Functional dissection of the C-terminal part of the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator alpha (PGC-1α)

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ABSTRACT

The superfamily of nuclear receptors is a class of transcriptional regulators that includes the receptors for steroid hormones, thyroid hormones, retinoids and vitamin D. The superfamily also includes so-called orphan receptors, for which an activating ligand is unknown or not required. They regulate diverse biological processes, such as homeostasis, reproduction, development, and metabolism. To exert their functions in the activation of transcription, they need to recruit so-called coactivator protein complexes, many of which remodel the chromatin structure in promoter regions and help to recruit the basal RNA polymerase II transcription machinery.

We and others identified the transcriptional coactivator PGC-1α. Its expression is induced by physiological signals such as cold, fasting, and exercise, and it turned out to be a central regulator of cellular energy homeostasis. The N-terminal part of PGC-1α harbors a strong transcriptional activation function and a nuclear receptor interaction domain. The C-terminal half of PGC-1α harbors several interesting motifs, including two serine/arginine-rich sequences, a putative RNA-binding domain, and an amino acid stretch containing a high percentage of glutamate residues.

In this work, we describe the identification of proteins interacting with the C-terminal part of PGC-1α and the functional interaction of two of the identified proteins with PGC-1α. We show that the acetyltransferase Tip60 has both positive and negative effects on PGC-1α-dependent transcription. In collaboration, we also show that the methyltransferase PRMT1 methylates PGC-1α and thereby enhances the activity of PGC-1α as a transcriptional coactivator.

CHAPTER I: INTRODUCTION

Transcription

In eukaryotes, transcription of genes is carried out by three different RNA polymerases. While RNA polymerase I is responsible for the production of a transcript that is processed to the 25S, 18S, and 5.8S RNA components of the ribosome (Grummt, 2003), RNA polymerase III transcribes the genes encoding the transfer RNAs, the 5S ribosomal RNA, and RNA components of the splicing apparatus (Geiduschek and Kassavetis, 2001; Huang and Maraia, 2001; Schramm and Hernandez, 2002). RNA polymerase II transcribes the protein-coding genes and some small nuclear RNAs (snRNAs) as well.

Eukaryotic RNA-polymerase II is a large multi-subunit enzyme, which comprises of at least 12 distinct subunits and possessing a molecular mass of 500 kilodaltons (Cramer, 2004; Dvir et al., 2001; Hahn, 2004). Transcription, like many other biological processes, can be divided into distinct steps, such as pre-initiation complex formation, open complex formation, initiation, promoter clearance, transcript elongation, and termination. Studies with purified proteins have revealed that transcription initiation requires, besides the RNA polymerase II, a minimum of five general initiation factors TFIIB, TFIID, TFIIE, TFIIF, and TFIIH. Transcription by RNA polymerase II begins with the assembly of the polymerase and the five general transcription factors at the promoter, followed by the ATP-dependent formation of the open complex and the subsequent transcription initiation. In the first step, the TATA-binding protein-containing TFIID complex binds a TA-rich sequence at the promoter and creates a recognition site for the RNA-polymerase II on the DNA. The other four TFIIs are then incorporated into the preinitiation complex in a defined order. Following formation of the fully assembled preinitiation complex, a DNA helicase activity, associated with TFIIH, catalyses the ATP-dependent unwinding of the DNA template at the transcriptional start site and forms the open complex. This is followed by the initiation and synthesis of the RNA transcript by RNA-polymerase II (Dvir et al., 2001; Hahn, 2004). Each protein-coding gene has a unique, specific program of transcriptional control. Much of this specificity of these programs is determined by sequence specific DNA-binding proteins that bind to the proximal promoter and distal transcriptional regulatory sequences. Hence, DNA-specific transcriptional regulators, also

called transcription factors, interpret and transmit the information encoded in the primary DNA sequence of genes to the RNA polymerase transcriptional machinery (Kadonaga, 2004).

The nuclear receptor superfamily of transcription factors

Nuclear receptors form a family of phylogenetically related proteins, encoded by 21 genes in the genome of the fly Drosophila melanogaster, 48 in humans, 49 in the mouse, and unexpectedly, more than 270 in the worm Caenorhabditis elegans (Robinson-Rechavi et al., 2003). Nuclear receptors are transcription factors that include the receptors for steroid hormones, thyroid hormones, retinoids and vitamin D . The superfamily also includes so-called orphan receptors for which an activating ligand is unknown or not required (Beato et al., 1995; Mangelsdorf and Evans, 1995; Mangelsdorf et al., 1995; Robinson-Rechavi et al., 2003). They regulate diverse biological processes, such as homeostasis, reproduction, development and metabolism. Nuclear receptors share a common structural organization (Mangelsdorf et al., 1995; Robinson-Rechavi et al., 2003; Warnmark et al., 2003). They contain a conserved DNA-binding domain in the central part of the protein, which allows them to bind specific DNA elements in the regulatory regions of target genes and which is also involved in the dimerization of nuclear receptors. This dimerization includes homodimers as well as heterodimers. Most nuclear receptors contain two transcriptional activation domains, AF-1 and AF-2, through which activation of transcription is achieved. The AF-1 generally resides in the N-terminal region, while AF-2 is localized in the C-terminal ligand binding domain (LBD). The LBD is structurally conserved between nuclear receptors and is formed by 11 to 12 α-helices, which are arranged together in an anti-parallel, three-layered sandwich with two to four β-strands included (Wurtz et al., 1996). All crystal structures of LBDs bound to agonists show that the ligand binds to a hydrophobic cavity buried within the core of the LBD (Bourguet et al., 2000). The crystal structures reveal that in the unliganded form the most C-terminal helix projects away from the core structure, whereas in the agonist-bound structures this helix is folded up against the core, creating a lid over the ligand-binding pocket.

How do activated nuclear receptors or other sequence-specific transcription factors activate transcription? In order to achieve activation of target genes, nuclear receptors recruit

transcriptional coactivators via protein-protein interactions (Kadonaga, 2004; Ptashne and Gann, 1997), which then act directly or indirectly to recruit and regulate the RNA polymerase II transcriptional machinery at the core promoter.

Coactivator proteins in most cases interact with the AF-1 or with the AF-2. Almost all coactivators that have been identified as AF-2 interacting proteins contain one or several conserved leucine-rich motifs with the consensus sequence LXXLL and display agonist-dependent interaction with nuclear receptors. The LXXLL motifs, also called NR boxes, have been demonstrated to be necessary and sufficient for the interaction between the receptor and the coactivator (Heery et al., 1997). In structures of nuclear receptors bound to peptides derived from coactivators, the LXXLL-motifs are part of an α-helix that binds to the hydrophobic AF-2 groove in the agonist-bound LBD (Darimont et al., 1998; Nolte et al., 1998; Shiau et al., 1998). The next section describes the structures and mechanisms of some transcriptional coactivators.

Nuclear receptor coactivators

Over the past decade, a large number of transcriptional coactivators has been identified and characterized, which act at different levels to achieve activation of gene expression. Some coactivators act as bridging factors between DNA-bound NRs and the basal transcription machinery and thereby stabilize the RNA polymerase II complex on gene promoters. Another class of coactivators are involved in the reorganization of chromatin. The chromatin structure of genes contributes significantly to the control of transcription. The basic unit of chromatin is the nucleosome, which consists of an octamer of histone proteins, around which the DNA is wrapped (Luger et al., 1997). The nucleosomes form an array that is ordered into a higher-order chromatin structure. The nucleosome structure and the histone-DNA interactions make the DNA of genes and their promoter regions difficult to access for the basal transcription machinery and thereby negatively regulate the transcription of genes. In a process, termed chromatin remodeling, the repressive chromatin structure is altered in a way that will allow the accessibility of the transcriptional machinery to gene promoters (Sif, 2004). ATP-dependent chromatin-remodeling complexes catalyze the mobilization or repositioning of nucleosomes or the alteration of histone-DNA contacts (Lusser and Kadonaga, 2003). Besides the chromatin-remodeling

complexes, many coactivators have been identified that catalyze post-translational modifications of histones. Such posttranslational modifications, including acetylation, methylation, phosphorylation, ubiquitination, and ADP-ribosylation control the association of histone binding proteins with chromatin, which can directly or indirectly lead to alterations in the chromatin structure or to the recruitment of the transcriptional machinery (Jenuwein and Allis, 2001; Shiio and Eisenman, 2003; Zhang, 2003).

1. ATP-dependent chromatin remodeling complexes

As mentioned above, chromatin structure represents a obstacle for DNA-binding proteins involved in transcriptional regulation or other processes like DNA repair and homologous recombination. It is therefore necessary to rearrange or mobilize the nucleosomes. This remodeling of the nucleosomes is achieved by the action of chromatin-remodeling complexes, which are a family of ATP-dependent molecular machines (Lusser and Kadonaga, 2003; Sif, 2004). Several such complexes have been identified in yeast, Drosophila, and human cells. Although the chromatin remodeling complexes differ in their composition and their biological function, they share the presence of a motor subunit that belongs to the Snf2-like family of ATPases. These ATPases are further subdivided, based on the presence of domains other than the ATPase domain. Members of the SNF2 subfamily are the ATPases in the yeast SWI/SNF chromatin remodeling complex and in its human and drosophila homologs (Swi2p/Snf2, hBRM, hBRG, dBRM). These complexes consist of 9 to 14 subunits. They have well established roles in the activation of transcription and are recruited to chromatin via DNA-binding transcription factors, which are able to bind to regulatory sequences in chromatinized DNA. Changes in the chromatin structure caused by the action of the chromatin remodeling complexes can then facilitate the binding of other transcription factors and the general transcription machinery.

2. Histone-modifying coactivators

Several coactivators contain enzymatic activities that allow the coactivators to modify histone proteins and therefore to change the chromatin structure, which then allows easier binding of the basal transcription machinery to DNA. It was observed that the levels of transcription of a specific gene were in relationship to the acetylation status of the histones in that region with hyperacetylated regions being more actively transcribed than

hypoacetylated regions. This idea that the acetylation status of histones has an influence on the transcription of a specific gene was supported by the identification of mammalian transcriptional coactivators that posses intrinsic histone acetyltransferase (HAT) activity. The main targets of the HATs are the N-terminal tails of the core histones H2A, H2B, H3, and H4, which protrude away from the DNA. Besides acetylation, other post-translational modifications of histone proteins have an influence on the chromatin structure and thereby contribute to the regulation of gene expression. Various histone modifying proteins that can act as transcriptional coactivators have been identified and are described here briefly.

CBP/p300

CBP and p300 are two closely related proteins, which can be found in many multicellular organisms including humans, rodents, flies, worms, and plants, but not in lower eukaryotes such as yeast. CBP and p300 can interact with the basal transcription factor TFIID and can form a complex with RNA-polymerase II. In addition, they bind to a number of DNA sequence-specific transcription factors. By interacting simultaneously with the basal transcription machinery and one ore more upstream transcription factors, CBP and p300 are believed to function as physical bridges or scaffolds and thereby stabilize the transcription complex. Moreover, CBP and p300 acetylate histones (Bannister and Kouzarides, 1996; Ogryzko et al., 1996) and other proteins involved in the regulation of transcription, such as transcription factors and coactivators, suggesting that CBP and p300 regulate transcription at multiple levels (Chen et al., 1999b; Gu and Roeder, 1997). Acetylation of non-histone substrates can have either positive or negative effect on transcription. For example, CBP negatively regulates the activity of the transcriptional coactivator ACTR by acetylating its NR-box motif and thereby weakening its interaction with nuclear receptors (Chen et al., 1999b).

GNAT-superfamily

The GNAT superfamily of acetyltransferases (Gcn5-related N-acetyltransferases) includes the mammalian coactivator proteins GCN5 and p/CAF (p300/CBP associated factor) and other proteins found in prokaryotes, arabidopsis, yeast, drosophila, and mammals (Vetting et al., 2005). Both human GCN5 and p/CAF have been found to be subunits of large protein complexes that are composed of more than 20 polypeptides (Ogryzko et al., 1998).

The GCN5 and the p/CAF complexes seem to be virtually identical, except for the acetyltransferase subunit. The SAGA complex is the yeast homologue of the mammalian GCN5 and p/CAF complexes (Grant et al., 1997). The presence of similar complexes in mammals and yeast suggests a strong evolutionary conservation of this type of acetyltransferase machineries. GCN5 and p/CAF are able to acetylate histone H3 and H4, suggesting a role for these two proteins in chromatin remodeling. The GCN5, p/CAF and SAGA complexes may also act as adapters or scaffolds, since single subunits have been shown to interact with components of the general transcription machinery (Sterner et al., 1999).

MYST-Family

Another group of evolutionarily conserved proteins is the MYST-family of acetyltransferases. This family of proteins is named after their founding members MOZ (Borrow et al., 1996), Ybf2/Sas3, Sas2 (Reifsnyder et al., 1996), and Tip60 (Kamine et al., 1996). Additional members have been identified, including the yeast Esa1 (Smith et al., 1998), the Drosophila MOF (Hilfiker et al., 1997), and the human MOF (Neal et al., 2000), HBO1 (Iizuka and Stillman, 1999), and MORF (Champagne et al., 1999). The MYST-domain, which contains the acetyltransferase activity, is highly conserved between members of this protein family. Diverse functions have been assigned to the different members of the MYST-family. Esa1p, an essential MYST-family protein from yeast, has been implicated in cell cycle progression (Clarke et al., 1999), DNA repair (Bird et al., 2002) and transcription regulation (Allard et al., 1999). The yeast proteins Sas2p and Sas3p have been shown to play a role in gene silencing (Reifsnyder et al., 1996), while dMOF was found to act in the dosage compensation of the X-chromosome (Hilfiker et al., 1997). The first human MYST protein that has been discovered and one of the best studied MYST-family members is Tip60 (Tat-interactive protein, 60 kDa) (Fig. 1.1). The MYST-domain of Tip60, which contains the acetyl-CoA-binding site, is located in the C-terminal half of the protein. The N-terminal region of Tip60 contains a so-called CHROMO-domain. Tip60 was identified as an interacting protein and coactivator of the HIV-Tat transactivator protein, hence its name (Kamine et al., 1996). Isolation of Tip60 cDNA in other studies identified a shorter form of Tip60 that misses exon 5 coding for 52 amino acids (Ran and Pereira-Smith, 2000; Sheridan et al., 2001). The longer, originally cloned variant was renamed Tip60α, while the shorter splice form is now know as Tip60β. Due to

its isolation as a interacting protein of cytosolic phospholipase A_2 ($cPLA_2$), Tip60 is also known as PLIP (cPLA-interacting protein) (Sheridan et al., 2001). A third splice form of Tip60 has been identified, whose hypethetical translation product contains a 33 amino acid insertion 4 amino acids after the first ATG codon (Legube and Trouche, 2003). No specific functions could be assigned to these additional 33 amino acids or the 52 amino acids encoded by exon 5. Tip60 is mainly localized in the cell nucleus, but it was detected in the cytosol and even at the plasma membrane as well (Halkidou et al., 2004; Lee et al., 2001; McAllister et al., 2002; Ran and Pereira-Smith, 2000; Sheridan et al., 2001; Sliva et al., 1999). Northern blot and RT-PCR analysis of different mammalian tissues suggest that Tip60 is relatively highly expressed in several tissues, such as testis, brain, heart, liver, kidney and also in all cell lines tested so far (McAllister et al., 2002; Ran and Pereira-Smith, 2000; Sheridan et al., 2001). A Tip60 complex consisting of at least 14 subunits could be biochemically purified from HeLa cells. Besides histone acetylase activity on chromatin, this complex possesses ATPase, DNA helicase, and strucural DNA-binding activities (Ikura et al., 2000). No difference in subunit composition between Tip60α and Tip60β complexes could be found (Doyon et al., 2004). Furthermore, the Tip60 complex has been demonstrated to be the mammalian homologue of the yeast NuA4 complex, which contains the yeast Tip60-homologue Esa1p as acetyltransferase (Doyon et al., 2004). *In vitro* acetylation assays have shown that free histones H2A, H3, and H4 are substrates for the acetyltransferase activty of Tip60 (Kimura and Horikoshi, 1998; Yamamoto and Horikoshi, 1997). In contrast to the Tip60 complex, monomeric Tip60 is not able to acetylate chromatin templates (Ikura et al., 2000). The demonstration that Tip60 is able to acetylate histones, like many coactivator proteins, suggests that Tip60 is involved in the regulation of transcription. A function of Tip60 in transcription was further supported by the finding that Tip60 interacts with and coactivates a number of nuclear receptors, including the androgen receptor (AR) (Brady et al., 1999; Gaughan et al., 2001; Gaughan et al., 2002). Tip60 was also shown to be recruited to the endogenous AR-regulated promoter of the prostate-specific antigen and endogenous RORα-induced genes during cerebellar development (Gold et al., 2003; Halkidou et al., 2003). An LXXLL-motif, which is also found in many other transcriptional coactivators, was identified in the very C-terminal part of Tip60 and has been shown to be essential for the interaction with the AR (Gaughan et al., 2001). Besides being coactivated by Tip60, the AR is also a substrate for the Tip60 acetyltransferase activity (Gaughan et al., 2002). The activity of Tip60 as a

