm SD of triplicates.

have created immortal preadipocyte cell lines derived from the brown adipose depot of mice lacking PGC-1 α . These cells have also allowed us to investigate the relative role of PGC-1 β , the closest homolog of PGC-1 α , in several important aspects of brown fat cell biology. This coactivator has been shown to promote mitochondrial biogenesis in gain-of-function experiments as effectively as PGC-1 α (Lin et al., 2002, 2003; St-Pierre et al., 2003). Hence, the relative importance of these two coactivators in a model of cellular mitochondrial biogenesis has not been analyzed. The data presented here indicate that PGC-1 α is not required for the differentiation of cells into the brown fat phenotype. However, these cells have a severe diminution of the thermogenic gene program, including poor induction of UCP1 by cyclic AMP signaling. These data further show that deficiency of either PGC-1 α or PGC-1 β causes a small but significant decrease in mitochondrial gene expression. However, deficiency of both PGC-1 α and PGC-1 β simultaneously causes a total loss of differentiation-linked mitochondrial biogenesis and mitochondrial respiration.

Results

Differentiation of brown preadipocytes lacking PGC-1 α is not impaired

In order to investigate the role of PGC-1 α in several aspects of brown fat cell function, we first generated immortalized preadipocyte cell lines from the interscapular BAT of wild-type (wt) and PGC-1 α KO mice.

We derived three wt and three PGC-1 α null preadipocyte cell lines from six newborn mice. Each of these cell lines was tested for its ability to differentiate into adipocytes using insulin, thyroid hormone T3, isobutylmethylxanthine, dexamethasone, and indomethacin, as described in [Experimental Procedures](#). After induction, cells of both genotypes rapidly accumulated fat, and a fully differentiated phenotype could be observed, with nearly 100% of the cells containing multilocular fat droplets (Figure 1A). This indicates that PGC-1 α is not required for the morphological changes and lipid accumulation associated with adipocyte differentiation.

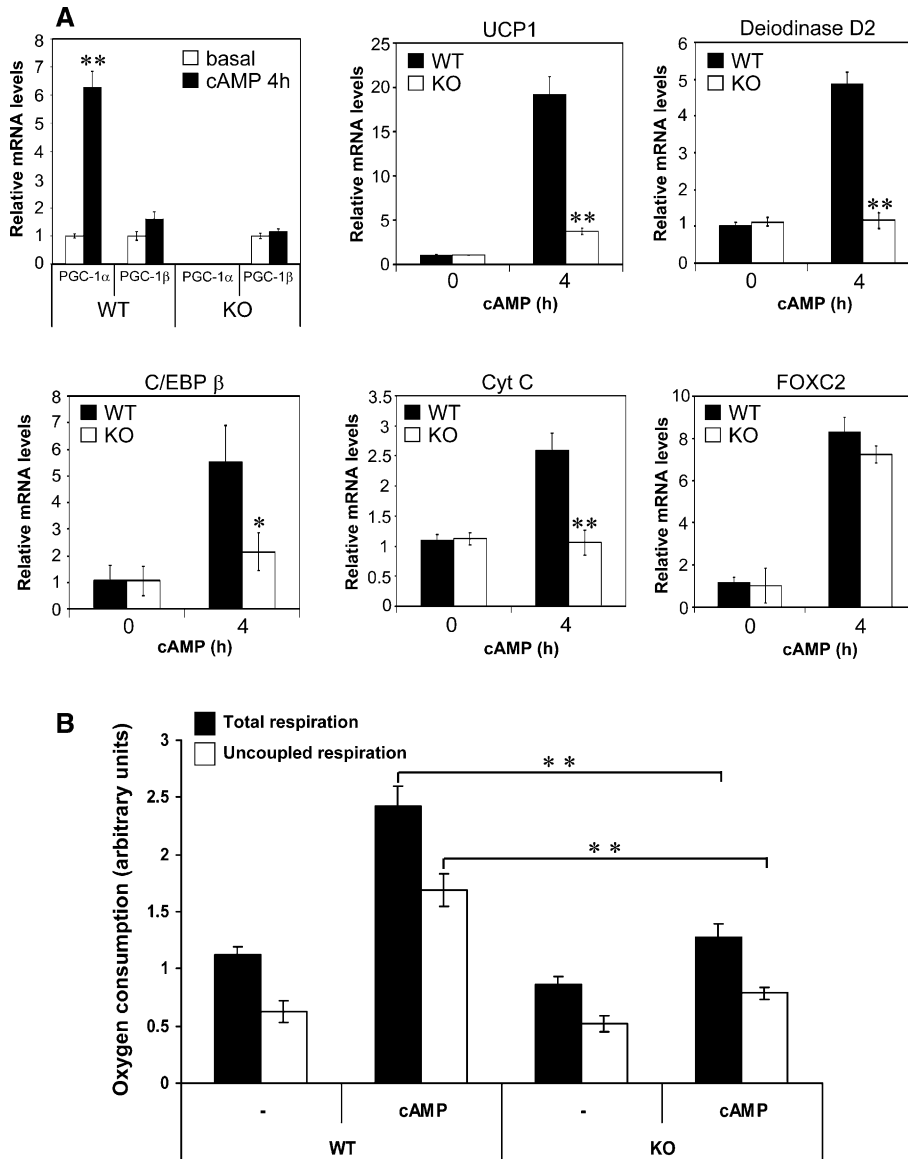


Figure 2. PGC-1 α is required for induction of the thermogenic gene program

A) PGC-1 α , PGC-1 β , UCP1, C/EBP β , deiodinase D2, cytochrome C, and FOXC2 mRNA levels were assessed by real-time PCR in differentiated wt and KO cells (day 7) with or without treatment with dbcAMP for 4 hr. * $p < 0.05$. ** $p < 0.01$. Data represent mean \pm SD of triplicates.

B) Total mitochondrial oxygen consumption and uncoupled respiration were measured in wt and KO brown adipocytes (day 7), with and without pretreatment with dbcAMP for 24 hr. Uncoupled respiration represents the fraction of mitochondrial respiration that is not coupled to ATP production and was ascertained through the addition of the ATP-synthase inhibitor oligomycin. ** $p < 0.01$. Data represent mean \pm SD of three independent experiments.