coactivator is not restricted nuclear receptors. It was also shown to enhance the transcriptional activity of NFκB transcription factors via indirect recruitment through Bcl-3 (Dechend et al., 1999). The c-myc protein is another transcription factor that recruits Tip60 and other components of the Tip60 complex to promoters, as shown by chromatin-immunoprecipitation experiments (Frank et al., 2003). In addition, Tip60 was found to form a transcriptionally active trimeric complex together with the C-terminal domain of the amyloid precursor protein (APP) and Fe65 (Cao and Sudhof, 2001). This complex seems to be recruited to the endogenous promoter of the cytokine-induced KAI1 gene upon stimulation of cells with interleukin-1β (Baek et al., 2002). In addition, Tip60 overexpression was shown to enhance the induction of the endogenous p53 target genes p21, GADD45, and Mdm2 after gamma irradiation of the cells. This effect could not be observed, when a acetyltransferase mutant of Tip60 was used. Tip60 not only seems to be an activator of transcription. There is also evidence that Tip60 is a negative regulator of transcription. Activation of CREB by protein kinase A has been shown to be inhibited by Tip60 (Gavaravarapu and Kamine, 2000). This inhibition seems to be mediated by direct interaction of Tip60 with CREB, since Tip60 binds to CREB *in vitro*. The negative effect of Tip60 on CREB-dependent transcription does not require the acetyltransferase activity of Tip60. In another study, Tip60 was found to repress STAT3-mediated transcription by the recruitment of the histone deacetylase HDAC7 (Xiao et al., 2003). Nordentoft and Jorgensen also provided evidence that Tip60 is a transcriptional corepressor. They demonstrated that overexpression of Tip60 enhanced the repressive effect of the transcriptional repressor protein TEL (Nordentoft and Jorgensen, 2003). The present knowledge about Tip60 suggest that this protein is a versatile transcriptional coregulator of different transcription factors. A significant number of reports have proposed an involvement of Tip60 and also the yeast homologue Esa1p in the regulation of apoptosis and DNA-repair. Ectopic expression of a mutated Tip60 lacking acetyltransferase activity resulted in cells with a defective double-strand DNA-break repair and a diminished apoptotic competence after gamma-irradiation (Ikura et al., 2000). In a similar study, overexpression of Tip60 enhanced the apoptosis induced by a intracellular carboxy-terminal fragment of APP, while a mutant Tip60 lacking acetyltransferase activity decreased apoptosis (Kinoshita et al., 2002). Tip60 was also shown to potentiate serum deprivation-induced apoptosis (Sheridan et al., 2001). In mammalian cells, the response to DNA damage is in part controlled by the tumor suppressor p53, which activates the

transcription of genes involved in cell-cycle regulation, apoptosis, and DNA-repair. The involvement of Tip60 in p53 dependent transcription is further supported by the fact that both proteins accumulate after UV-irradiation and are targeted for proteosomal degradation by the ubiquitin-ligase Mdm2 (Legube et al., 2002) and, as mentioned above, overexpression of Tip60 enhances the expression of the p53-regulated p21, GADD45, and Mdm2 (Doyon et al., 2004). Finally, a role in the activation of RNA polI-regulated 45S ribosomal pre-RNA has been assigned toTip60. Tip60 localized to the nucleolus and was associated with the ribosomal pre-RNA promoter after release of cells from serum-starvation (Halkidou et al., 2004).

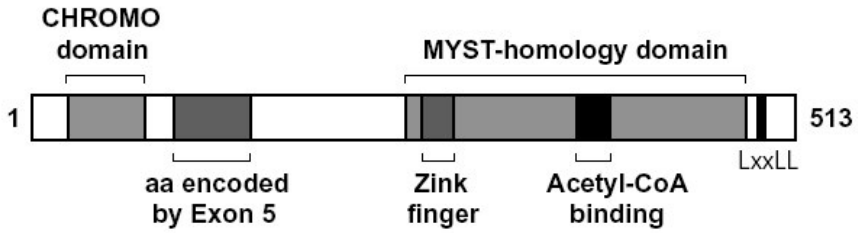

Figure 1.1. Schematic presentation of the structure of Tip60.
Regions and protein motifs identified in Tip60 are highlighted. The protein domain encoded by exon 5 is present in Tip60α but not Tip60β.

p160-coactivators

The p160-family of transcriptional coactivators consists of the proteins SRC-1 (NcoA-1) (Onate et al., 1995), SRC-2 (GRIP1, TIF2, NcoA-2) (Hong et al., 1997; Voegel et al., 1996), and SRC-3 (p/CIP, RAC3, ACTR, TRAM-1, AIB1) (Anzick et al., 1997; Chen et al., 1997; Li et al., 1997; Takeshita et al., 1997; Torchia et al., 1997) . All three members of the p160 family are able to interact with and coactivate many nuclear receptors and other transcription factors. Human and rodent SRC proteins are all approximately 160 kDa in size and have an overall 50-55% sequence similarity and a 43-48% sequence identity (Sterner and Berger, 2000; Xu and Li, 2003). The relatively conserved central region of the SRC members contains three LXXLL motifs that are responsible for the interaction with ligand-bound nuclear receptors. In the C-terminal part of each of the three proteins there

are two independent transcription activation domains that serve as platforms for the recruitment of other coactivators. The first activation domain interacts with the general transcriptional cointegrators CBP, p300, and p/CAF (Chen et al., 1997; Torchia et al., 1997) . The second activation domain lies at the very C terminus. This part of SRC-1 has been shown to mediate the interaction with the methyltransferase CARM1 (Chen et al., 1999a). The C-terminal domains of SRC-1 and SRC-3 have been demonstrated to harbor weak histone acetyltransferase activity (Chen et al., 1997; Spencer et al., 1997) raising the possibility that they play a direct role in chromatin remodeling.

Methyltransferases PRMT1 and CARM1

As mentioned earlier, methylation also seems to play an important role in the regulation of transcription. PRMT1 and CARM1 belong to the family of methyltransferases and have been demonstrated to act as coactivators of nuclear receptors (Koh et al., 2001) and other transcription factors, such as p53 (An et al., 2004). PRMT1 and CARM1 are quite different in size, but they share high sequence similarity in the central region, which contains the arginine-specific protein methyltransferase activity (Koh et al., 2001). Substrates of PRMT1 include RNA-binding proteins (Cote et al., 2003; Lin et al., 2002; Liu and Dreyfuss, 1995; Rajpurohit et al., 1994) and histone H4 (Strahl et al., 2001; Wang et al., 2001), while CARM1 can methylate histone H3 (Chen et al., 1999a; Ma et al., 2001). One can envision many scenarios, in which methylation contributes to transcriptional regulation. PRMT1 and CARM1 might methylate other coactivators, thereby changing their transcriptional activity or creating new interaction surfaces for the interaction with downstream acting factors. It has been shown that PRMT1-methylated histone H4 is a better substrate for the acetyltransferase activity of p300 (Wang et al., 2001). On the other hand, acetylation of histone H3 by CBP seems to be a signal for the subsequent methylation by CARM1 (Daujat et al., 2002).

3. The mediator complex

The main function of the coactivators described so far is to permit access of the general transcription machinery to the DNA. Besides coactivators that harbor nucleosome remodeling or histone modifying activities, an other type of coregulator has been identified. A complex consisting of more than a dozen of proteins has been purified from

different organisms (Lusser and Kadonaga, 2003; Rachez and Freedman, 2001). The so-called mediator complex enhances the transcriptional activities of nuclear receptors and other signal-dependent transcription factors in vitro. A 220 kDa subunit mediates the interaction of the mediator complex with ligand-activated nuclear receptors via two alternatively utilized LXXLL nuclear receptor interaction motifs (Yuan et al., 1998). The remaining components of the complex have no known enzymatic functions but are interacting with the RNA polymerase II and are thought to recruit the RNA polymerase II holoenzyme to promoters marked by transcription factors.

Interplay between coactivators

Besides the mentioned, well studied coactivators, many more cofactors with the ability to enhance transcription have been described. In respect of this large number of proteins and protein complexes, it is possible that different coactivator complexes are recruited to a specific promoter in an sequential fashion. One possible scenario would be that Brg/Brhm-like complexes remodel chromatin to improve the access for other coactivator complexes. In a next step, p160 family coactivators together with histone acetyltransferases, such as CBP/p300 and p/CAF, or methyltransferases like CARM1 and PRMT1, would further modify the chromatin structure. Finally, recruitment of the mediator complex may bring RNA polymerase II to the promoter (Rosenfeld and Glass, 2001).

The transcriptional coactivator PGC-1α

Biological function of PGC-1α

The transcriptional coactivator PGC-1α (peroxisome proliferator-activated receptor gamma coactivator alpha) was originally cloned as an interacting protein of the nuclear receptor PPARγ in a yeast two-hybrid screen, and as a protein enhancing GR signaling in a functional screen (Knutti et al., 2000; Puigserver et al., 1998). Northern blot analysis revealed a tissue specific expression of PGC-1α. High levels of PGC-1α mRNA can be found in skeletal muscle, heart, liver, brain, and brown adipose tissue (BAT) (Esterbauer et al., 1999; Knutti et al., 2000; Puigserver et al., 1998). The expression levels of PGC-1α in those tissues are highly regulated. PGC-1α is strongly induced at the transcriptional level in BAT and skeletal muscle when mice are exposed to cold (Puigserver et al., 1998). This

leads to a strong increase in the expression levels of respiratory chain uncoupling proteins (UCPs) (Puigserver et al., 1998), which makes the mitochondrial membrane permeable for protons. A leaky mitochondrial membrane does not allow the creation of a protein gradient for the generation of ATP. Instead, the energy released by the flow of the electrons in the respiratory chain is converted to heat (Puigserver and Spiegelman, 2003). Expression of UCP-1 is known to be regulated by the nuclear receptors PPARα, PPARγ, RAR, and TRα (Barbera et al., 2001; Cassard-Doulcier et al., 1994; Sears et al., 1996). Since PGC-1α can coactivate all those transcription factors, it is likely that PGC-1α is recruited to the UCP-1 promoter through one or several of those receptors (Puigserver et al., 1998; Wu et al., 1999). The production of heat in response to cold exposure, also called adaptive thermogenesis, is also increased by an enhanced mitochondrial biogenesis. Ectopic expression of PGC-1α leads to an increase in the number of mitochondria per cell (Lehman et al., 2000). In agreement with this observation, PGC-1α induces the expression of a number of mitochondrial proteins encoded in the nucleus, including cytochrome c, ATP-synthase β, cytochrome c-oxidase, and mtTFA (Wu et al., 1999). Many of those genes contain binding sites for the transcription factors NRF-1, NRF-2, and ERRα, all three of which have been shown to be induced and coactivated by PGC-1α (Schreiber et al., 2003; Wu et al., 1999). Not only exposure to cold, but also physical exercise induces the expression of PGC-1α in muscle of mice and humans (Baar et al., 2002; Goto et al., 2000; Pilegaard et al., 2003). The cellular responses triggered by PGC-1α require an increase in the uptake and metabolism of fuels, like glucose and fat. PGC-1α has been shown to stimulate the expression of genes of fatty acid oxidation in cardiac cells, which is associated with an increase in fatty acid oxidation. PPARα seems to play an important role, since overexpression of this transcription factor increased the effect of PGC-1α (Vega et al., 2000). PGC-1α also induces expression of the insulin-sensitive glucose transporter GLUT4 and increases glucose uptake. This is partially mediated by coactivation of the muscle transcriptional regulator MEF2C by PGC-1α (Michael et al., 2001). Although humans don't have a clear depot of brown fat, it could be shown that human white adipocytes acquired features of brown adipocytes upon adenoviral overexpression of PGC-1α, which induced the expression of UCP1, respiratory chain proteins, and fatty acid oxidation enzymes (Tiraby et al., 2003). If the energy consumed by adaptive thermogenesis and physical exercise is not replenished, the blood glucose levels drop and trigger the release of the hormones glucagon and glucocorticoids, which signal the liver to

release glucose. The liver has to pathways to produce glucose. The first is glycogenolysis, the breakdown of glycogen, that occurs after a relatively short term fast. The second pathway is glyconeogenesis, the *de novo* synthesis of glucose from precursors such as lactate, pyruvate, glycerol, or alanine. This pathway comes into play when all stored glycogen has been consumed. It has been shown that fasting of mice, induces the expression of PGC-1α in liver, which then helps to induce the expression of the key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphatase, and glucose-6-phosphatase (Yoon et al., 2001). It could be shown that the expression of PGC-1α is directly activated by cAMP response element-binding protein (CREB), a transcription factor activated by glucagon and cAMP, through binding to the PGC-1α promoter (Herzig et al., 2001). The mechanistic basis for the activation of the gluconeogenic enzymes by PGC-1α was studied using the promoter of PEPCK. Two transcription factors, hepatocyte nuclear factor 4-alpha (HNF4α) and the glucocorticoid receptor, seem to play important roles in the activation of the gluconeogenic enzymes by PGC-1α (Rhee et al., 2003; Yoon et al., 2001). The importance of PGC-1α in the regulation of energy metabolism prompted many researchers to investigate whether aberrant levels of PGC-1α can be a factor in the development of type II diabetes mellitus and obesity. Type II diabetes mellitus is characterized by disturbances in the regulation of the blood glucose level, which can be caused by diminished insulin-stimulated glucose uptake by skeletal muscle and fat cells, by increased hepatic glucose production, or impaired insulin secretion by pancreatic β cells. Constantly elevated blood glucose levels can lead to atherosclerotic vascular disease, blindness, amputation, and kidney failure. Support for a role of PGC-1α in the development of diabetes comes from a report that shows elevated PGC-1α levels in the liver of diabetic mice (Lane et al., 2002). Several studies have shown that the oxidative phosphorylation genes are coordinately downregulated in muscles of diabetic patients. Interestingly, the PGC-1α mRNA was also reduced, suggesting that the repressed PGC-1α levels contribute to decreased energy expenditure and to the development of diabetes (Mootha et al., 2003; Patti et al., 2003). Mutations in the PGC-1α gene locus have been associated with an increase in susceptibility to type II diabetes (Ek et al., 2001; Hara et al., 2002).

Molecular mechanisms of PGC-1α

As described in a previous section, many coactivators enhance transcription by having enzymatic activities that serve to remodel chromatin. PGC-1α does not have significant homology to other coactivator families. In addition, it does not harbor a recognizable HAT domain and does not seem to have this intrinsic enzymatic activity. PGC-1α is a protein build of 798 amino acids. Database searches for sequences with similarities to PGC-1α have identified the coactivators PRC and PGC-1β (also called PERC) and defined a new, small family of coactivators (Andersson and Scarpulla, 2001; Kressler et al., 2002). Figure 1.2 shows a schematic presentation of PGC-1α. By definition, transcriptional coactivators do not bind regulatory DNA elements directly, rather are they recruited by DNA-binding transcription factors. PGC-1α uses several motifs or domains to interact with transcription factors. PGC-1α contains three so-called L-motifs (L1, L2, and L3) of which L2 matches the consensus sequence LXXLL present in many proteins interacting with the ligand-binding domain of nuclear receptors. L2 lies between amino acids 144 and 148 and mediates the interaction with all nuclear receptors tested so far (Schreiber et al., 2003; Tcherepanova et al., 2000; Vega et al., 2000). The atypical L-motif (L3) between amino acids 210 and 214 has been shown to strongly contribute to the interaction with the orphan nuclear receptor ERRα (Schreiber et al., 2003). A proline-rich domain between amino acids 180-403 interacts with PPARγ and NRF-1 (Wu et al., 1999). Finally, a domain ranging from amino acids 400 to 570 interacts with the transcription factor MEF2C (Michael et al., 2001). PGC-1α is a transcriptional coactivator that enhances transcription from promoters where it has been recruited to. Early studies demonstrated that the N-terminal part PGC-1α harbors a potent transcription activation domain, which when fused to the yeast GAL4-DNA-binding domain, strongly activates the expression of GAL4-responsive reporter constructs (Knutti et al., 2000). Activation of transcription may occur through the recruitment of the trancriptional coactivators SRC-1 and CBP/p300 or the RNA polymerase machinery (Monsalve et al., 2000; Puigserver et al., 1999). The L-motifs and the transcription activation domain of PGC-1α described above are typical for transcriptional coactivators. What distinguishes PGC-1α from most known coactivators are the serine/arginine-rich domains (SR) and the putative RNA recognition motif (RRM) located in its C-terminal part. Such protein motifs are characteristic for proteins acting in RNA processing (Blencowe et al., 1999; Graveley, 2000). It has also been shown that proteins containing paired SR and RRM motifs can interact with the C-terminal domain of

the RNA polymerase II, suggesting a link between transcription and pre-RNA processing (Hirose and Manley, 2000). It is now widely accepted that the SR-domains of SR proteins function as protein interaction domains. SR-domains can be extensively phosphorylated. Such phosphorylation has been shown to regulate the subnuclear localization of SR-proteins or the interaction with other proteins, both of which may change the ability of SR proteins to regulate splicing. A possible role for PGC-1α in the regulation of alternative splicing is supported by the observation that PGC-1α expression can alter the processing of a model mRNA, but only when it is loaded onto the promoter of the gene by interacting with a transcription factor (Monsalve et al., 2000). In addition to the role in the regulation of splicing, the C-terminal domains have also been shown to interact with the mediator complex (Wallberg et al., 2003).