We also assayed mRNA expression of several genes selective for the brown fat phenotype during the differentiation process. As expected, the expression of PGC-1 α itself was highly induced in wt cells but not in KO cells (data not shown). PGC-1 β was induced early and robustly during differentiation of the brown adipocytes (Figure S1; Lin et al., 2002). Expression of UCP1 mRNA, encoding the classic brown fat thermogenic protein, was similarly induced during differentiation in the KO cells compared to wt cells (Figure 1B). In addition, expression of mRNA for CIDEA, another molecular marker of the brown fat phenotype (Zhou et al., 2003), was expressed similarly in mature wt and KO cells, (Figure 1C). We also assessed the mRNA expression level of the key genes that regulate white or brown adipogenesis, including PPAR γ and C/EBP α . Both were similarly induced during differentiation of wt and KO cells (Figure 1C). Importantly, expression of PGC-1 β was not significantly different in mature KO adipocytes. Finally, the expression of mitochondrial genes was slightly diminished in fully differentiated KO cells compared to their wt counterparts (Figure 1D). Taken together,

these results clearly show that PGC-1 α is not required for molecular aspects of brown fat cell differentiation.

Expression of the thermogenic gene program requires PGC-1 α

Cold-induced thermogenesis in vivo is mediated through a signaling cascade involving the sympathetic nervous system and the β 3-adrenergic receptor activation in brown fat, resulting in increased cytoplasmic cAMP levels in brown adipocytes. Figure 2A shows that 4 hr of treatment with dibutyl-3-cyclic AMP (dbcAMP) strongly induced PGC-1 α mRNA expression in wt cells; comparable results were obtained with isoproterenol and forskolin treatment (not shown). We then measured mRNA expression of several key genes involved in the thermogenic response after 4 hr of dbcAMP treatment. UCP1 mRNA was strongly induced in wt cells (about 20-fold), but about 80% of this response was lost in PGC-1 α KO cells. The UCP1 protein was maximally induced at 24 hr of dbcAMP treatment for the wt cells, as assessed by Western blot analysis (Figure S2). KO

cells had an equivalent UCP1 protein level to wt cells under basal condition, but no increase in UCP1 protein expression was observed after dbcAMP treatment.

The induction of several mRNAs encoded by other genes involved in thermogenesis was either severely impaired in KO cells (C/EBP β) or was even completely blunted (deiodinase D2, cytochrome C) after dbcAMP treatment (Figure 2A). Importantly, some genes such as FOXC2 were still normally inducible by dbcAMP in the KO cells, indicating that cAMP sensitivity per se is not affected in KO cells; these data suggest that PGC-1 α is required for the regulation of some but not all cAMP-regulated genes.

The requirement for PGC-1 α in dbcAMP-stimulated respiration was investigated with an oxygen-sensing electrode. Figure 2B shows that wt cells increased their oxygen consumption 2.2-fold after 24 hr of dbcAMP treatment, versus only 1.4-fold for the KO cells. Furthermore, after dbcAMP treatment, uncoupled respiration, as a fraction of total respiration, was increased from 56% to 71%, but this fraction was not changed in the PGC-1 α -deficient cells. Thus, the loss of PGC-1 α causes a profound reduction of several aspects of the thermogenic gene and physiological program under cAMP treatment.

In order to assess the specific requirement for PGC-1 α in the global transcriptional response to cAMP, we used Affymetrix arrays to compare the sets of genes induced in response to a 4 hr dbcAMP treatment in differentiated wt and KO cells. This analysis revealed that 88 genes (Table S1) were induced more than 3-fold in the wt cells; of these, 54 (61% of total) were similarly increased in both wt and KO. However, 28 genes (32% of total) were decreased by at least 50% in the KO cells compared to wt cells (Figure S3). Among genes requiring PGC-1 α are (as expected) UCP1, deiodinase D2, and C/EBP β . Finally 6 (7% of total) genes, such as Fos (FBJ osteosarcoma oncogene) or IER3 (Immediate early response 3), were *more* strongly induced in KO cells than in wt cells. These data were confirmed by quantitative PCR for a subset of genes (Figure S4). These data indicate that PGC-1 α is required for proper expression of approximately one third of the genes induced in response to cAMP in brown fat cells, but this set of sensitive genes is enriched in those involved in adaptative thermogenesis.

Acquisition of the brown fat phenotype is impaired in cells deficient in both PGC-1 α and PGC-1 β

PGC-1 β is strongly induced during brown fat cell differentiation (Lin et al., 2002), and, as shown in Figure 1C, this induction does not require PGC-1 α . However, the role of PGC-1 β in BAT or brown adipose cells is unknown. To address this question, we used retroviral vectors to express a siRNA that specifically knocks down PGC-1 β levels or a control siRNA of random sequence. The four different cell lines that we generated (wt ctr siRNA, wt PGC-1 β siRNA, KO ctr siRNA, KO PGC-1 β siRNA) were all able to accumulate lipid during differentiation, with nearly 100% of the cells accumulating fat droplets in their cytoplasm (Figure S5). Western blot analysis on mature adipocytes showed a 90% reduction of PGC-1 β protein level for the cell lines that express the specific PGC-1 β siRNA, compared to the cells that express the control siRNA (Figure 3A).

To examine the integrity of the brown fat differentiation program with deficiencies in one or both PGC-1 proteins, we analyzed mRNA expression of several genes at days 0 and 7 of the differentiation protocol. As shown in Figure 3B, genes known

to be expressed in both mature white or brown adipocytes, such as aP2 or GLUT4 (Assimacopoulos-Jeannet et al., 1991; Kopecky et al., 1995), were induced identically in all four cell lines, regardless of PGC-1 α and PGC-1 β expression. However, while induction of the UCP1 gene during differentiation was normal in cells lacking either PGC-1 α or PGC-1 β , expression of this gene was reduced by 90% during differentiation in cells with deficiency in *both* PGC-1 α and β . Western blot analysis showed that UCP1 protein expression was also reduced by 90% in the PGC-1 α / β -deficient cells but was equivalent in the wt cells and the cells lacking only one of the PGC-1 isoforms (Figure 3A).