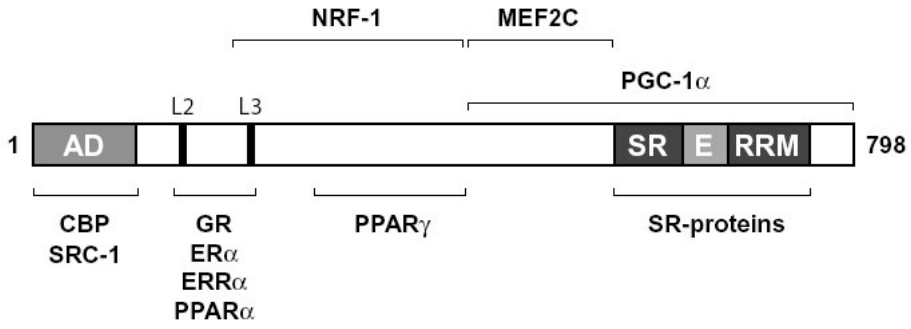

Figure 1.2. Schematic presentation of the structure of PGC-1α.
Parenthesis indicate the interaction domains with different proteins. The activation domain (AD), the LxxLL-motifs (L2 and L3), the SR-rich region (SR), the E-rich region (E), and the RNA-recognition motif (RRM) are indicated by gray or black boxes.

Post-translational regulation of PGC-1α

As described above, a number of pathways are switched on by the induction of PGC-1α in animals or by the overexpression of PGC-1α in cell culture systems. The activity of PGC-1α as transcriptional coactivator is definitively regulated at the transcriptional level. It is now becoming evident that the activity of PGC-1α is also regulated at the posttranscriptional level. It has been shown that cytokines like interleukin 1α, interleukin 1β, and tumor necrosis factor alpha increase the transcriptional activity of PGC-1α through direct phosphorylation by the mitogen activated protein kinase p38, resulting in the stabilization and activation of the PGC-1α protein (Puigserver et al., 2001). Displacement of a molecular repressor protein was suggested as the mechanism for the increase of the PGC-1α activity by the p38 phosphorylation (Knutti et al., 2001). Further studies led to the identification of p160 myb binding protein ($p160^{MBP}$) as a PGC-1α interacting protein, whose overexpression decreased the PGC-1α activity. Most importantly, the interaction of $p160^{MBP}$ with PGC-1α was weaker when PGC-1α was phosphorylated by p38 (Fan et al., 2004).

Aim of this study

Transcriptional coactivators can use several different mechanisms to translate the signal of DNA-bound transcription factors into activation of gene expression. Coactivators can recruit the basal transcription machinery to promoters by acting as bridges between transcription factors and the transcription machinery. Many coactivators posses ATP-dependent chromatin remodeling activity or other enzymatic activities for the modification of histones or other proteins involved in transcription with acetyl-groups, methyl-groups, phosphate, ubiquitin, or SUMO . At the same time, the activity of many coactivators has been shown to be modulated by post-translational modifications. Understanding the mechanisms used by the coactivator PGC-1α to enhance transcription and how its activity is regulated is still a major challenge.

Previous work in our laboratory has suggested that the C-terminal part of PGC-1α, which encodes two SR-rich domains and a putative RNA recognition motif, contributes to the coactivation of nuclear receptor responses (Knutti et al., 2000). We hypothesized that PGC-1α recruits, via its C-terminal domains, proteins that allow PGC-1α to reach its full

activity by either being a subunit of a PGC-1α complex or by posttranslationally modifying and regulating PGC-1α. A yeast two-hybrid screen was chosen to identify proteins interacting with the C-terminal domains of PGC-1α. Such proteins may explain the importance of the PGC-1α C-terminal domains in the regulation of transcription. In addition, they may help to identify unknown functions of PGC-1α or possible mechanisms for the regulation of the PGC-1α transcriptional activity.

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CHAPTER II: EXPERIMENTAL PROCEDURES

Plasmids

PGC-1α constructs: In frame fusions of amino acids 189-798, 294-798, 408-798, or 506-798 of PGC-1α to the yeast GAL4-DBD were generated by cloning either an EcoRI/SalI, XbaI/SalI, StuI/SalI or NcoI/SalI fragments from pBS/HA-hPGC-1α (Knutti et al., 2000) into pGBKT7 (Clontech). If necessary, the ends were treated with Klenov polymerase and/or mung bean nuclease before ligation to obtain the correct reading frame. For the construction of the in frame fusion of amino acids 91-798 to the GAL4-DBD a NdeI/EcoRI fragment from pcDNA3/HA-PGC-1α.91-408 coding for amino acids 91-189 of PGC-1α and an EcoRI/SalI fragment from pBS/HA-PGC-1α (Knutti et al., 2000) coding for amino acids 190-798 of PGC-1α were ligated into pGBKT7. Plasmid pBS/2xFlag-PGC-1α was constructed by replacing the NdeI/XhoI fragment of pBS/2xFlag-Clk1 with the NdeI/XhoI fragment of pBS/HA-PGC-1α (Knutti et al., 2000). A BamHI/NotI fragment from pBS/2xFlag-PGC-1α containing the PGC-1α cDNA downstream of the two Flag-tag-coding sequence was subcloned into pcDNA3 to give pcDNA3/2xFlag-PGC-1α. Plasmid pcDNA3/HA-PGC-1α for the expression of HA-tagged PGC-1α in mammalian cell has been described (Knutti et al., 2000). For the generation of the PGC-1 deletion variants Δ6, ΔSR, ΔE, ΔRRM, N754, and ΔSR/E/RRM the plasmid pBS/HA-PGC-1α (Knutti et al., 2000) was used as template. Variant Δ6 was made by PCR-amplification of a DNA fragment encoding amino acids 566-798 of PGC-1 using primers CAG TCT AGA CGC TCT CGT TCA AGG TCC and GTA ATA CGA CTC ACT ATA GGG C. The PCR-fragment was then digested with XbaI and XhoI and used to replace the wildtype XbaI/XhoI-fragment of pBS/HA-PGC-1α. Variant N754 was constructed in a similar manner using primers GGT GAC CAT GAC TAT TGC and GAC TGA CTC GAG TTA CTT GCG TCC ACA AAA GTA C. To make PGC-1α construct SR/E/RRM (aa 565-754), primers TAC GCC GGT CAT ATG CGC TCT CGT TCA AGG TCC and GAC TGA CTC GAG TTA CTT GCG TCC ACA AAA GTA C were used to PCR-amplify a DNA-fragment encoding amino acids 565-754 of PGC-1α. The PCR-product was digested with NdeI and XhoI and used to replace the NdeI/XhoI fragment of pBS/HA-PGC-1α. PGC-1α variants ΔSR, ΔE, ΔRRM, and ΔSR/E/RRM were constructed by a two-step PCR-method.

In the first round, primer C8/1190 was used in combination with primers ATA CCT GGG CAT CCT TTG GGG TCT TTG (for ΔSR), CAC ACG GCG CCG ACG GCT GTA GGG CGA TC (for ΔE), GAA AAA TTG CTC TTC AAT TGC CTT CTG (for ΔRRM), and GAA AAA TTG CAT CCT TTG GGG TCT TTG (for ΔSR/E/RRM) to amplify the 5'-flanking sequences of the sequences to be deleted. Primer GTA ATA CGA CTC ACT ATA GGG C was used with primers CAA AGG ATG CCC AGG TAT GAC AGC TAC (for ΔSR), AGC CGT CGG CGC CGT GTG ATT TAT GTC (for ΔE), ATT GAA GAG CAA TTT TTC AAG TCT AAC (for ΔRRM), and CAA AGG ATG CAA TTT TTC AAG TCT AAC (for ΔSR/E/RRM) to amplify the 3'-flanking-sequences of the sequences to be deleted. Corresponding pairs of 5'- and 3'-flanking regions generated in the first round were used as template for the second PCR-round with primers GGT GAC CAT GAC TAT TGC and GTA ATA CGA CTC ACT ATA GGG C. The generated PCR-products harboring the deletions were digested with XbaI and XhoI and used to replace the wildtype XbaI/XhoI-fragment of pBS/HA-PGC-1α. For the construction of the expression vectors for the PGC-1 variants having lysine to arginine substitutions in the putative SUMOylation motifs, a similar two-step PCR-method was used. To introduce the K184R mutation, a PCR was performed with primer pairs GCT TTC TGG GTGGAC TCA AGT GG / GCA ATT GTG CGC ACT GAG AAT TCA TGG AGC and CTC AGT GCG CAC AAT TGC AGG GTT TGT TC / GTG GTG GGT GGA GTT AGG CCT GCA G and pBS/2xFlag-PGC-1α as template. In the second step, the two PCR-products of the first step, which overlap by 18 nucleotides, were used as template for a PCR with primers GCT TTC TGG GTG GAC TCA AGT GG and GTG GTG GGT GGA GTT AGG CCT GCA G. The resulting amplification product, containing the K184R mutation in the putative SUMOylation motif, was cut with NheI and EcoRI to replace the corresponding wildtype fragment in pBS/2xFlag-PGC-1α. To introduce the K646R mutation, PCR was performed with primer pairs GAC TAG CCA TGG ATG GCC / AGG CTG CGT CGC GAA GAA TAT CGC AGA GAG and TTC TTC GCG ACG CAG CCT CTC GTG CTG ATA TTC C / GTA ATA CGA CTC ACT ATA GGG C and pBS/2xFlag-PGC-1α as template. In the second step, the two PCR-products of the first step, which overlap by 18 nucleotides, were used as template for primers GAC TAG CCA TGG ATG GCC and GTA ATA CGA CTC ACT ATA GGG C. The resulting amplification product, containing the K646R mutation in the putative SUMOylation motif, was cut with AgeI and XhoI to replace the corresponding wildtype fragment in pBS/2xFlag-PGC-1α. To confirm the presence of the right deletions

and to exclude unwanted mutations, all constructs were sequenced. For expression in mammalian cells, the HA-tagged or 2xFlag-tagged PGC-1α variants were subcloned as BamHI/NotI fragments into pcDNA3 to generate pcDNA3/HA-PGC-1α.Δ6, pcDNA3/HA-PGC-1α.ΔSR, pcDNA3/HA-PGC-1α.ΔE, pcDNA3/HA-PGC-1α.ΔRRM, pcDNA3/HA-PGC-1α.ΔSR/E/RRM, pcDNA3/HA-PGC-1α.N754, pcDNA3/HA-PGC-1α.SR/E/RRM, pcDNA3/2xFlag-PGC-1α.184R, and pcDNA3/2xFlag-PGC-1α.646R. To make pcDNA3/GAL4DBD-PGC-1α.91C, a DNA sequence coding for amino acids 91-798 of PGC-1α was subcloned in frame behind the GAL4-DBD of plasmid pcDNA3/GAL4DBD (Kressler et al., 2002).

Constructs for proteins identified to interact with PGC-1α. To make pcDNA3/2xFlag-Clk1, the complete ORF of human Clk1 was PCR amplified with the oligonucleotides GGA TCC ATG GAC TAC AAG GAT CAC GAC GGT GAC TAC AAG GAT GAC GAT GAC AAG CAT ATG AGA CAC TCA AAG AGA AC (containing BamHI, NcoI sites, two Flag-tags, NdeI site and 27 nucleotides of the Clk1 sequence; the underlined ATG is the translation initiation codon of Clk1) and oligonucleotide GAC TGA CTC GAG CTA TAT ACT TTT CTT CAG (containing an XhoI site and 15 nucleotides of Clk1; natural stop codon of Clk1 is underlined) using the yeast two-hybrid clone 1 as template, and cloned into BamHI/XhoI restricted pBS/HA-PGC-1α, which gave rise to the plasmid pBS/2xFlag-Clk1. The sequence of the cloned Clk1 cDNA and the Flag-tag was verified by sequencing. The full length 2xFlag-Clk1 was then subcloned as a BamHI/NotI fragment into pcDNA3 (Invitrogen) downstream of the cytomegalovirus enhancer to give pcDNA3/2xFlag-Clk1. The full length cDNA of HM-1 was PCR-amplified using oligonucleotides CGA ATT CAT ATG AAC GAT TGG ATG CCC and GAC TGA CTC GAG CTA CTT GCC TCT TTC CTT C and yeast two-hybrid clone 39 as template. Tip60β was amplified with oligonucleotides CGA ATT CAT ATG GCG GAG GTG GGG GAG ATA ATC GAG and GAC TGA CTC GAG TCA CCA CTT CCC CCT CTT G and yeast two-hybrid clone 15 as template. Tip60α was amplified with the same oligonucleotides but with HepG2 cDNA as template. For the amplification of the Skip ORF, oligonucleotides CGA ATT CAT ATG GCG CTC ACC AGC TTT TTA C and GAC TGA CTC GAG CTA TTC CTT CCT CCT CTT C were used with yeast two-hybrid clone 251 as template. The full length cDNAs of HM-1, Tip60α, Tip60β and Skip were cut

with NdeI and XhoI and used to replace the NdeI/XhoI fragment of pBS/2xFlag-Clk1 to generate pBS/2xFlag-HM-1, pBS/2xFlag-Tip60α, pBS/2xFlag-Tip60β, and pBS/2xFlag-Skip, which were all verified by sequencing. The 2xFlag-tagged version of the HM-1, Tip60α, Tip60β, and Skip cDNAs were then subcloned as BamHI/NotI fragments into pcDNA3 (Invitrogen) to give pcDNA3/2xFlag-HM-1, pcDNA3/2xFlag-Tip60α, pcDNA3/2xFlag-Tip60β, and pcDNA3/2xFlag-Skip. For the *in vitro* transcription/translation of the PGC-1α-interacting proteins, the inserts of the isolated library plasmids were cut out as NcoI/XhoI fragments and used to replace the NcoI/SalI fragment of pGEM4/HA-PGC-1α.

Tip60 constructs. Construction of the Tip60α and Tip60β expression constructs have been described above. The Tip60 point mutants were generated by fusion PCR. To introduce the mutations in the acetyl-CoA binding motif (Q377E,G380E), two PCRs were performed with primer pairs CGA ATT CAT ATG GCG GAG GTG GGG GAG ATA ATC GAG / GTA CTC GCG ACG CTC GTA GGG AGG CAG GGT TAG and TAC GAG CGT CGC GAG TAC GGC AAG CTG CTG ATC GAG TTC AGC / GAC TGA CTC GAG TCA CCA CTT CCC CCT CTT G and pBS/2xFlag-Tip60α as template. In the second step, the two PCR-products of the first step, which overlap by 18 nucleotides, were used as template for primers CGA ATT CAT ATG GCG GAG GTG GGG GAG ATA ATC GAG and GAC TGA CTC GAG TCA CCA CTT CCC CCT CTT G. The resulting amplification product, containing the Q377E and G380E, was cut with BglI and PstI to replace the corresponding wildtype fragment in pBS/2xFlag-Tip60α. To introduce the mutations in the LXXLL motif (L489/492/493A), two PCRs were performed with primer GTC ATG ACA GAG TAT GAC / CCG AGC TGC CCG CTT GGC CAT GGC CCG CTC ATG GCC ATC and ATG GCC AAG CGG GCA GCT CGG ATC GAC TCC AAG TGT CTG / GTA ATA CGA CTC ACT ATA GGG C and pBS/2xFlag-Tip60α as template. In the second step, the two PCR-products of the first step, which overlap by 18 nucleotides, were used as template for primers GTC ATG ACA GAG TAT GAC and GTA ATA CGA CTC ACT ATA GGG C. The resulting amplification product, containing the L489A, L492A and L493A mutations, was cut with PstI and SalI to replace the corresponding wildtype fragment in pBS/2xFlag-Tip60α. To make the Tip60 variants N147 and 148C, fragments coding for amino acids 1-147 or 148-513 of Tip60α were amplified with primer pairs CGA ATT CAT ATG GCG GAG GTG GGG GAG ATA ATC

GAG/ GAC TGA CTC GAG TCA CGT TGA GCG GTG GTT GGG or CGA ATT CAT ATG AAA CGG AAG GTG GAG GTG/ GAC TGA CTC GAG TCA CCA CTT CCC CCT CTT G. The two PCR products were cut with NdeI/XhoI, and used to replace the NdeI/XhoI fragment of pBS/2xFlag-Tip60α. All Tip60 variants were verified by sequencing and cloned into pcDNA3 (Clontech) (BamHI/NotI), pGBKT7 (Clontech) (NdeI/SalI), pcDNA3/VP16 (BamHI/NotI), pGEX-4T-3 (Pharmacia) (BamHI/SalI), and pAdlox (BamHI/XhoI). Oligonucleotide pairs GAT CCC CAC GTC TGG ATG AAT GGG TGT TCA AGA GAC ACC CAT TCA TCC AGA CGT TTT TTG GAA A / AGC TTT TCC AAA AAA CGT CTG GAT GAA TGG GTG TCT CTT GAA CAC CCA TTC ATC CAG ACG TGG G and GAT CCC CGC CAC GAC GAC ATC GTC ACT TCA AGA GAG TGA CGA TGT CGT CGT GGC TTT TTG GAA A / AGC TTT TCC AAA AAG CCA CGA CGA CAT CGT CAC TCT CTT GAA GTG ACG ATG TCG TCG TGG CGG G were annealed and cloned into BglII/HindIII-restricted pAdSUPER (Schreiber et al., 2003). The resulting plasmids pAdSUPER/Tip60-4 and pAdSUPER/Tip60-19 were used for the generation of adenoviral vectors expressing siRNAs directed against Tip60.

Other constructs. Expression vectors for the rat glucocorticoid receptor (p6RGR) (Godowski et al., 1988), p52 (pMT2T-p52) (Chang et al., 1994), APP , Fe65 (pCMV5/Fe65) (Cao and Sudhof, 2001), and Bcl-3 (pFlag-CMV2-Bcl-3) (Westerheide et al., 2001) as well as the luciferase reporter constructs pTAT3-luc, pTAT1-luc, pMMTV-luc (Iniguez-Lluhi et al., 1997), and pGK-1 (Webb et al., 1995), and p2xκBSV40 (Pan and McEver, 1995) have been described. To construct the luciferase reporter pERRα-luc, the ERRα-responsive sequences of the ESRRA promoter (nt –537 to –829 nt, relative to transcription initiation site) were amplified by PCR and subcloned upstream of the minimal alcohol dehydrogenase promoter of pΔLuc (Iniguez-Lluhi et al., 1997).

Yeast two-hybrid screen

Yeast strain AH109 (Clontech) was simultaneously transformed with pGBKT7/PGC-1α.294C or pGBKT7/PGC-1α.506C and a human skeletal muscle cDNA library (Clontech) according to the lithium acetate method and then plated on selection medium (SD –Trp, -Leu, -His, -Ade, 15mM 3-AT). Library plasmids from 401 positive clones were recovered and reintroduced into yeast strain AH 109 to confirm the interactions. Strength

of the interactions was determined by measuring β-galactosidase activity in yeast strain CG 187 [CG1945 x Y187 (Clontech)]. Positive clones were sequenced using an ABI prism sequencer and primer TAC CAC TAC AAT GGA TG.

Adenoviruses

Adenoviral vectors were generated by CRE-lox mediated recombination in CRE8 cells (Hardy et al., 1997). 90% confluent CRE8 cells (Hardy et al., 1997), grown in a 10 cm tissue culture plate, were transfected with 3 µg of purified Ψ5 adenovirus DNA and 10 µg of the corresponding pAdlox or pAdSUPER shuttle plasmids according to the calcium phosphate protocol. After 5-8 days and the formation of a few plaques, cells were washed from the plate and collected by centrifugation. The supernatant was removed except for 1 ml that was used to resuspend the cells. 10 µl of this cell suspension was used to infect fresh CRE8 cell grown on a 10 cm plate. The rest was frozen at –70°C. After most of the infected cells had detached from the plate, they were harvested and resuspended as described above. The previous infection step was repeated one more time. The resulting cell suspension was used for the isolation of single adenovirus clones by the plaque assay.

For the plaque assay, CRE8 cells were split into 6-well tissue culture plates and grown to about 90% confluence. Serial dilutions of an adenoviral stock solution were prepared in 1 ml media each. The media on the CRE8 cells were removed and replaced with the adenovirus dilutions. After a one hour incubation at 37°C, the media were aspirated and the cells were overlaid with 2 ml of a 45°C warm agar solution (growth media containing 0.6% agar). To let the agar cool down the cells were incubated at room temperature for 30 min. Finally, 4 ml media was added to each well on top of the agar layer. Plaques were counted and isolated at 5-7 days after infection.

Adenoviral vectors for HA-PGC-1α and GFP have been described (Schreiber et al., 2003).

Cell Lines, Transfections, and Infections

COS7, U2OS, and SAOS2-GR(+) (Rogatsky et al., 1997), and CRE8 (Hardy et al., 1997) cells were grown in Dulbecco's modified Eagle's medium supplemented with 9% fetal calf

serum. For SAOS2-GR(+) cells, charcoal stripped serum was used and the medium was supplemented with 200µg/ml G418.

For transfections, cells were incubated with a calcium phosphate/DNA precipitate. For the transfection of cells grown in 35 mm wells, the DNA to be transfected was mixed, and water was added to a final volume of 180 µl. 60 µl of a 1 M $CaCl_2$ Solution were added to the DNA mix. After a 15 min incubation, 240 µl of a 2 x HBS (pH 6.98) solution was added. The solution was mixed well by pipetting up and down and then divided into two 35 mm wells containing 1.5 ml media each. For luciferase assays, transfections included 200 ng of p6R-lacZ (Pearce and Yamamoto, 1993) for normalization of transfection efficiency. About 16 h after transfection, the precipitate was removed by two washes with PBS (w/o Ca^{2+} and Mg^{2+}) and fresh medium was added to the cells. Cell lysates were prepared 40 hr after transfection using 100 µl reporter lysis buffer (Promega) and assayed for luciferase and β-galactosidase activity. Luciferase values normalized to the β-galactosidase activity are referred to as luciferase units.

For infections, SAOS2 (GR+) or U2OS cells were plated at 1.0 x 10^5 cells per well in a six-well plate or at 10^6 cells per 10 cm dish. Cells were infected with viruses at a multiplicity of infection as indicated in the figure legends. 2 hr later, cells were washed and replenished with fresh media.

Western blot analysis

Whole cells extracts were prepared by boiling the cells in TSD-buffer (50 mM Tris-HCl pH7.5, 1% SDS, 5 mM DTT) for 5 min. The insoluble material was removed by cetrifugation at RT for 5 min and the supernatant was diluted 1:10 in NP-40 lysis buffer (50mM Tris-HCl pH 8, 150 mM NaCl, 0.5 % NP-40, 1 mM EDTA, 1mM PMSF, protease inhibitors). After the addition of SDS-PAGE loading buffer the samples were boiled for 5 min. For the separate analysis of NP-40-soluble and NP-40-insoluble cellular components, cells extracts were prepared in NP-40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8, 1 mM EDTA, 0.5% NP-40, protease inhibitors). Insoluble material was pelleted by centrifugation for 10 min at 4°C. The supernatant was transferred to a fresh tube and boiled for 5 min after the addition of SDS-PAGE loading buffer. The pelleted material was

solubilized by boiling for 5 min. in NP-40 lysis buffer supplemented with SDS-PAGE loading buffer. The resulting samples were resolved by SDS-PAGE and transferred to nitrocellulose or PVDF membranes. Western blot analysis was performed using antibodies against PGC-1α (Schreiber et al., 2003) and the Flag-epitope (Sigma).

Co-immunoprecipitation

To study protein-protein interactions in cells, various expression plasmids were transfected into COS7 cells according to the calcium phosphate method. When SAOS-GR(+) cells were used, they were infected with adenoviruses expressing either GFP, HA-PGC-1α, 2xFlag-Tip60α or 2xFlag-Tip60α.mHAT. 24 h after infection, cell extracts were prepared by incubating cells in 1 ml NP-40 lysis buffer (50mM Tris-HCl pH 8, 150 mM NaCl, 0.5 % NP-40, 1 mM EDTA, 1 mM PMSF, 1 x protease inhibitors, 1x phosphatase inhibitors) for 15 min. Extracts were spun at 14000 rpm for 10 min. The pellet was discarded. For the immunoprecipitation of PGC-1α complexes, the cell extracts were first precleared with a mixture of protein A/G sepharose for 1 h. Extracts were then incubated with anti-PGC-1α antibody (1:500) (Schreiber et al., 2003) for 2 hr followed by an incubation with a mixture of protein A/G sepharose for 1 hr. Sepharose with the bound proteins was washed three times with lysis buffer. For the immunoprecipitation of Tip60 complexes, 5 µl anti-Flag-agarose (Sigma) diluted with 15 µl sepharose were mixed with the cell extracts which were then rotated at 4°C for 2 hours. Beads with the bound proteins were washed three times with lysis buffer. Immunocomplexes were released by boiling in SDS-PAGE loading buffer, resolved by SDS-PAGE and transferred to a PVDF or nitrocellulose membrane. PGC-1α was detected using anti-PGC-1α antiserum (Schreiber et al., 2003). The Flag-tagged proteins Tip60, HM-1, Skip, and Clk1 were detected with an anti-Flag antibody (Sigma).

RNA Analysis

Total RNA was isolated using the TRIzol reagent (Invitrogen) and checked for its integrity by agarose gel electrophoresis and ethidium bromide staining. RNA (400 ng) was converted to cDNA using the Superscript Reverse Transcriptase Kit (Invitrogen) and random hexamer primers according to manufacturer's instructions. Real-time PCR with the

Chromo4 (MJ Research) was used for the amplification and quantification of Cyt c, ERRα, IL22R, BBS1, p21, and GAPDH cDNA. PCR reactions were performed in 20 µl reactions using 2 µl cDNA, 500 nM specific primers, and the SYBRgreen kit (ABI). The sequences of the primers and the sizes of the products were: CCA GTG CCA CAC CGT TGA A (exon 2) and TCC CCA GAT GAT GCC TTT GTT (exon 3) for Cyt c (product 136 bp), AAG ACA GCA GCC CCA GTG AA (exon 4/5) and ACA CCC AGC ACC AGC ACC T (exon 6) for ERRα (product 254 bp), CGT GAA ATT CCA GTC CAG CAA (exon 2) and CGG TGA CCC TGG CAT AGT AGA (exon 3) for IL22R (product 212 bp), CAC ACC TTT TCT GCC TGCC T (exon 2) and TTT CGG TCA TCA CCA GTG GTC (exon 4) for BBS1 (product 136 bp), CGG GAT GAG TTG GGA GGA G (exon 1) and CGG CGT TTG GAG TGG TAG AA (exon 2) for p21 (product 212 bp), and GAA GGT GAA GGT CGG AGT C (exon 2) and GAA GAT GGT GAT GGG ATT TC (exon 4) for GAPDH (product 226 bp). A melting curve from 65°C to 95°C was at the end of the reaction was used to check the purity and nature of the products.

In vitro protein interaction assays

Single colonies of E. coli strain BL21 transformed with plasmids expressing GST or the GST-PGC-1α fusions were grown overnight in liquid LB medium containing 50 µg/ml ampicillin. The next morning, the cultures were diluted to an OD_{600} of 0.1 in LB medium containing ampicillin and 2% glucose and grown to an OD_{600} of approximately 0.5. Expression of the GST-fusion proteins was then induced by the addition of IPTG to a final concentration of 0.5 mM and by incubation for 2 to 3 hours more, shaking and at RT. Bacteria were pelleted at 4°C, washed once with ice-cold PBS, and resuspended in PBS (1/20 of the original culture volume). The cell suspension was sonicated 5 x 30 sec on ice. NP-40 to a final concentration of 0.5% was added to the cell suspension, which was then incubated on ice for 30 min. Insoluble cell debris was pelleted at 9000 g for 30 min at 4°C. The supernatant was incubated with glutathione sepharose (1/1000 of original culture volume, Amersham) for 20 min at room temperature. The beads with the bound GST-fusions were washed twice with PBS containing 0.5% NP-40 and once with binding buffer (20 mM Tris, pH8, 75 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 10 % glycerol, 0.05 % NP-40, 1 mM PMSF, and protease inhibitors). After the three wash steps the beads were resuspended in ice-cold binding buffer and the concentration of the GST-fusion proteins

was determined using the Bradford assay. [^{35}S]methionine-labeled proteins were produced with the TNT reticulocyte lysate *in vitro* transcription and translation kit (Promega) using 0.5 µg of template plasmid in a final volume of 20 µl. About 1 µg of GST or GST-fusion proteins bound to the glutathione beads were mixed with 5 µl of in vitro-translated [^{35}S]methionine-labeled proteins in binding buffer. The reaction volumes were 150 µl. After binding for 1 hour at room temperature, the beads were washed three times with wash buffer buffer (20 mM Tris, pH8, 75 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 10 % glycerol, 0.05 % NP-40, 1 mM PMSF, and protease inhibitors). Bound proteins were then released by boiling in SDS-sample buffer and resolved by SDS-PAGE. The gel was incubated in fixing solution (45% methanol, 10% acetic acid) for 30 min and in 1 M sodium salicylate for 20 min and finally dried. Bound proteins were detected by autoradiography.

In vitro acetylation assay

Expression of maltose binding protein (MBP), the MBP-PGC-1α fusions, GST-Tip60α, and GST-Tip60α.mHAT was induced in E. coli strain BL21 with 0.5 mM IPTG for 3 hours at RT. MBP and the MBP-PGC-1α fusions were purified on amylose beads (NEB). The GST-Tip60 fusions were purified on glutathione beads (Amersham Biosciences). Bound GST-fusions were eluted twice with elution buffer (50 mM Tris pH8, 150 mM NaCl, 0.1 mM EDTA, 10 mM glutathione). About 1 µg of MBP or the MBP-PGC-1α constructs bound to the amylose beads were mixed with about 2 µg GST-Tip60α or GST-Tip60α.mHAT in the reaction buffer containing 50 mM Tris, pH 8, 150 mM NaCl, 0.1 mM EDTA, 5 mM sodium butyrate, 1 mM PMSF, and 0.5 µl [^{3}H]-acetyl-coenzyme A (Amersham) and incubated at 30°C for 1 hour. Proteins were resolved by 5-15% gradient SDS-PAGE. The gel was then incubated in fixing solution (45% methanol, 10% acetic acid) for 30 min and in the amplify solution (Amersham) for 20 min and finally dried. Acetylated proteins were detected by autoradiography.

Pulse-chase experiment

On day 1, COS7 cells were transfected with expression plasmids for Flag-tagged PGC-1α, HA-tagged Tip60α, or vector alone in 10 cm tissue culture plates. The next morning, cells

were split into 3 cm plates (1/6 of a 10 cm plate into a 3 cm plate). On day three, β-galactosidase activity of one 3 cm plate of each condition was measured to confirm similar transfection efficiencies. The cells of the remaining plates were washed once with PBS (without Ca^{2+} and Mg^{2+}) for 5 min before they were incubated with 500 µl starvation medium (DMEM w/o methionine and cysteine) per 3 cm plate for 30 min. The starvation medium was removed and replaced with 500 µl labeling medium (starvation medium containing [^{35}S]-labeled methionine and cysteine). After one hour incubation with labeling medium, cells were washed once with warm PBS (without Ca^{2+} and Mg^{2+}). Cells were then incubated with 2 ml chase medium (DMEM, 10 % FBS, 2 mM methionine, 2 mM cysteine) per 3 cm plate. 0, 1, 2, 3, or 4 hours after addition of the chase medium, cells of one plate of each condition were washed once with cold PBS and harvested in 500 µl PBS (without Ca^{2+} and Mg^{2+}), transferred to a 1.5 ml screw cap tube and centrifuged for 1 min at 4°C. The supernatant was removed and the cell pellets were frozen in liquid nitrogen and stored at –70°C. The frozen cells were resuspended in 50 µl TSD-buffer (50 mM Tris pH 7.5, 1% SDS, 5 mM DTT) and boiled for 5 min. The cell lysates were spun at RT for 5 min to pellet the insoluble material. The supernatant was transferred to a fresh tube and diluted with 600 µl of TNN-buffer (50 mM Tris pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.5% NP-40). 5 µl anti-Flag-agarose (Sigma) diluted with 15 µl sepharose were then added to each sample, which were then rotated at 4°C for 2 hours. The beads were washed 4 times with TNN-buffer. Bound proteins were released with SDS-PAGE sample buffer and resolved on a 7.5 % SDS-PAGE gel. The gel was then incubated in fixing solution for 30 min and in enhancing solution (1 M sodium salicylate) for 20 min and finally dried. [^{35}S]-labeled PGC-1α was detected by autoradiography.