The expression of several other genes was also affected. ERR α was far less induced (1.6-fold) during differentiation in the absence of PGC-1 α and β , compared to the other cell lines (4-fold) (Figure 3B). Similarly, cells deficient in both PGC-1 α and β expression showed significantly lower induction of several mitochondrial genes during differentiation such as Cox4i or Cox5b. In addition, induction of cytochrome C was completely lost in the cells deficient in both PGC-1 α and β as compared to the other cell lines. These results clearly show that while PGC-1 α or β are not individually necessary for adipogenic differentiation, the presence of at least one of those is essential for development of the brown fat phenotype.

PGC-1 α does not regulate thermogenic genes induced by insulin and retinoic acid

As shown above, the induction of UCP1 in response to cAMP is reduced 80% in the absence of PGC-1 α , but its induction is not completely lost. We asked whether PGC-1 β could be responsible for the remaining induction. Figure 4 shows that wt cells and cells with reduced PGC-1 β expression were able to equivalently induce UCP1 mRNA after 4 hr of dbcAMP treatment. In cells deficient for both PGC-1 α and β , the baseline level of UCP1 mRNA is 10 times lower, but the fold increase in UCP1 expression in response to dbcAMP remains similar to that in PGC-1 α null cells. These results strongly suggest that PGC-1 β is not required for cAMP-induced UCP1 gene expression.

Although cAMP is believed to play a crucial role in BAT-mediated adaptative thermogenesis *in vivo*, UCP1 has been shown to be regulated, at least in cultured cells, by insulin and retinoic acid (Alvarez et al., 1995; Puigserver et al., 1996; Teruel et al., 1998; Valverde et al., 2003). We tested the requirements of PGC-1 α and β in UCP1 induction by insulin and retinoic acid. UCP1 was induced about 4-fold in wt cells treated with either retinoic acid or insulin. However, when combined together, these two compounds had synergistic effects, leading to a 17-fold increase of UCP1 mRNA expression as compared to non-treated cells. Identical results were also seen in cells deficient in either PGC-1 α or β . In addition, despite the fact that the basal level of UCP1 expression is severely reduced in cells deficient in both PGC-1 α and β , the fold induction of UCP1 mRNA in response to insulin and retinoic acid was nearly identical.

These results suggest that neither PGC-1 α nor PGC-1 β are required for insulin or retinoic acid regulation of UCP1.

Differentiation-linked increased respiration and mitochondrial biogenesis are both ablated in cells lacking both PGC-1 α and β

Fully mature brown adipocytes have very high mitochondrial density and a strong capacity for fatty-acid oxidation. Brown fat differentiation is one of the best cellular models for

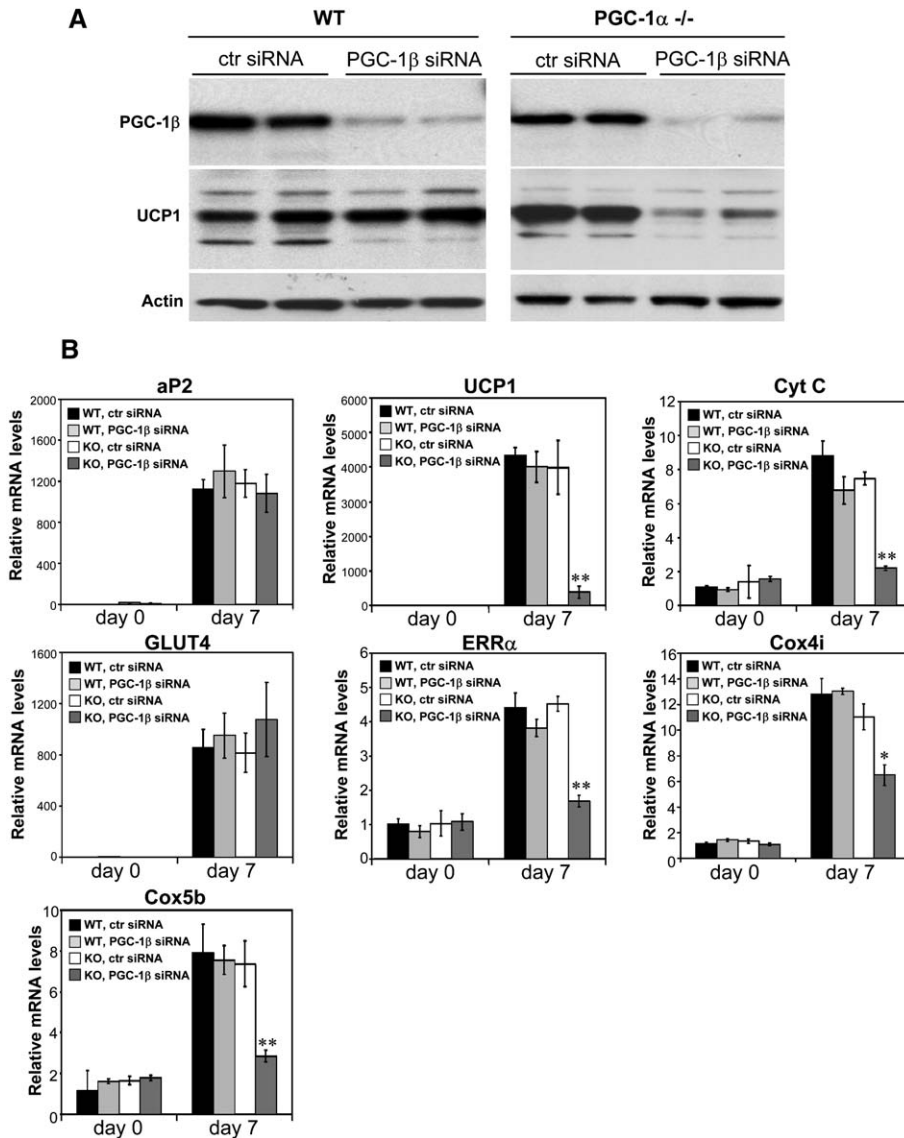


Figure 3. Acquisition of the brown fat phenotype is impaired in cells deficient in both PGC-1 α and PGC-1 β

A) Western blot analysis of PGC-1 β and UCP1 proteins in mature wt and PGC-1 α KO adipocytes (day 7) expressing either an RNAi specific for PGC-1 β knockdown or a control RNAi (ctr RNAi) of random sequence.