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CHAPTER III: IDENTIFICATION OF PROTEINS INTERACTING WITH THE C-TERMINAL PART OF PGC-1α

The C-terminal part of PGC-1α contributes to the full activity of PGC-1α as a transcriptional coactivator

PGC-1α was identified as a transcriptional coactivator for nuclear receptors (Knutti et al., 2000; Puigserver et al., 1998). When this work started, little was know about the mechanisms used by PGC-1α to enhance transcription. A potent transcription activation domain has been mapped to its first approximately 200 amino acids, which were shown to bind the coactivators and acetyltransferases CBP and SRC-1 (Knutti et al., 2000; Puigserver et al., 1999; Puigserver et al., 1998). Our laboratory has shown that deletion of this region completely abolishes the ability of PGC-1α to enhance the activity of the glucocorticoid receptor (GR) on a reporter plasmid controlled by three glucocorticoid responsive enhancer elements derived from the tyrosine aminotransferase (TAT) promoter (TAT3-luc) in COS7 cells, suggesting that the N-terminal 200 amino acids contain the only autonomous transcription activation domain of PGC-1α (Knutti et al., 2000). A PGC-1α variant consisting of only the first 408 amino acids (N408) displayed a reduced ability to coactivate the GR compared to wild type PGC-1α (Fig. 3.1B). This reduced activity could neither be attributed to a lower expression level (Fig. 3.1D) nor to a defective nuclear localization of this PGC-1α variant (Knutti et al., 2000). When a luciferase reporter under control of the mouse mammary tumor virus promoter (MMTV-luc) was used, the contribution of the C terminus to the PGC-1α activity seemed even more pronounced, while the C terminus seemed to be dispensable for the coactivation of a luciferase reporter under control of only one GR-responsive enhancer element from the TAT promoter (TAT1-luc) (Fig. 3.1B).

The glutamine-rich region contributes to the coactivator function of PGC-1α

To narrow down the region in the PGC-1α C terminus that contributes to the coactivator function, we constructed five PGC-1α deletion variants, each lacking a specific domain in the C-terminal part (Fig. 3.1A). An expression plasmid for the GR and the luciferase reporter TAT3-luc were cotransfected into COS7 along with control vector or the different PGC-1α constructs. The results shown in Figure 3.1B demonstrate that deletion of the E-rich region reduces the coactivator function of PGC-1α to a similar extent as deletion of the whole C-terminal half, while deletion of the other domains did not affect the PGC-1α function significantly. We repeated the above experiment with the luciferase reporter under control of the mouse mammary tumor promoter (MMTV-luc) and with the luciferase reporter under control of only one GR-responsive element of the TAT promoter (TAT1-luc). The contribution of the C-terminal part and also the E-rich region to the PGC-1α coactivator function was even more pronounced with the MMTV-luc reporter (Fig. 3.1B). No reduced coactivator activity of the PGC-1α constructs lacking the whole C-terminal half or only the E-rich domain was observed when the TAT1-luc reporter was used (Fig. 3.1B). To confirm that all PGC-1α deletion constructs are expressed at levels that are at least as high as those of wild type PGC-1α, we performed a western blot analysis. Deletion constructs Δ6, ΔE, and N754 show similar expression levels as wild type PGC-1α, while the deletion variants ΔSR and ΔRRM show significantly higher expression levels (Fig. 3.1C). These result suggests that the E-rich region of PGC-1α plays a significant role in the coactivation of the GR-dependent transcription of the TAT3 and MMTV luciferase reporters.

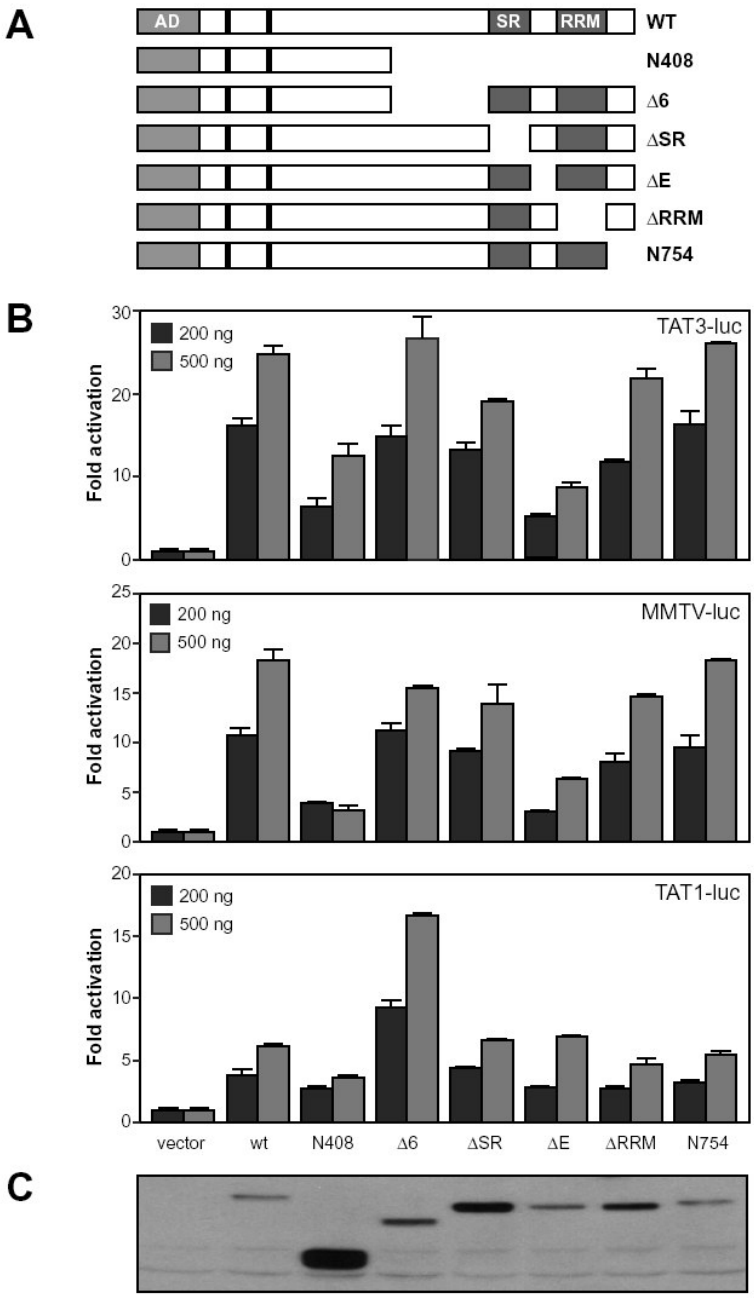

Figure 3.1. Ability of various PGC-1α deletion constructs to coactivate the glucocorticoid receptor (GR).

(A) Schematic presentation of the PGC-1α constructs used.

(B) A plasmid expressing the rat GR was cotransfected into COS7 cells along with a control vector or the indicated PGC-1α deletion constructs and one of the luciferase reporters TAT3-luc, MMTV-luc , or TAT1-luc. Cells were treated with 50 nM corticosterine for 24 hours and assayed for luciferase activity. Results are expressed as fold enhancement by the PGC-1α constructs. Results in the absence of PGC-1α was set equal to 1.

(C) Western blot showing the expression levels of the different PGC-1α constructs.

The SR-rich region, the E-rich region, and the RRM mediate PGC-1α dimerization

No function had been assigned to the E-rich region of PGC-1α so far. Our lab found that PGC-1α can homodimerize via the C-terminal half in a yeast two-hybrid assay (Knutti et al., 2000). This prompted us to test whether the E-rich region forms the protein surface that mediates the homodimerization of PGC-1α. To narrow down the region required for the PGC-1α homodimerization, we performed a yeast two-hybrid interaction assay with amino acids 294-798 of PGC-1α fused to the GAL4 DBD and the PGC-1α deletion constructs used in the previous experiments fused to the GAL4 AD (Fig. 3.2A). As shown in figure 3.2B, coexpression of amino acids 294-798 of PGC-1α fused to the GAL4 DBD and full length PGC-1α fused to the GAL4-AD led to a nice induction of the GAL4-responsive β-galactosidase reporter. As mentioned above, deletion of PGC-1α amino acids 409-798 in the GAL4-AD-fusion construct completely abolished the interaction with the bait construct. Also, deletions of the SR-rich, the E-rich region, or the RRM strongly reduced the ability of PGC-1α to homodimerize, while constructs Δ6 and N754 still allowed dimerization. Having shown this, we wanted to know if a PGC-1α fragment encompassing the SR-rich, E-rich, and RRM regions is sufficient for interaction with the bait construct. Figure 3.2B shows that a fragment of PGC-1α encompassing those three domains is indeed sufficient for interaction with the bait construct. We also tested the interaction of a fragment containing the SR-rich, E-rich, and RRM domain fused to the GAL4-DBD with either full length PGC-1α or only a fragment containing the SR-rich, E-rich, and RRM domains fused to the GAL4-AD. The results confirmed that the region of PGC-1α encompassing the SR-rich, E-rich, and RRM domains is indeed sufficient for dimerization (Fig. 3.2B). Since the SR-rich, the E-rich, and RRM regions are all required for the homodimerization of PGC-1α, but only the deletion of the E-rich region significantly reduces the ability of PGC-1α to coactivate the GR in the transient transfection experiments, we conclude that lack of dimerization is not sufficient to explain the reduced coactivator activity of a PGC-1α construct lacking the E-rich region. Thus one or several other functions must be encoded by the E-rich region that are required for the full activity of PGC-1α in the transient transfection assays.

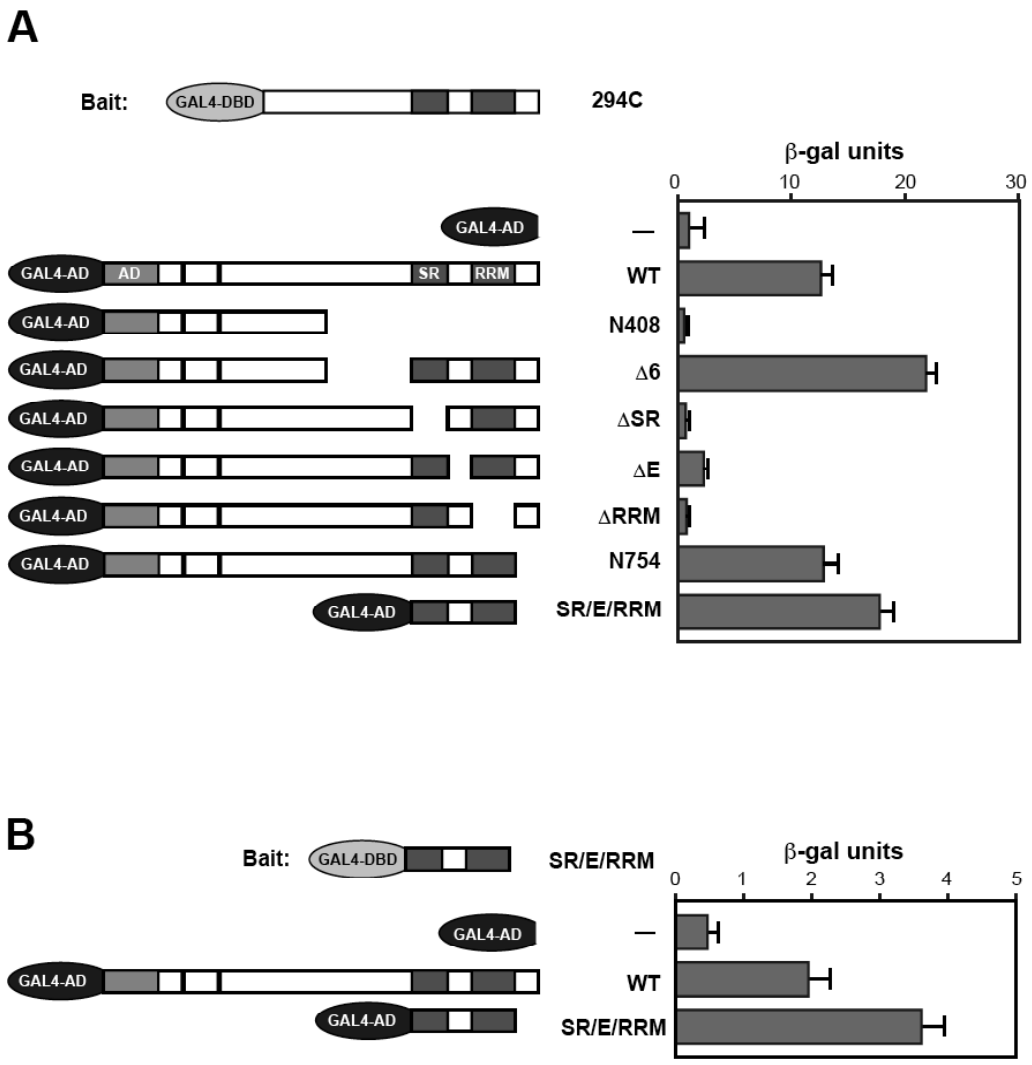

Figure 3.2. Mapping of the PGC-1α dimerization domain.
(A) Yeast strain GC187 carrying a GAL4-responsive b-galactosidase reporter was transformed with a plasmid expressing a fusion of the yeast GAL4-DBD to amino acids 294-798 of PGC-1α and a plasmid expressing the GAL4-AD, either alone, or fused to one of the indicated PGC-1α deletion constructs.
(B) Same as in A, exept that a PGC-1α fragment encompassing the SR-rich, E-rich, and RRM domains fused to the GAL4-DBD was used as bait.

Identification of proteins interacting with the C-terminal domains of PGC-1α

The coactivators CBP, SRC-1, and several transcription factors were known to interact with PGC-1α at the beginning of these studies. All these proteins make contact with N-terminal activation domain or the L-motif L2 of PGC-1α. We knew that the C-terminal domains of PGC-1α are important for full transcriptional activity PGC-1α in certain experimental setups (Fig. 3.1). Since no proteins were known to interact with the C-terminal domains of PGC-1α and could point to a function of this part of the protein, we decided to perform a yeast two-hybrid screen to identify proteins interacting with the C-terminal part of PGC-1α. The yeast strain AH109 (Clontech) was chosen for this screen, because it harbors four different GAL4-regulated reporter constructs that allow to detect and measure protein-interaction. The next step was to construct a GAL4-DBD fusion of a PGC-1α fragment, for which we wanted to find interacting proteins. Such a bait construct had to fulfill several criteria: (i) since the goal was to identify proteins interacting with the C-terminal domains of PGC-1α, the bait construct had to contain those PGC-1 domains, (ii) the PGC-1α domains fused to the GAL4-DBD should be in a conformation that would allow specific interactions, (iii) the bait construct should not significantly activate the reporter constructs by itself. To find a bait construct that fulfills these criteria, expression vectors for five different PGC-1α fragments [91C (aa 91-798), 189C (aa 189-798), 294C (aa 294-798), 408C (aa 408-798), and 506C (aa 506-798)] fused to the GAL4 DBD were constructed. They all contain the C-terminal half of PGC-1α and therefore fulfill the first criteria. To test for their ability to activate transcription of the GAL4-regulated HIS3 reporter, they were transformed into yeast strain AH109 along with the empty library vector (pACT2) and spotted on medium selecting for the induction of the GAL4-regulated HIS3 reporter. All five bait constructs contained some residual transcription activation activity, indicated by the appearance of small yeast colonies after a few days. The chemical compound 3-amino-triazole (3-AT) can be used to inhibit small amounts of His3p protein resulting from weak transcriptional activity of the GAL4 fusion proteins used as bait. We tested different 3-AT concentrations and found that we could inhibit the growth of small colonies due to background transcriptional activity of the bait constructs 189C, 294C, and 506C with 3-AT concentrations of 15 mM or higher. It was not possible to inhibit the background transcriptional activities of the bait-constructs 91C, and 408C by 3-AT sufficiently. To test for a correct conformation of the five bait constructs, they were

transformed into yeast strain AH109 along with the empty library vector (pACT2) or an expression vector for a fusion of full length PGC-1α to the GAL4 activation domain (pACT2/HA-PGC-1) and plated on medium selecting for the induction of the GAL4-regulated HIS3 reporter. Cotransformation of the five bait constructs together with full length PGC-1α fused to the GAL4-AD allowed nice growth of the yeast cells, which is an indication for a dimerization of PGC-1α and therefore a correct conformation of all five bait-constructs. Based on the background activities of the tested GAL4-DBD-PGC-1α fusions, we decided to use the constructs 294C and 506C as baits for the yeast two-hybrid screen.