B) Real-time PCR analysis of mRNA levels for brown fat-selective genes (UCP1, ERR α), mitochondrial genes (CytC, Cox4i, Cox5b), and adipocytes genes (AP2, GLUT4) in wt and PGC-1 α KO cells expressing an RNAi specific for PGC-1 β knockdown or a control RNAi. Expression is shown at day 0 (preadipocytes) and day 7 (fully mature adipocytes) of the differentiation protocol. * $p < 0.05$. ** $p < 0.01$. Data represent mean \pm SD of triplicates.

mitochondrial biogenesis and cellular respiration. In order to investigate the importance of PGC-1 α and β in the high respiration capacity of brown fat cells, we measured the total and uncoupled respiration rate of fully differentiated wt cells and equivalent cells deficient for PGC-1 α and/or PGC-1 β .

As shown in Figure 5, the highest respiration rate was seen for wt cells, and 52% of this oxygen consumption was uncoupled. In either PGC-1 α ^{-/-} cells or PGC-1 β -deficient cells, the respiration rate was reduced by about 20%, compared to wt cells. The uncoupled respiration rate was 56% of total respiration for those two latter cell lines. Importantly, a drastic decrease of oxygen consumption was observed in cells with defective expression of both PGC-1 α and β , where the total respiration rate was decreased by about 57% compared to wt cells. Furthermore, the fraction of uncoupled respiration in those cells was 44%, a ratio significantly lower than measured in all three other cell lines. Thus, decreased expression of PGC-1 α or PGC-1 β leads to a slightly decreased total respiration rate, while loss of both results in a massive decrease in both total and uncoupled respiration rate. PGC-1 α and β are thus critical for acquisition of

elevated respiration rates, a key characteristic needed for heat production.

We assessed whether brown fat differentiation-induced mitochondrial biogenesis per se was impaired in absence of the PGC-1s, by analyzing mitochondrial density via quantitative transmission electron microscopy (Figure 6). Preadipocytes that were wt or deficient for both PGC-1 α and β showed no significant difference in their mitochondrial volume density. As expected, there was a great increase (2.4-fold) in mitochondria content after differentiation of wt cells compared to preadipose cells. In striking contrast, adipocytes deficient for both PGC-1 α and β expression showed no increase in mitochondrial volume density. Deficiency of either PGC-1 α or PGC-1 β led only to a small but significant decreased mitochondrial volume density in fully mature adipocyte. The expression of mitochondrial-selective genes is in accord with these analyses (Figure S6).

These data demonstrate that while differentiation-linked mitochondrial biogenesis can occur without PGC-1 α or PGC-1 β , there is an absolute requirement for at least one of these proteins to increase mitochondrial density.

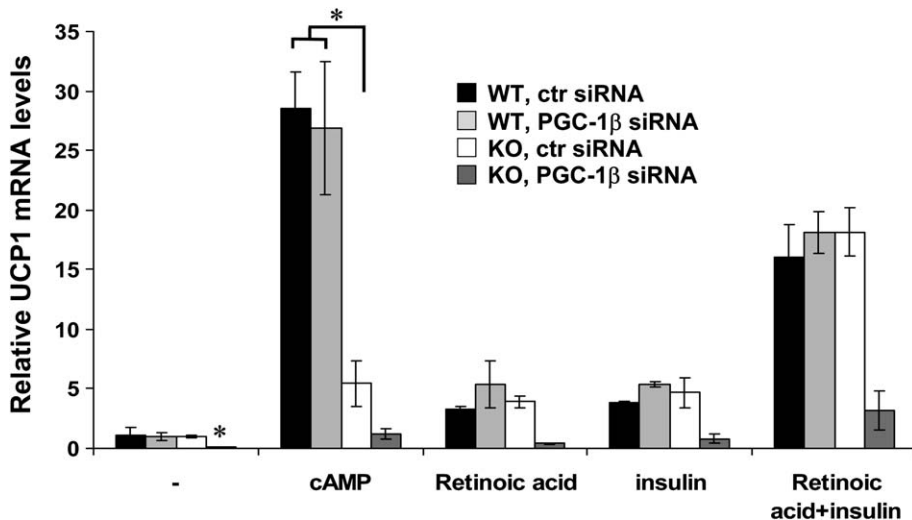


Figure 4. PGC-1 α and PGC-1 β are not involved in UCP1 induction by retinoic acid and insulin

Real-time PCR analysis of mRNA levels for UCP1 in wt and PGC-1 α KO mature adipocytes (day 7) expressing either an RNAi specific for PGC-1 β knock-down or a control RNAi and treated with dbcAMP, retinoic acid, or insulin for 4 hr. * $p < 0.01$. Data represent mean \pm SD of triplicates.

Discussion

Mitochondrial number and mitochondrial-based respiration is highly adaptive in most eukaryotes, responding to environmental conditions such as diet, physical activity, and temperature. The PGC-1 transcriptional coactivators appear to be major regulators of many aspects of mitochondrial biology (Lin et al., 2005a). These proteins activate mitochondrial biogenesis and the genes of mitochondrial function that are encoded in both the mitochondrial and nuclear genomes. They do this by docking on and coactivating transcription factors such as ERR α (Huss et al., 2002; Mootha et al., 2004) and nuclear respiratory factors (NRF) 1 and 2 (Kelly and Scarpulla, 2004; Puigserver and Spiegelman, 2003; Wu et al., 1999a). Coactivation of the NRFs, in particular, regulates expression of TFAM (also known as mTFA), which is encoded in the nuclear genome but enters the mitochondria and stimulates replication and transcription of the mitochondrial genome (Clayton, 1991).

Genetic ablation of PGC-1 α causes quite profound disturbances in several pathways of energy homeostasis, and functional abnormalities in multiple tissues such as liver, brown fat, brain, and heart (Lin et al., 2004). Because mitochondrial biology is absolutely central to the major physiological function of brown fat, thermogenesis, this tissue is particularly appropriate for detailed mechanistic studies of the PGC-1 coactivators. These cellular studies are essential because the phenotype of the KO mice is very complicated. For example, there is extensive control of brown fat function by CNS circuits, and the apparent CNS defects in the KO mice made it impossible to determine whether the poor thermogenic function of the brown fat was due to defective sympathetic nervous system activation of the brown fat, cell-autonomous problems with the differentiation of brown fat cells, or both. Because mitochondrial biogenesis is such a crucial part of the brown fat cell phenotype, this cell is also an excellent model to explore the requirements for both PGC-1 α and β in mitochondrial biogenesis.

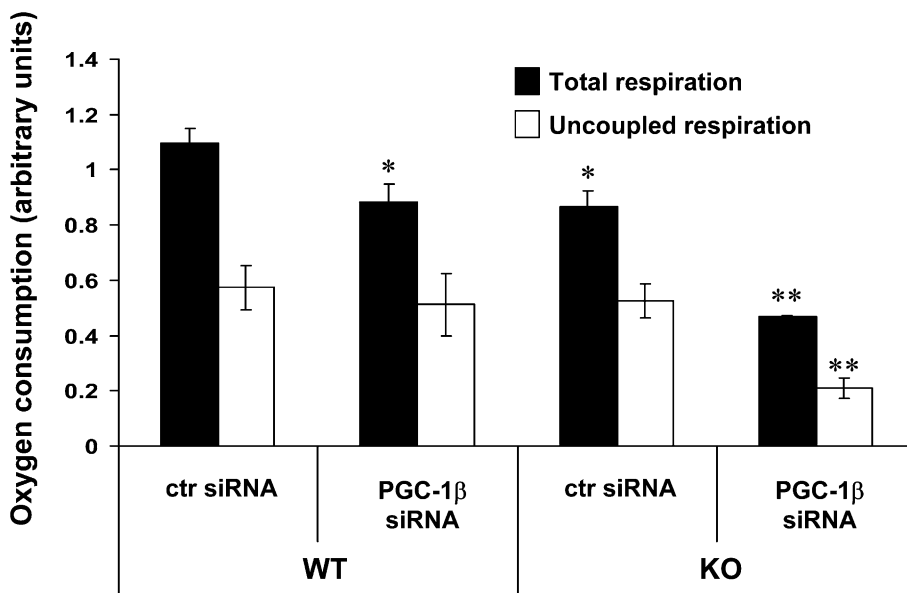


Figure 5. Respiration is severely decreased in cells deficient in both PGC-1 α and β

Total mitochondrial oxygen consumption and uncoupled respiration were measured in basal condition in mature wt and KO brown adipocytes (day 7) expressing either an RNAi that knocks down PGC-1 β levels or a control RNAi. * $p < 0.05$. ** $p < 0.01$. Data represent mean \pm SD of three independent experiments.

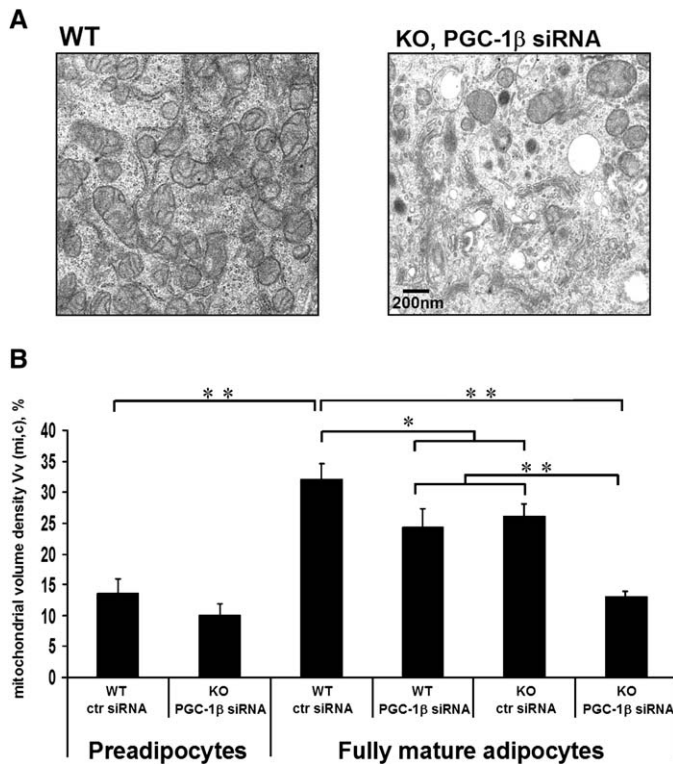


Figure 6. Requirement for PGC-1 α or PGC-1 β for mitochondrial biogenesis during brown fat differentiation

A) Representative electron microscopy micrographs from wt mature adipocytes expressing a control RNAi or PGC-1 α KO mature adipocytes expressing an RNAi that knocks down PGC-1 β levels.

B) Comparison of mitochondrial volume densities between preadipocytes and mature brown adipocytes of wt or PGC-1 α ^{-/-} genotype, expressing either an RNAi that specifically knocks down PGC-1 β expression or a control RNAi. **p* < 0.05. ***p* < 0.01. Data represent mean \pm SD of quantification of ten micrographs.