Having found convenient bait constructs and the appropriate screen conditions, we had to decide what tissue we wanted to screen for PGC-1α-interacting proteins. Since PGC-1α seems to be highly expressed in skeletal muscle, proteins that cooperate with PGC-1α in the regulation of transcription should be expressed in this tissue as well (Esterbauer et al., 1999; Knutti et al., 2000; Puigserver et al., 1998). We therefore decided to screen a skeletal muscle library fused to the GAL4 AD (Clontech) for PGC-1α-interacting proteins. In a first round of transformation, we cotransformed yeast strain AH109 with the bait construct 294C and the skeletal muscle cDNA library and plated them on growth medium selecting for protein interactions. In a second round of transformation, we used the bait construct 506C. The first round of transformation resulted in approximately 150000 transformant. The first 210 colonies that grew on the plates were picked and restreaked on selective medium. The second round of transformation resulted in approximately 1 million transformants. The first 250 colonies that grew were picked and restreaked on selective medium. The library plasmids from the picked colonies were isolated, amplified, retransformed into the yeast strain AH109 together with the respective bait construct, and spotted on the same medium that was used for the screen. This procedure eliminated about half of the isolated clones as false positives. The positive clones were transformed into the yeast strain CG187 [CG1945 x Y187 (Clontech)] together with the bait construct 294C or 506C and analyzed for the expression of the GAL4-responsive β-galactosidase reporter. Around 400 nucleotides of the cDNA-inserts of 80 positive clones were sequenced and blasted against the NCBI database to determine the identity of the isolated cDNAs.

Identified proteins

Table 3.1 lists the names of the proteins identified in the screen and how many times their encoding cDNAs were isolated. The isolated genes are grouped according to their function. The largest group consists of proteins that have been implicated in the regulation of gene expression. A second group is formed by proteins implicated in pre-mRNA processing. The identification of these proteins is in agreement with a report attributing such a function to PGC-1α (Monsalve et al., 2000). A third group consists of proteins that have a connection to ubiquitin- or SUMO-related processes. The remaining identified proteins have either structural roles (fourth group) or are implicated in processes other than the proteins of the first four groups. The following section briefly describes some of the identified proteins.

Group 1: Proteins implicated in transcriptional regulation

NRF-1 is a transcription factor that has been shown to interact with PGC-1α in previous studies (Wu et al., 1999).

ERRα is a member of the nuclear receptor family of transcription factors and was already known to interact with PGC-1α (Schreiber et al., 2003). The identification of ERRα in this screen could not be expected, since the region of PGC-1α that contains the L-motifs 2 and 3 and mediates the interaction with ERRα was not present in the bait constructs. This suggests that ERRα also interacts with a region of PGC-1α other than the L-motifs.

Tip60 was first identified as protein interacting with the HIV-1 Tat transactivator (Kamine et al., 1996). It has been shown to act as a positive and also as a negative transcriptional coregulator of steroid receptors and other families of transcription factors. Tip60 has acetyltransferase activity and is part of a large protein complex with ATPase and DNA helicase activities (Ikura et al., 2000).

Table 1. Proteins identified in the yeast two-hybrid screen

Protein	Symbol	Gene symbol	Times isolated
Transcription Regulation			
Estrogen-related receptor alpha	ERRα	ESRRA	2
Nuclear respiratory factor 1	NRF-1	NRF1	1
Tat interactive protein 60 kDa	Tip60	HTATIP	1
SKI-interacting protein	Skip	SKIIP	2
Hematopoietic PBX-interacting protein	HPIP	PBXIP1	3
Testis-specific protein Y-encoded-like	TSPYL	TSPYL1	5
Splicing			
Cdc2/Cdc28-like kinase 1	Clk1	CLK1	9
Cdc2/Cdc28-like kinase 3	Clk3	CLK3	1
Cdc2/Cdc28-like kinase 4	Clk4	CLK4	3
U1 snRNP binding protein homolog	HM-1	U1SNRNPBP	3
Scaffold attachment factor B	SAF-B	SAFB	2
Ubiquitination/SUMOylation			
RAD23 homolog A	hHR23A	HHR23A	5
Ubiquitin-specific protease 25	USP25	USP25	3
Ubiquitin-conjugating enzyme E2I	UBC9	UBE2I	3
Protein inhibitor of activated STAT 2	PIASx	PIAS2	1
Structural Proteins			
Troponin T1, skeletal, slow	ssTn1	TNNT1	15
Troponin T3, skeletal, fast	TNNT3	TNNT3	2
myosin heavy polypeptide 2, skeletal muscle, adult	MYH2A	MYH2	2
myosin-binding protein C, slow type	MyBP-C	MYBPC1	1
Pericentrin 1	PCNT	PCNT1	1
Titin	TTN	TTN	1
Others			
Signal recognition paarticle 68kDa	SRP68	SRP68	4
Glycine cleavage system protein H	GCSH	IQWD1	2
Activator of HSP90 ATPase homolog 1	AHA1	AHSA1	1
xeroderma pigmentosum (complementation group A)	XP1	XPA	1
VAMP-associated protein A (33 kDa)	VAP33	VAPA	1
Guanidinoacetate N-metyltransferase	GAMT	GAMT	1
Mitochondrial ribosomal protein L3	MRPL39	MRPL39	1
ORF (LOC51035)			2
Chromosome 14 genomic sequence			1

Skip/NCoA-62 has been identified as an interacting protein of the protooncogene SKI and as a transcriptional regulator in a variety of signal transduction pathways including TGF-β, nuclear receptors, Notch, and retinoblastoma signaling (Dahl et al., 1998; Figueroa and Hayman, 2004a; Leong et al., 2001; Prathapam et al., 2002). However, Skip has also been identified in purified spliceosomal complexes (Neubauer et al., 1998). A possible role of SKIP in the process of pre-mRNA splicing is supported by the demonstration that human Skip can functionally substitute for the mRNA splicing gene PRP45 of S. cerevisiae (Figueroa and Hayman, 2004b). Thus, this protein also belongs to the group 2 of the identified PGC-1α interacting proteins.

HPIP is an interacting protein of the homeodomain transcription factor PBX1. HPIP and PBX1 are thought to regulate transcription (Abramovich et al., 2002; Abramovich et al., 2000).

TSPYL was cloned based on its sequence similarity to TSPY, a member of the TSPY/SET/NAP1L1 family of nucleosome assembly proteins (Vogel et al., 1998). Members of this protein family have been shown to assemble chromatin during cell division but also to regulate transcription. Their ability to bind histone proteins and their interaction with transcriptional coactivators, such as p300, has led to the hypothesis that they may act as integrators between chromatin and transcriptional coactivator proteins (Shikama et al., 2000).

Group 2: Proteins implicated in the regulation of pre-mRNA processing

Clk1, **Clk3**, and **Clk4** are three closely related dual-specificity kinases. Members of this family of proteins have been shown to regulate splicing, possibly by interacting with and phosphorylating a series of SR-domain splice factors (Colwill et al., 1996; Duncan et al., 1997; Duncan et al., 1998; Hartmann et al., 2001; Prasad et al., 1999).

HM-1 is another protein with a putative role in pre-mRNA splicing. It has been identified as a single-strand DNA-binding protein. The aminoterminal half of HM-1 displays high amino acid sequence similarity with the U1-70K splicing factor a component of the U1

snRNP complex. The C-terminal half of HM-1 includes several Arg/Glu, Arg/Asp, and Arg/Ser dipeptides, which are often found in splicing factors (Adams et al., 1998).

SAF-B was first isolated as a protein that binds to scaffold/matrix attachment regions, i.e. DNA-sequences that mediate the anchoring of chromatin to the nuclear matrix. It harbors domains characteristic of RNA-processing proteins, interacts with RS-containing splicing factors and hnRNPs, and can alter splice site selection *in vivo* (Arao et al., 2000; Li et al., 2003; Nayler et al., 1998; Nikolakaki et al., 2001; Renz and Fackelmayer, 1996; Weighardt et al., 1999). Like the above described Skip, SAF-B is involved in both splicing regulation and also transcription regulation. SAF-B has been shown to bind to and regulate the HSP27 promoter (Oesterreich et al., 1997).

Group 3: Proteins playing a role in ubiquitination of SUMOylation

hHR23A, one of two human homologues of the yeast Rad23 protein, has two UBA (ubiquitin-associated) domains, both of which can bind ubiquitin. It also contains an N-terminal UBL (ubiquitin-like) domain that binds S5a and S2, two components of the 26S proteasome, suggesting that hHR23A is involved in the delivery of polyubiquitinated proteins to the proteasome (Arao et al., 2000; Li et al., 2003; Nayler et al., 1998; Nikolakaki et al., 2001; Renz and Fackelmayer, 1996; Weighardt et al., 1999).

USP25 is a protein with high homology to members of the ubiquitin protease family (UBP). UBPs belong to a complex family of deubiquitinating enzymes that specifically cleave ubiquitin conjugates on a great variety of substrates. These enzymes have an essential role in protein degradation via the 26S proteasome and thus regulate many cellular pathways (Valero et al., 2001; Valero et al., 1999).

UBE2I, also known as **UBC9**, and **PIASx** stimulate the attachment of SUMO (small ubiquitin-like modifiers) to target proteins by acting as the E2 conjugating enzyme and the E3 ligase, respectively. Many different proteins, among them nuclear receptors and nuclear receptor coactivators have been suggested to be SUMOylated by UbE2I and PIASx (Kotaja et al., 2002; Ohshima et al., 2004; Tian et al., 2002).

Interestingly, some of these proteins cannot be clearly grouped into one of the above groups. For example, Skip is an abundant protein of spliceosomes but can also coactivate the nuclear receptor VDR. Similarly, SAF-B can affect alternative splicing, but also influence the transcriptional activity of the estrogen regulated HSP27 promoter. Given that many of these proteins have not been extensively studied, the classification presented above may change upon further characterization.

Confirmation of the interactions identified in the two-hybrid screen

To determine which of the proteins identified in the yeast two-hybrid screen are likely to interact with PGC-1α, we tested their ability to bind PGC-1α in an *in vitro* binding assay. An in frame GST-fusion of PGC-1α and GST control protein alone were expressed in bacteria, bound to glutathione beads and incubated with the candidate proteins, which had been *in vitro* translated and [^{35}S]methionine-labeled. As shown in Figure 3.3A, all tested proteins except for PIASx bound to GST-PGC-1α but not to control GST alone, confirming the interactions found in the yeast two-hybrid screen. To test if the proteins identified in the yeast two-hybrid screen are also able to form stable complexes with PGC-1α in mammalian cells, we performed co-immunoprecipitation experiments with a selected subset of the identified protein. I focused on proteins having proposed functions in the regulation of gene transcription or the regulation of splicing. Using the polymerase chain reaction, we amplified the complete open reading frames of Clk1, HM-1, Tip60α, Tip60β, and Skip and cloned them into mammalian expression vectors. To be able to detect the translation products of those genes, a double Flag-tag was inserted in frame upstream of the first ATG codon of each cDNA. COS7 cells were transfected with each of the five proteins alone, with PGC-1α alone, or cotransfected with PGC-1α and each of the five proteins. Protein complexes containing the PGC-1α-interacting proteins were immunoprecipitated using anti-Flag sepharose and analyzed by western blot for the presence of PGC-1α. As shown in figure 3.3B, only immunoprecipitation of Tip60α and Tip60β reproducibly coimmunoprecipitated a significant percentage of the PGC-1α protein. This coimmunoprecipitation of PGC-1α was specific, since no PGC-1α was detected in the absence of overexpressed Tip60α or Tip60β. No PGC-1α could be detected that coimmunoprecipitated with Clk1, HM-1 or Skip (data not shown). The strong

interaction of PGC-1α and the two Tip60 splice forms prompted us to study the functional interaction of those proteins in more detail. The results are described and discussed in the next chapter.

The fact that not all tested proteins interacted with PGC-1α *in vitro* or in the coimmunoprecipitation assays does not mean that they would not interact with PGC-1α under different conditions. Further experiments will be necessary to find a possible interaction between these proteins and PGC-1α. It will also be interesting to determine whether PGC-1α interacts with one of the proteins of groups four and five, which do not have an obvious functional connection to PGC-1α. Demonstration of an interaction between one or several of these proteins with PGC-1α may identify new biological functions of PGC-1α.

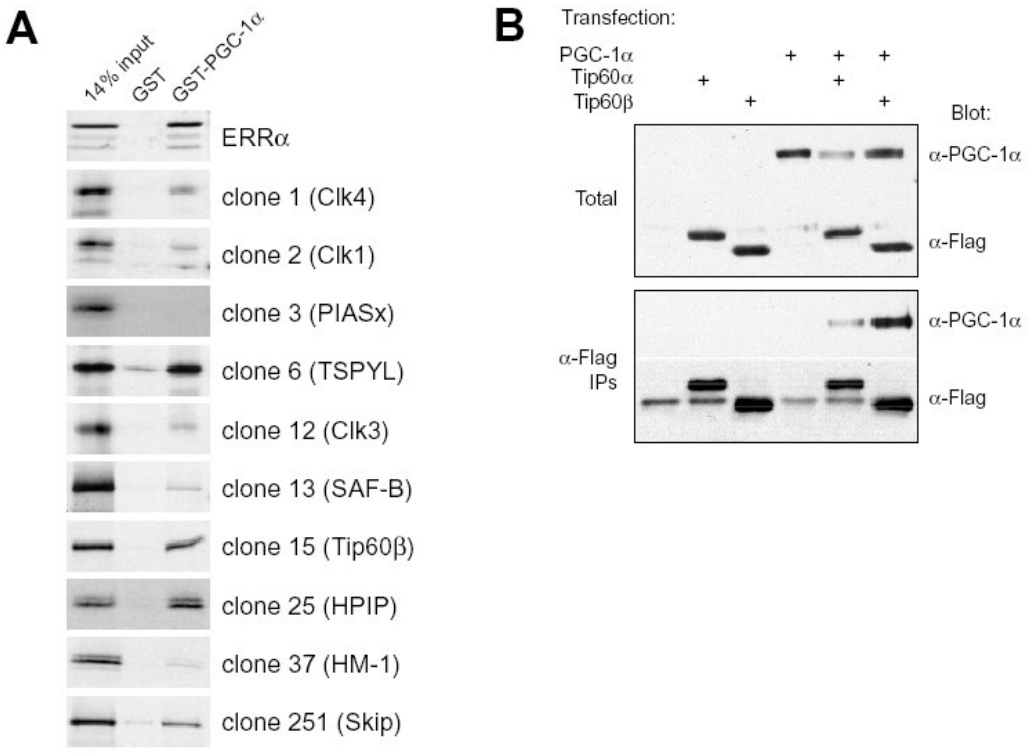

Figure 3.3. Interaction between PGC-1α and identified proteins *in vitro* and *in vivo* .
(A) GST-pulldown experiments confirm interactions found in the yeast two-hybrid screen. Control GST protein alone or PGC-1α fused to GST were immobilized on glutathione agarose beads and incubated with different *in vitro*-translated and [^{35}S]-methionine-labeled proteins. After the beads were washed, bound proteins were eluted, resolved by SDS-PAGE and visualized by autoradiography.
(B) PGC-1α forms a complex with Tip60α and Tip60β in mammalian cells.
COS7 cells were transfected with the indicated proteins. 40 h after transfection, cell lysates were prepared and subjected to immunoprecipitation using anti-Flag agarose. Purified protein complexes were resolved by SDS-PAGE and analysed by western blot using anti-Flag and anti-PGC-1α antibodies.

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CHAPTER IV: PGC-1α INTERACTS WITH THE ACETYLTRANSFERASE TIP60

(Manuscript in preparation)

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Abstract

The transcriptional coactivator PPAR gamma coactivator 1 alpha (PGC-1α) is a key regulator of metabolic processes such as mitochondrial biogenesis and respiration in muscle and gluconeogenesis in the liver. We describe here the identification of the MYST-family acetyltransferase Tip60 as a PGC-1α-interacting protein. Tip60 has been shown to be a positive and negative coregulator for many transcription factors such as nuclear receptors, p53, c-myc, and others. We mapped the interaction surface for Tip60 to a region encompassing amino acids 482-565 of PGC-1α. In previous studies, this domain has also been shown to mediate the interaction of PGC-1α with nuclear respiratory factor 1 (NRF-1). Overexpression of Tip60 by transfection or by infection using adenoviral vectors repressed the PGC-1α-dependent expression of all exogenous reporter constructs and endogenous genes tested. We also found that PGC-1α enhances expression of the endogenous cell cycle regulator p21, whose expression has previously been shown to be enhanced by Tip60. The PGC-1α-dependent induction of p21 was strongly reduced by the expression of Tip60 lacking acetyltransferase activity or by siRNA-mediated knock-down of endogenous Tip60, suggesting a requirement for the Tip60 acetytransferase activity in the PGC-1α-dependent transcription of p21. Our results also suggest a gene specific functional interaction between PGC-1α and Tip60.