The data presented here, using brown fat cells lacking PGC-1 α , clarify several of these issues. The adipogenic differentiation of brown fat cells *does not* require PGC-1 α ; these cells express many RNA markers of brown fat differentiation quite normally, including UCP1, PGC-1 β , CIDEA, ERR α , and type 2 deiodinase. However, in the absence of PGC-1 α , there are huge defects in the ability to activate the program of gene expression linked to thermogenesis, upon stimulation of the cyclic AMP pathway, the major pathway through which brown fat heat production is turned on. The cAMP stimulation of UCP1, type 2 deiodinase, C/EBP β , and cytochrome C are all severely blunted in cells mutant in the PGC-1 α gene. Consistent with these expression studies, both total respiration and uncoupled respiration induced by cAMP are blunted in PGC-1 α KO cells compared to controls. Interestingly, the expression of UCP1 can still be induced in cells lacking PGC-1 α by insulin and retinoic acid. While the physiological significance of these pathways is not clear, these data do indicate that the UCP1 gene can be induced without the involvement of PGC-1 α .

The PGC-1 α KO cells also allowed us to do a more global analysis of the role of this coactivator in cAMP-induced gene expression. Figure S3 illustrates that PGC-1 α is involved in a substantial portion (32%) of cAMP-induced gene expression, but many genes controlled by cAMP in the brown fat cells are expressed quite normally without this protein. However, expres-

sion of those genes specifically involved in thermogenesis is defective. It is thus very likely that the morphological changes in brown fat of PGC-1 α KO mice, which display abnormally large lipid droplets, are due to decreased lipid oxidation and are not due to a defect in brown fat development.

Both PGC-1 α and PGC-1 β can stimulate mitochondrial biogenesis in gain-of-function experiments. While some quantitative defects in mitochondrial gene expression are obvious in the PGC-1 α KO mice, these defects in most tissues are in the range of approximately 20%. Whether this reflects the full extent of the involvement of this protein in mitochondrial gene expression and biogenesis, or whether the absence of PGC-1 α can be compensated for by the presence of PGC-1 β or other proteins has not been previously analyzed. By combining the cells genetically deficient in PGC-1 α with an effective RNAi directed against PGC-1 β RNA, we show here that there is a very strong requirement for either PGC-1 α or β to maintain mitochondrial gene expression, mitochondrial density, and respiration. Mature brown adipocytes with no PGC-1 α and 10% of normal PGC-1 β protein have only 35% of the mitochondrial density of control cells, which corresponds to the mitochondrial density of preadipocytes. These data show unambiguously that the PGC-1 coactivators are essential for mitochondrial biogenesis linked to brown fat cell differentiation.

In addition, cells deficient in both PGC-1 α and β expression showed significantly lower induction of the brown fat-selective genes UCP1 and ERR α during differentiation, whereas lipid accumulation and expression of aP2 and GLUT4 were not affected. These results clearly show that neither PGC-1 α nor β are necessary for adipogenic differentiation; however, the presence of at least one of these is essential for development of the brown fat phenotype.

The data presented here also have implications for manipulation of PGC-1 coactivator levels as a target for the development of a novel class of therapeutics. Agents that elevate levels of either PGC-1 can be expected to increase mitochondrial gene expression, mitochondrial biogenesis, and probably mitochondrial-based respiration. This could conceivably be valuable in diseases associated with mitochondrial abnormalities such as type 2 diabetes or neurodegenerative conditions like Parkinson's disease or Huntington's disease. On the other hand, diminution of mitochondrial activity via inhibition of the expression or activity of either PGC-1 separately may be of limited effectiveness. Simultaneous inhibition of both might be necessary to see major effects on mitochondrial density and respiration. Fortunately, it is an increase in activity of these molecules that is most clearly warranted for therapeutic intervention, at least at this early state of our knowledge of mitochondria and disease.

Experimental procedures

Cell culture

Brown preadipocytes were isolated from newborn wild-type and PGC-1 α KO mice (Lin et al., 2004) by collagenase digestion as described previously (Fasshauer et al., 2001; Klein et al., 2002; Tseng et al., 2004). Preadipocytes were immortalized by infection with the retroviral vector pBabe encoding SV40T antigen and selection with puromycin (2 μ g/ml). Preadipocytes were grown to confluence in culture medium supplemented with 20 nM insulin and 1 nM T3 (differentiation medium) (day 0). Adipocyte differentiation was induced by treating confluent cells for 48 hr in differentiation medium further supplemented with 0.5 mM isobutylmethylxanthine, 0.5 μ M dexamethasone,

and 0.125 mM indomethacin. After this induction period (day 2), the medium was washed and cells were incubated in differentiation medium. Medium was changed every 2 days. Full differentiation was achieved after 7 days. PGC-1 β knockdown cell lines were generated using retroviral-mediated expression of shRNA specific for PGC-1 β (Lin et al., 2005b) and scrambled shRNA as a control. Infected cells were selected with G418 (50 mg/ml).

To induce the thermogenic gene program, fully differentiated brown adipocytes were submitted to the different following treatments: 500 μ M of dibutyryl cAMP (Sigma) for 4 hr and 6 hr, 10 μ M forskolin (Sigma) for 4 hr, 10 μ M isoproterenol (Sigma) for 4 hr, 1 μ M retinoic acid (Sigma) for 4 hr, or 100 nM insulin (Sigma) for 4 hr.

RNA and protein analysis

Total RNA was isolated from cultured brown fat cultured cell lines using QIAshredder and RNeasy kits from Qiagen. For real-time PCR analysis, RNA samples were reverse transcribed using iScript cDNA synthesis kit (Bio-rad) and used in quantitative PCR reactions in the presence of a fluorescent dye (Cybergreen, Bio-rad). Relative abundance of mRNA was calculated after normalization to 18S ribosomal RNA. Sequences for the primers used in this study are shown in Table S2. For microarray analyses, RNA samples were evaluated with Affymetrix 430A chips using the DFCI core facility. All subsequent analyses were performed with dCHIP software.

For the detection of PGC-1 β and UCP1 protein, cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl). Thirty micrograms of lysates were analyzed by immunoblotting using rabbit polyclonal antibodies raised against PGC-1 β , or using a UCP1 polyclonal antibody from Sigma. Immunoblots were hybridized with mouse monoclonal actin antibody as a loading control (Santa Cruz Biotechnology).