Introduction

Proteins regulating gene expression have to integrate information from many different signaling pathways to adjust the amount of a specific gene product to the physiological conditions. Alterations in gene expression are in many cases regulated by changes in the amount or the activity of DNA-binding transcription factors but also transcriptional coactivators. The transcriptional coactivator PGC-1α was originally cloned as an interacting protein of the nuclear receptor PPARγ in a yeast two-hybrid screen, and as a protein to enhance GR signaling in a functional screen (Knutti et al., 2000; Puigserver et al., 1998). It has been shown to interact and coactivate several members of the nuclear receptor family, as well as other transcription factors (Boustead et al., 2003; Delerive et al., 2002; Hentschke et al., 2002; Knutti et al., 2000; Michael et al., 2001; Oberkofler et al., 2003; Schreiber et al., 2003; Tcherepanova et al., 2000; Vega et al., 2000; Wu et al., 1999). The expression PGC-1α is induced in a tissue selective manner and in response to metabolic needs. Exposure to cold leads to the induction of PGC-1α in brown fat and in muscle (Puigserver et al., 1998), starvation induces PGC-1α expression in heart and liver (Herzig et al., 2001; Yoon et al., 2001), and physical exercise upregulates the expression of PGC-1α in muscle (Baar et al., 2002; Goto et al., 2000; Norrbom et al., 2004; Pilegaard et al., 2003). Under these condtions PGC-1α induces the expression of genes that increase the ability of cells to mobilize and utilize stored energy (Barbera et al., 2001; Herzig et al., 2001; Lehman et al., 2000; Vega et al., 2000; Wu et al., 1999; Yoon et al., 2001). PGC-1α contains a transcriptional activation domain in the N-terminal 200 amino acids (Knutti et al., 2000). The binding sites for serveral transcription factors resides in the central part of PGC-1α (aa 200-465). The C-terminal 350 amino acids of PGC-1α harbor two SR-rich regions and and RNA recognition motif, which have been shown to be important for RNA processing by PGC-1α (Monsalve et al., 2000).

Tip60, a protein localized mainly to the nucleus, is a member if the MYST family of acetyltranferases, which are highly conserved from yeast to humans. Two splice variants have been identified, Tip60α and Tip60β, resulting from differential splicing of exon 5 (Ran and Pereira-Smith, 2000; Sheridan et al., 2001). Tip60 can acetylate histones, as well as transcritpion factors, such as AR and UBF1, suggesting a role of Tip60 in chromatin remodeling and regulation of gene expression (Gaughan et al., 2002; Halkidou et al., 2004;

Kimura and Horikoshi, 1998; Yamamoto and Horikoshi, 1997). Indeed, it acts as a cofactor for a variety of transcription regulators, like HIV-1 Tat (Kamine et al., 1996), nuclear receptors (Brady et al., 1999), p53 (Doyon et al., 2004), and the cytoplasmic domain of APP complexed to Fe65 (Cao and Sudhof, 2001). On the other hand, Tip60 has been shown to act as a repressor of the transcritipional factors CREB, STAT3, TEL, and E2F (Gavaravarapu and Kamine, 2000; Nordentoft and Jorgensen, 2003; Taubert et al., 2004; Xiao et al., 2003). Tip60 and its yeast homologue Esa1p have both been linked to DNA double strand break repair (Bird et al., 2002; Ikura et al., 2000). DNA damage response is mediated in part by the transcription factor p53. Tip60 has recently been shown to enhance the transcription of p53 target genes after gamma-irradiation (Doyon et al., 2004). Tip60 also interacts with plasma membrane associated receptors for IL-9 and endothelin, suggesting a role in the signal transduction in response to extracellular stimuli (Lee et al., 2001; Sliva et al., 1999).

We describe here the identification of Tip60α and Tip60β as PGC-1α-interacting proteins. We map the binding site for Tip60α to a region between amino acids 482-565 of PGC-1α. We show that overexpression of Tip60 by transfection or by infection using adenoviral vectors repressed the PGC-1α-dependent expression of several exogenous reporter constructs and the endogenous PGC-1α target genes ERRα and cytochrome c. This repressive effect of Tip60 depended partially on its acetyltransferase activity. We also found that PGC-1α enhances p53-dependent expression of the endogenous cell cycle regulator p21 after gamma-irradiation. This PGC-1α-mediated transcription was not affected by overexpression of wildtype Tip60, but was dramatically reduced by the expression of a Tip60 lacking acetyltransferase activity or by siRNA-mediated knock-down of endogenous Tip60, suggesting a requirement for Tip60 and its acetytransferase activity in the PGC-1α-dependent transcription of p21. These results demonstrate that Tip60 can cooperate with PGC-1α in gene expression, but that Tip60 can also negatively regulate the transcriptional activity of PGC-1α.

Experimental Procedures

Plasmids and Adenoviral Vectors

In frame fusions of amino acids 294-798 or 506-798 of PGC-1α to the yeast GAL4-DBD used for the two-hybrid screen were generated by cloning either StuI/SalI or NcoI/SalI fragments from pBS/HA-hPGC-1α (Knutti et al., 2000) into pGBKT7 (Clontech). PGC-1α variants Δ6, ΔSR/E/RRM, 409C, N482, N565, N754, 565-754, 294-565, and 341-565 used in the yeast two-hybrid interaction assay were generated by standard PCR methods in pBS/HA-hPGC-1α (Knutti et al. 2000) and verified by sequencing. Deletion variants 294C, N408 have been described (Knutti et al. 2000). All PGC-1α deletion contructs were cloned into pGADT7 (Clontech) in frame behind the yeast GAL4 activation domain. Expression plasmids pcDNA3/HA-hPGC-1 (Knutti et al., 2000) and p6RGR (Godowski et al., 1988) as well as the luciferase reporter constructs pTAT3-luc (Iniguez-Lluhi et al., 1997), pGK-1 (Webb et al., 1995) have been described. To construct the luciferase reporter pERRα-Luc, the ERRα-responsive sequences of the ESRRA promoter (nt –537 to –829 nt, relative to transcription initiation site) were amplified by PCR and subcloned upstream of the minimal alcohol dehydrogenase promoter of pΔLuc. To make pcDNA3/GAL4DBD-hPGC-1α.91C, a DNA sequence coding for amino acids 91-798 of PGC-1α was subcloned in frame behind the GAL4-DBD of plasmid pcDNA3/GAL4DBD (Kressler et al., 2002). All Tip60 expression vectors are derived from plasmids pBS/2xFlag-Tip60α and pBS/2xFLAG-Tip60β, which carry the Tip60α and Tip60β cDNAs downstream of the 2xFlag epitope-encoding sequence. To make pBS/2xFlag-Tip60β, the Tip60β open reading frame was PCR-amplified using the positive yeast two-hybrid cDNA clone as template and primers CGA ATT CAT ATG GCG GAG GTG GGG GAG ATA ATC GAG and GAC TGA CTC GAG TCA CCA CTT CCC CCT CTT G and then cloned as NdeI/XhoI fragment into pBS/2xFLAG. The same primers were used to obtain full length Tip60α from HepG2 cDNA. The Tip60 acetyltransferase mutant (Q377E, G380E) was generated by standard PCR methods. All Tip60 variants were verified by sequencing and were cloned into pcDNA3 (Clontech) (BamHI/NotI), pGBKT7 (Clontech) (NdeI/SalI), pcDNA3/VP16 (BamHI/NotI), pGEX-4T-3 (Pharmacia) (BamHI/SalI), and pAdlox (BamHI/XhoI). More information on the plasmids is available on request. Adenoviral vectors for HA-PGC-1α and GFP have been described (Schreiber et al., 2003). Flag-tagged Tip60α and Tip60α.377/80E expressing adenoviruses were constructed by the same method.

Oligonucleotide pairs GAT CCC CAC GTC TGG ATG AAT GGG TGT TCA AGA GAC ACC CAT TCA TCC AGA CGT TTT TTG GAA A / AGC TTT TCC AAA AAA CGT CTG GAT GAA TGG GTG TCT CTT GAA CAC CCA TTC ATC CAG ACG TGG G and GAT CCC CGC CAC GAC GAC ATC GTC ACT TCA AGA GAG TGA CGA TGT CGT CGT GGC TTT TTG GAA A / AGC TTT TCC AAA AAG CCA CGA CGA CAT CGT CAC TCT CTT GAA GTG ACG ATG TCG TCG TGG CGG G were annealed and cloned into pAdSUPER (Schreiber et al., 2003). The resulting plasmids pAdSUPER/Tip60-4 and pAdSUPER/Tip60-19 were used for the generation of adenoviral vectors expressing siRNAs for Tip60.

Yeast two-hybrid screening

Yeast strain AH109 (Clontech) was transformed with pGBKT7/hPGC-1α.294C or pGBKT7/hPGC-1α.506C and a human skeletal muscle cDNA library according to the lithium acetate method and then plated on selection medium (SD –Trp, -Leu, -His, -Ade, 15 mM 3-AT). Library plasmids from 401 positive clones were recovered and reintroduced into yeast strain AH 109 to confirm the specificity of the interactions. Strength of the interactions was determined by measuring β-galactosidase activity in yeast strain CG 187 (CG1945 x Y187 (Clontech)). 80 Positive clones were sequenced using an ABI prism sequencer.

Protein interaction assays

GST-fusion proteins expressed in E. coli were purified on glutathione beads. [^{35}S]methionine-labelled PGC-1α was produced with the TNT reticulocyte lysate in vitro transcription and translation kit (Promega) with pcDNA/HA-PGC-1α as template. About 1 μg of GST , GST-Tip60α, or GST-Tip60β bound to the glutathione beads were mixed with 5 μl of in vitro translated PGC-1α in binding buffer containing 20 mM Tris, pH8, 75 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 10 % glycerol, 0.05 % NP-40, 1 mM PMSF, and protease inhibitors. After binding for 1 hour at room temperature, the beads were washed three times with binding buffer. Bound proteins were then released in SDS-sample buffer and resolved by SDS-PAGE. The gel was dried and bound PGC-1α was detected by autoradiography.

Yeast two-hybrid interaction assay

Plasmids expressing GAL4 DBD and GAL4 AD fusions were introduced into yeast diploid cells (CG1945 X Y187; Clontech), which carry a GAL4-driven *his3* reporter. Transformants were grown in 96 well plates, washed once with water, and diluted to an OD_{600} of 0.1. Five microliters of each yeast culture were spotted on SD medium lacking Trp and Leu to check for viability of the yeast cells and on SD medium lacking Trp and Leu as well as His and containing 5 mM 3-aminotriazol to select for protein-protein interactions.

Cell Lines, Infections, and Transfections

COS7, SAOS2-GR(+) (Rogatsky et al., 1997) and U2OS cells were grown in Dulbecco's modified Eagle's medium supplemented with 9% fetal calf serum. For SAOS2-GR(+) cells, charcoal stripped serum was used and the medium was supplemented with 200mg/ml G418. For infection, 1.5 x 10^5 cells per well in a six-well dish or 10^6 cells per 10 cm dish were plated. Cells were infected with viruses at a multiplicity of infection as indicated in the figure legends. 2 hr later, cells were washed and replenished with fresh media. For transfections, cells were incubated with a calcium phosphate/DNA precipitate. Transfections included 200 ng of p6R-lacZ for normalization of transfection efficiency. Cell lysates were prepared 40 hr after transfection and assayed for luciferase and β-galactosidase activity. Luciferase values normalized to the β-galactosidase activity are referred to as luciferase units.

Western blot analysis

Protein extracts of SAOS2-(GR+) cells were prepared in RIPA buffer containing 50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 0.1% SDS, 1 mM PMSF, protease inhibitors, and phosphatase inhibitors. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. PGC-1α and Tip60 were detected using an anti-PGC-1α antibody (Schreiber et al., 2003) or an anti-Flag antibody (Sigma), respectively.

Co-immunoprecipitation

Approximately 10^6 SAOS-GR(+) cells on a 10 cm dish were infected with adenoviruses expressing either GFP, HA-PGC-1α, 2xFlag-Tip60α or 2xFlag-Tip60α.mHAT. 24 h after

infection, cell extracts were prepared by incubating cells in 1 ml NP-40 lysis buffer (50mM Tris-HCl pH 8, 150 mM NaCl, 0.5 % NP-40, 1 mM EDTA, 1mM PMSF, protease inhibitors, phosphatase inhibitors) for 15 min. Extracts were spun at 14000 rpm for 10 min. The pellet was discarded. The extracts were precleared with a mixture of protein A/G sepharose beads. Extracts were incubated with anti-PGC-1α antibody (Schreiber et al., 2003) for 2 hr followed by an incubation with a mixture of protein A/G sepharose beads for 1 hr. Beads were washed three times with lysis buffer. Immunocomplexes were resoloved by SDS-PAGE and transferred to a PVDF membrane. PGC-1α and Tip60 were detected using a-PGC-1α (Schreiber et al., 2003) and anti-Flag (Sigma) antibodies.

RNA Analysis

Total RNA was isolated using the TRIzol reagent (Invitrogen) and checked for its integrity by agarose gel electrophoresis and ethidium bromide staining. RNA (400 ng) was converted to cDNA using the Superscript Reverse Transcriptase Kit (Invitrogen) and random hexamer primers according to manufacturer's instructions. Realtime PCR with the Chromo4 (MJ Research) was used for the amplification and quantitation of Cyt c, ERRα, p21, and GAPDH cDNA. PCR ractions were performed in 20 µl reaction using 2 µl cDNA, 500 nM specific primers, and the SYBRgreen kit (ABI). The sequences of the primers and the sizes of the products were: CCA GTG CCA CAC CGT TGA A (exon 2) and TCC CCA GAT GAT GCC TTT GTT (exon 3) for Cyt c (product 136 bp), AAG ACA GCA GCC CCA GTG AA (exon 4/5) and ACA CCC AGC ACC AGC ACC T (exon 6) for ERRα (product 254 bp), CGG GAT GAG TTG GGA GGA G (exon 1) and CGG CGT TTG GAG TGG TAG AA (exon 2) for p21 (product 212 bp), and GAA GGT GAA GGT CGG AGT C (exon 2) and GAA GAT GGT GAT GGG ATT TC (exon 4) for GAPDH (product 226 bp). A melting curve from 65°C to 95°C at the end of each reaction was used to check the purity and nature of the products.

Results

Identification of Tip60 as a PGC-1α-interacting protein

To identify proteins that cooperate with PGC-1α in gene expression or that regulate the activity of PGC-1α, we performed a yeast two-hybrid screen. A region of PGC-1α encompassing residues 294-798 fused to the GAL4-DBD was used to screen a human skeletal muscle cDNA expression library fused to the GAL4-activation domain for PGC-1α interacting partners. Clone 15 interacted specifically with GAL4-PGC-1α.294-798, since no interaction was observed with the GAL4-DBD alone. Sequence analysis of clone 15 revealed a 100% identity to Tip60β, a splice variant of the MYST family acetyltransferase Tip60α. We tested the interaction of clone 15, full length Tip60α, and Tip60β with PGC-1α.294-798 in a quantitative yeast two-hybrid assay. As shown in figure 4.1A, clone 15, Tip60α, and Tip60β interacted with PGC-1α. To confirm the interaction between PGC-1α and Tip60 found in the yeast two-hybrid system, we performed *in vitro* binding assays. GST-fusions of Tip60α and Tip60β were immobilized on glutathione sepharose beads and incubated with *in vitro* translated, [^{35}S]methionone-labeled PGC-1α. As shown in figure 4.1B, PGC-1α associates with GST-Tip60α and GST-Tip60β, but fails to bind to the control GST protein. We next wanted to know whether PGC-1α and Tip60α form a complex in mammalian cells. To this end, we expressed a Gal4DBD fusion of amino acids 91-798 of PGC-1α and a VP16AD fusion of full length Tip60α in COS7 cells and tested for PGC-1α – Tip60α interaction using the mammalian two-hybrid assay. Figure 4.1C shows that coexpression of the Gal4DBD-PGC-1α.91C and VP16AD-Tip60α lead to a higher expression of the GAL4 responsive luciferase reporter than coexpression of Gal4DBD-PGC-1α.91C and VP16AD alone, Gal4DBD and VP16AD-Tip60α, or Gal4DBD and VP16AD, suggesting a specific interaction between PGC-1α and Tip60α. To determine whether PGC-1α and Tip60α form a stable complex in mammalian cells, we infected SAOS2-GR(+) cells with adenoviruses expressing PGC-1α and Flag epitope tagged Tip60α. After 24 hours, cell extracts were prepared and immunoprecipitated with anti-PGC-1α antibody. Western blot analysis with anti-PGC-1α and anti-Flag antibody showed that Tip60 associates specifically with PGC-1α, since no Tip60 could be detected in the immunoprecipitate, when the cells were infected with GFP instead of PGC-1α (Fig. 4.1D).