Oxygen consumption assays

Cells were isolated by washing with PBS and trypsinizing for 5 min, resuspended in DMEM with 10% FBS, and spun twice at 1000 rpm for 5 min. Cells were then resuspended in DPBS supplemented with 25 mM glucose, 1 mM pyruvate, and 2% BSA. Total and uncoupled respiration were measured as published (Mootha et al., 2004; St-Pierre et al., 2003). For normalization of the respiration rates, an aliquot of the resuspended cells was lysed and protein concentration was measured by Bradford assay. For cAMP-induced respiration assays, fully differentiated brown fat cells were incubated for 24 hr with 1 mM of dibutyryl cyclic AMP prior to oxygen consumption assay.

Electronic microscopy

Cellular samples were fixed for 1 hr in a mixture of 2.5% glutaraldehyde, 1.25% paraformaldehyde, and 0.03% picric acid in 0.1 M sodium cacodylate buffer (pH 7.4), washed in 0.1 M cacodylate buffer, postfixed with 1% osmium tetroxide/1.5% potassium ferrocyanide for 1 hr, washed in water, and stained in 1% aqueous uranyl acetate for 30 min followed by dehydration in grades of alcohol (5 min at 70%, 5 min at 90%, and 2 \times 5 min at 100%). The samples were then infiltrated and embedded in TAAB Epon (Marivac Canada Inc., St. Laurent, Canada). Ultrathin sections (about 60 nm) were cut on a Reichert Ultracut-S microtome, picked up on to copper grids stained with uranylacetate and lead citrate, and examined in a JEOL 1200EX. To calculate mitochondrial volume density, a grid was laid on randomly selected micrographs, and the number of points falling onto mitochondria was expressed as a fraction of those falling onto the cell area, as described previously (St-Pierre et al., 2003).

Retroviral basal expression of PGC-1 β RNAi

The RNAi construct for PGC-1 β was generated using one sequence in the coding region of PGC-1 β : 5'-GTACGGAACTGCATAAGCA-3' as described (Lin et al., 2005b). Oligonucleotides containing this sequence or random sequence were subcloned into the retroviral vector pSUPER-retro neomycin (Brummelkamp et al., 2002). For retrovirus production, Φ NX packaging cells (Force et al., 1997) were transfected at 70% confluence by calcium phosphate coprecipitation with 10 μ g of retroviral vectors, and viral supernatants were harvested 48 hr after transfection. Preadipocytes were infected at 60% confluence with polybrene (4 μ g/ml)-supplemented virus overnight. Selection with 1 mg/ml G418 (Sigma) was started 48 hr after infection.

Supplemental data

Supplemental data include six figures and two tables and can be found with this article online at <http://www.cellmetabolism.org/cgi/content/full/3/5/333/DC1/>.

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References

- Alvarez, R., de Andres, J., Yubero, P., Vinas, O., Mampel, T., Iglesias, R., Giralt, M., and Villarroya, F. (1995). A novel regulatory pathway of brown fat thermogenesis. Retinoic acid is a transcriptional activator of the mitochondrial uncoupling protein gene. *J. Biol. Chem.* 270, 5666–5673.
- Assimacopoulos-Jeannet, F., Cusin, I., Greco-Perotto, R.M., Terretaz, J., Rohner-Jeanrenaud, F., Zarjevski, N., and Jeanrenaud, B. (1991). Glucose transporters: structure, function, and regulation. *Biochimie* 73, 67–70.
- Bouillaud, F., Ricquier, D., Thibault, J., and Weissenbach, J. (1985). Molecular approach to thermogenesis in brown adipose tissue: cDNA cloning of the mitochondrial uncoupling protein. *Proc. Natl. Acad. Sci. USA* 82, 445–448.
- Brummelkamp, T.R., Bernards, R., and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296, 550–553.
- Butow, R.A., and Bahassi, E.M. (1999). Adaptive thermogenesis: orchestrating mitochondrial biogenesis. *Curr. Biol.* 9, R767–R769.
- Cannon, B., and Nedergaard, J. (2004). Brown adipose tissue: function and physiological significance. *Physiol. Rev.* 84, 277–359.
- Clayton, D.A. (1991). Replication and transcription of vertebrate mitochondrial DNA. *Annu. Rev. Cell Biol.* 7, 453–478.
- Fasshauer, M., Klein, J., Kriauciunas, K.M., Ueki, K., Benito, M., and Kahn, C.R. (2001). Essential role of insulin receptor substrate 1 in differentiation of brown adipocytes. *Mol. Cell Biol.* 21, 319–329.
- Force, W.R., Cheung, T.C., and Ware, C.F. (1997). Dominant negative mutants of TRAF3 reveal an important role for the coiled coil domains in cell death signaling by the lymphotoxin-beta receptor. *J. Biol. Chem.* 272, 30835–30840.
- Himms-Hagen, J. (1990). Brown adipose tissue thermogenesis: interdisciplinary studies. *FASEB J.* 4, 2890–2898.
- Huss, J.M., Kopp, R.P., and Kelly, D.P. (2002). Peroxisome proliferator-activated receptor coactivator-1alpha (PGC-1alpha) coactivates the cardiac-enriched nuclear receptors estrogen-related receptor-alpha and -gamma. Identification of novel leucine-rich interaction motif within PGC-1alpha. *J. Biol. Chem.* 277, 40265–40274.
- Kelly, D.P., and Scarpulla, R.C. (2004). Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. *Genes Dev.* 18, 357–368.
- Klein, J., Fasshauer, M., Klein, H.H., Benito, M., and Kahn, C.R. (2002). Novel adipocyte lines from brown fat: a model system for the study of differentiation, energy metabolism, and insulin action. *Bioessays* 24, 382–388.

- Klingenberg, M., and Huang, S.G. (1999). Structure and function of the uncoupling protein from brown adipose tissue. *Biochim. Biophys. Acta* *1415*, 271–296.
- Kopecky, J., Clarke, G., Enerback, S., Spiegelman, B., and Kozak, L.P. (1995). Expression of the mitochondrial uncoupling protein gene from the aP2 gene promoter prevents genetic obesity. *J. Clin. Invest.* *96*, 2914–2923.
- Leone, T.C., Lehman, J.J., Finck, B.N., Schaeffer, P.J., Wende, A.R., Boudina, S., Courtois, M., Wozniak, D.F., Sambandam, N., Bernal-Mizrachi, C., et al. (2005). PGC-1alpha deficiency causes multi-system energy metabolic derangements: muscle dysfunction, abnormal weight control and hepatic steatosis. *PLoS Biol.* *3*, e101. 10.1371/journal.pbio.0030101.
- Lin, J., Handschin, C., and Spiegelman, B.M. (2005a). Metabolic control through the PGC-1 family of transcription coactivators. *Cell Metab.* *1*, 361–370.
- Lin, J., Puigserver, P., Donovan, J., Tarr, P., and Spiegelman, B.M. (2002). Peroxisome proliferator-activated receptor gamma coactivator 1beta (PGC-1beta), a novel PGC-1-related transcription coactivator associated with host cell factor. *J. Biol. Chem.* *277*, 1645–1648.
- Lin, J., Tarr, P.T., Yang, R., Rhee, J., Puigserver, P., Newgard, C.B., and Spiegelman, B.M. (2003). PGC-1beta in the regulation of hepatic glucose and energy metabolism. *J. Biol. Chem.* *278*, 30843–30848.
- Lin, J., Wu, P.H., Tarr, P.T., Lindenberg, K.S., St-Pierre, J., Zhang, C.Y., Mootha, V.K., Jager, S., Vianna, C.R., Reznick, R.M., et al. (2004). Defects in adaptive energy metabolism with CNS-linked hyperactivity in PGC-1alpha null mice. *Cell* *119*, 121–135.
- Lin, J., Yang, R., Tarr, P.T., Wu, P.H., Handschin, C., Li, S., Yang, W., Pei, L., Uldry, M., Tontonoz, P., et al. (2005b). Hyperlipidemic effects of dietary saturated fats mediated through PGC-1beta coactivation of SREBP. *Cell* *120*, 261–273.
- Lindberg, O., de Pierre, J., Rylander, E., and Afzelius, B.A. (1967). Studies of the mitochondrial energy-transfer system of brown adipose tissue. *J. Cell Biol.* *34*, 293–310.
- Lowell, B.B., and Spiegelman, B.M. (2000). Towards a molecular understanding of adaptive thermogenesis. *Nature* *404*, 652–660.
- Mootha, V.K., Handschin, C., Arlow, D., Xie, X., St Pierre, J., Sihag, S., Yang, W., Altshuler, D., Puigserver, P., Patterson, N., et al. (2004). Erralpha and Gabpa/b specify PGC-1alpha-dependent oxidative phosphorylation gene expression that is altered in diabetic muscle. *Proc. Natl. Acad. Sci. USA* *101*, 6570–6575.
- Puigserver, P., and Spiegelman, B.M. (2003). Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. *Endocr. Rev.* *24*, 78–90.
- Puigserver, P., Vazquez, F., Bonet, M.L., Pico, C., and Palou, A. (1996). In vitro and in vivo induction of brown adipocyte uncoupling protein (thermogenin) by retinoic acid. *Biochem. J.* *317*, 827–833.
- Puigserver, P., Wu, Z., Park, C.W., Graves, R., Wright, M., and Spiegelman, B.M. (1998). A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* *92*, 829–839.
- Rosen, E.D., Hsu, C.H., Wang, X., Sakai, S., Freeman, M.W., Gonzalez, F.J., and Spiegelman, B.M. (2002). C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. *Genes Dev.* *16*, 22–26.
- Rosen, E.D., Sarraf, P., Troy, A.E., Bradwin, G., Moore, K., Milstone, D.S., Spiegelman, B.M., and Mortensen, R.M. (1999). PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. *Mol. Cell* *4*, 611–617.
- Rosen, E.D., and Spiegelman, B.M. (2000). Molecular regulation of adipogenesis. *Annu. Rev. Cell Dev. Biol.* *16*, 145–171.
- St-Pierre, J., Lin, J., Krauss, S., Tarr, P.T., Yang, R., Newgard, C.B., and Spiegelman, B.M. (2003). Bioenergetic analysis of peroxisome proliferator-activated receptor gamma coactivators 1alpha and 1beta (PGC-1alpha and PGC-1beta) in muscle cells. *J. Biol. Chem.* *278*, 26597–26603.
- Teruel, T., Valverde, A.M., Navarro, P., Benito, M., and Lorenzo, M. (1998). Inhibition of PI 3-kinase and RAS blocks IGF-I and insulin-induced uncoupling protein 1 gene expression in brown adipocytes. *J. Cell. Physiol.* *176*, 99–109.
- Tseng, Y.H., Kriacunas, K.M., Kokkotou, E., and Kahn, C.R. (2004). Differential roles of insulin receptor substrates in brown adipocyte differentiation. *Mol. Cell. Biol.* *24*, 1918–1929.
- Valverde, A.M., Arribas, M., Mur, C., Navarro, P., Pons, S., Cassard-Doulcier, A.M., Kahn, C.R., and Benito, M. (2003). Insulin-induced up-regulated uncoupling protein-1 expression is mediated by insulin receptor substrate 1 through the phosphatidylinositol 3-kinase/Akt signaling pathway in fetal brown adipocytes. *J. Biol. Chem.* *278*, 10221–10231.
- Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R.C., and Spiegelman, B.M. (1999a). Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* *98*, 115–124.
- Wu, Z., Rosen, E.D., Brun, R., Hauser, S., Adelmant, G., Troy, A.E., McKeon, C., Darlington, G.J., and Spiegelman, B.M. (1999b). Cross-regulation of C/EBP alpha and PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity. *Mol. Cell* *3*, 151–158.
- Zhou, Z., Yon Toh, S., Chen, Z., Guo, K., Ng, C.P., Ponniah, S., Lin, S.C., Hong, W., and Li, P. (2003). Cidea-deficient mice have lean phenotype and are resistant to obesity. *Nat. Genet.* *35*, 49–56.