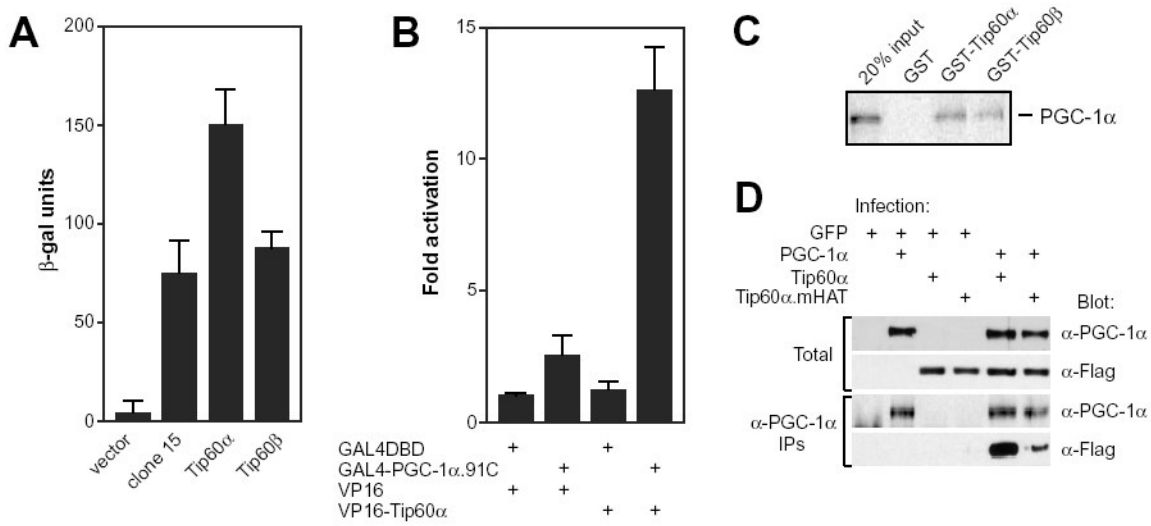

Figure 4.1. PGC-1α and Tip60 interact *in vitro* and *in vivo*.
(A) Yeast two-hybrid strain GC187 was transformed with plasmids expressing GAL-DBD-PGC-1α.294C and the GAL4-AD, either alone, fused to clone 15, Tip60α, or Tip60β and then assayed for β-gal activity.
(B) COS7 cells were transfected with the GAL4 responsive luciferase reporter pGK-1 and the indicated plasmids. 40 h after infection, cells were assayed for luciferase activity.
(C) Control GST alone or either Tip60α or Tip60β fused to GST were immobilized on glutathione sepharose beads and incubated with in vitro translated, [^{35}S]-methionine labeled PGC-1α. After the beads were washed, bound PGC-1α was eluted, resolved by SDS-PAGE and visualized by autoradiography.
(D) SAOS2-GR(+) cells were infected with the indicated combinations of adenoviruses expressing GFP, HA-PGC-1α, 2xFlag-Tip60α and Tip60α.mHAT at an moi of 10. 24h after infection, cell extracts were prepared and subjected to immunoprecipitation with anti-PGC-1α antibody. PGC-1α and Tip60 were detected by western blot using anti-PGC-1α and anti-Flag antiserum respectively.

Mapping of the Tip60 interaction domain in PGC-1α

To map the region of PGC-1α required for the interaction with Tip60α, a yeast two-hybrid assay was performed using full length Tip60α fused to the GAL4-DBD and either full length PGC-1α or PGC-1α deletion variants fused to the GAL4 AD shown in figure 4.2A. None of the GAL4AD-PGC-1α fusions activated the expression of the *his3* reporter when coexpressed with the GAL4-DBD alone. To confirm that all GAL4-PGC-1α fusions are expressed, we checked the interaction of the C-terminal PGC-1α deletion variants with the GR-LBD fused to the GAL4-DBD and the interaction of the N-terminal PGC-1α deletion constructs with GAL4-DBD-PGC-1α.294C. Interaction of PGC-1α with the GR-LBD and PGC-1α homodimerization in a yeast two-hybrid assay have been described (Knutti et al., 2000). Tip60α fused to the GAL4-DBD did not interact with the GAL4-AD but interacted with full length PGC-1α showing that Tip60α and PGC-1α interact independent of which of the two proteins is fused to the GAL4-DBD or the GAL4-AD (compare to Fig 4.1A). As shown in figure 4.2B, C-terminal truncations of PGC-1α up to amino acid 565 did not affect the growth of the yeast cells on the selective medium. Only yeast cells containing the PGC-1α construct N482 or N408 lost their ability to grow under the selective conditions. When we tested N-terminal PGC-1α truncations, we found that the first 408 amino acids of PGC-1α are dispensable for the interaction with Tip60α. When the PGC-1α constructs 409C was tested, no interaction with Tip60α could be detected. These results suggest that amino acids 409-565 of PGC-1α contain a domain that is required for the interaction with Tip60α. We also tested three internal PGC-1α deletion constructs for the interaction with Tip60α. The only deletion in PGC-1α that completely abolished the interaction with Tip60α is Δ6, which lacks amino acids 409-565. This is in agreement with the results above. Unexpectedly, also PGC-1α construct Δ4 showed a strongly reduced interaction with Tip60α. This might be attributed to a unfavorable conformation of constructs Δ4, although the interaction with the GR-LBD does not seem to be disturbed by this deletion. Finally, we could show that amino acids 294-565 and even the amino acids 341-565 of PGC-1α are sufficient for the interaction with Tip60α, while a PGC-1α fragment harboring the SR and RRM domains could not interact with Tip60α.

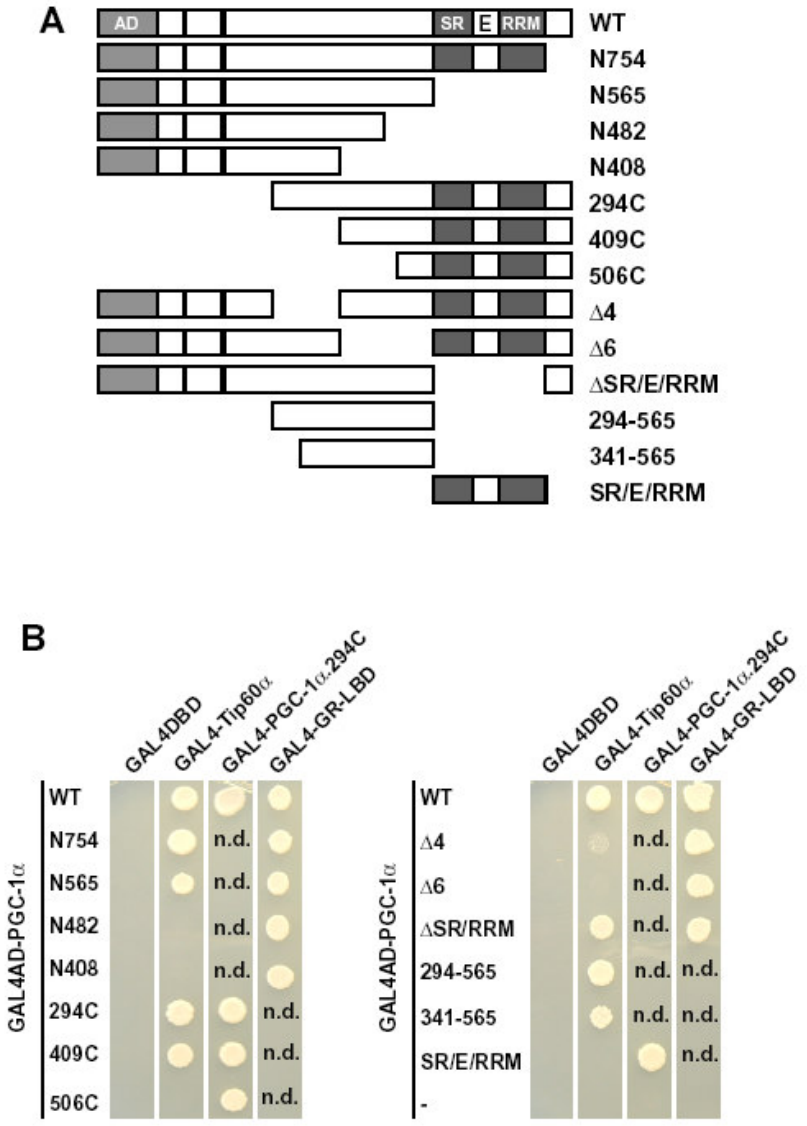

Figure 4.2. Mapping of the Tip60α interaction domain in PGC-1α.
(A) Schematic representation of the PGC-1α constructs fused to the GAL4-AD used for the yeast two-hybrid protein-protein interaction assay.
(B) Indicated combinations of GAL4-DBD and GAL4-AD fusion-constructs were transformed into yeast strain CG187. Transformants were grown in liquid media overnight, washed in water and spotted on selective media containing 5 mM 3-aminotriazole and lacking histidine, tryptophan, and leucine. For the assay of the PGC-1α – GR-LBD interaction, the media contained 25 mM corticosterone.

Tip60a represses PGC-1a dependent gene expression.

Since Tip60 can be a coactivator and harbors acetyltransferase activity, we next tested whether Tip60 cooperates with PGC-1α in PGC-1α-dependent gene regulation. For this, we first transfected COS7 cells with expression vectors for the glucocorticoid receptor (GR) and PGC-1α, along with a GR-responsive luciferase reporter, as well as expression vectors for Tip60α, Tip60β, and an acetyltransferase mutant of Tip60α (Tip60α.mHAT). 40 hours post-transfection, cells were treated with 50 nM corticosterone for 4 hours and then lysed for luciferase assays. If Tip60 collaborated with PGC-1α, we would expect a positive effect on the PGC-1α-dependent expression of the luciferase reporter by overexpressed wildtype Tip60 and a negative effect by overexpressed Tip60 acetyltransferase mutant. However, overexpression of both Tip60α and Tip60β markedly reduced PGC-1α transcriptional activity, while the Tip60 variant that has no acetyltransferase activity did not repress the PGC-1α transcriptional activity (Fig. 4.3A). We also performed similar experiments with two other luciferase reporter constructs. As shown in figure 4.3B, Tip60α also repressed the transcriptional activity of PGC-1α, when a luciferase reporter under control of the ERRα promoter was used. In contrast to the previous experiment, the Tip60α acetyltransferase mutant also repressed the PGC-1α activity, but to a lesser extend than wildtype Tip60α. Furthermore, we repeated the experiment with a third luciferase reporter under control of the cytochrome c promoter, which has been shown to be upregulated by PGC-1α. In this experiment both Tip60α wildtype and acetyltransferase mutant repressed the PGC-1α-dependent expression of the luciferase reporter to a similar extent (Fig. 4.3C). To show in a different assay that Tip60 represses the transcriptional activity of PGC-1α, we assayed the expression of a luciferase reporter under control of GAL4-UAS by full-length PGC-1α fused to the GAL4-DBD with or without overexpressed Tip60α or Tip60α acetyltransferase mutant. As observed in the previous experiments, Tip60α strongly repressed the PGC-1α-dependent expression of the luciferase reporter (Fig. 4.3D). Also the Tip60α acetyltransferase mutant reduced the PGC-1α transcriptional activity, but about 10 times less than wildtype Tip60α. The repressive effect of Tip60α on the PGC-1α transcriptional activity was specific, since overexpression of Tip60 did not affect the GAL4-VP16 dependent expression of the luciferase reporter (Fig. 4.3D).

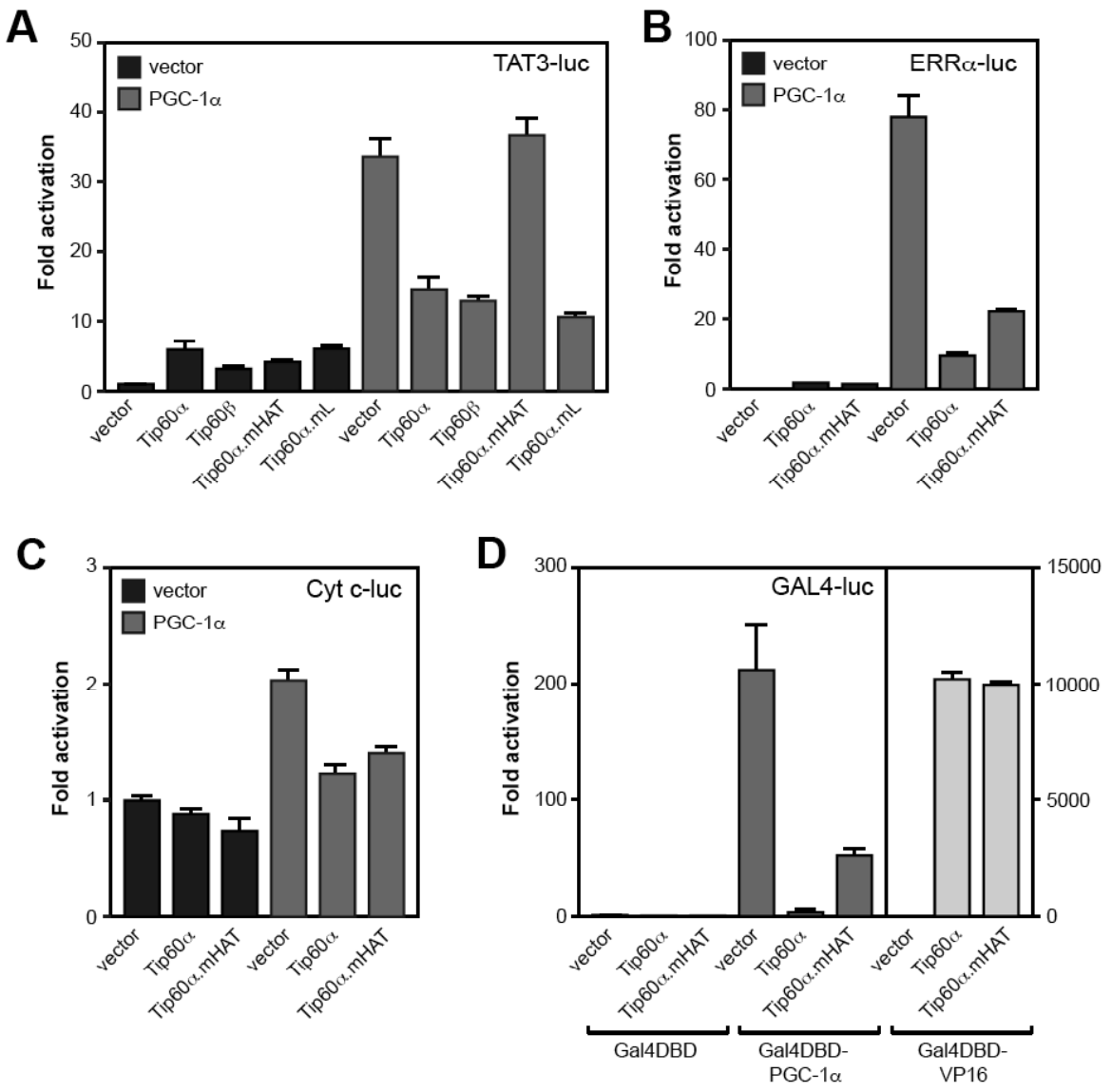

Figure 4.3. Tip60 represses PGC-1α activity.
(A) COS7 cells were transfected with a GR-responsive luciferase reporter TAT3-luc (100 ng), an expression plasmid for rat GR (100 ng), a PGC-1α expression vector (500 ng) or control vector pcDNA3, and the indicated Tip60 constructs (1 μg). 40 h after transfection, cells were treated with 50 nM corticosterone for 4 h and then assayed for luciferase activity.
(B) COS7 cells were transfected with 100 ng of a luciferase reporter regulated by the ERRa-promoter and vectors expressing PGC-1α (100 ng) and Tip60α (100 ng). Luciferase activity was measured 40 h post-transfection.
(C) As in B, except, that cells were transfected with a luciferase reporter regulated by the cytochrome c promoter.
(D) Transcriptional activities of PGC-1α or VP16 fused to the GAL4-DBD in the presence or absence of Tip60 were measured in COS7 cells using a luciferase reporter regulated by 5 copies of the GAL4-UAS. 50 ng of the GAL4-PGC-1α expression plasmid, 10ng of the GAL4-VP16, and 100 ng of the Tip60 expression vectors were transfected. Luciferase activity was determined 40 h after transfection.

Tip60 represses PGC-1a-dependent expression of endogenous genes.

We next asked, if Tip60 has also a repressive effect on the expression of endogenous PGC-1α target genes. To test this, we infected SAOS2-GR(+) cells with adenoviruses expressing GFP, PGC-1α, Tip60α, and Tip60α.mHAT. Cells were infected with the Tip60 viruses or the control virus expressing GFP on day one. The next day, cells were infected with the viruses expressing PGC-1α or GFP, respectively. We looked at the expression of cytochrome c and ERRα, two well established PGC-1α targets, under the different conditions. Overexpression of both Tip60α wildtype and acetyltransfease mutant almost completely prevented the PGC-1α-dependent induction of the cytochtome c mRNA, while there was a weaker but still significant inhibition of the ERRα mRNA induction (Fig. 4.4A). Tip60α and the Tip60α acetyltransferase mutant did not significantly reduce the Cyt c and ERRα mRNA level in the absence of PGC-1α, showing that the repressive effect was specific for PGC-1α. Figure 4.4B shows that the expression levels of PGC-1α are not reduced by the overexpression of Tip60.

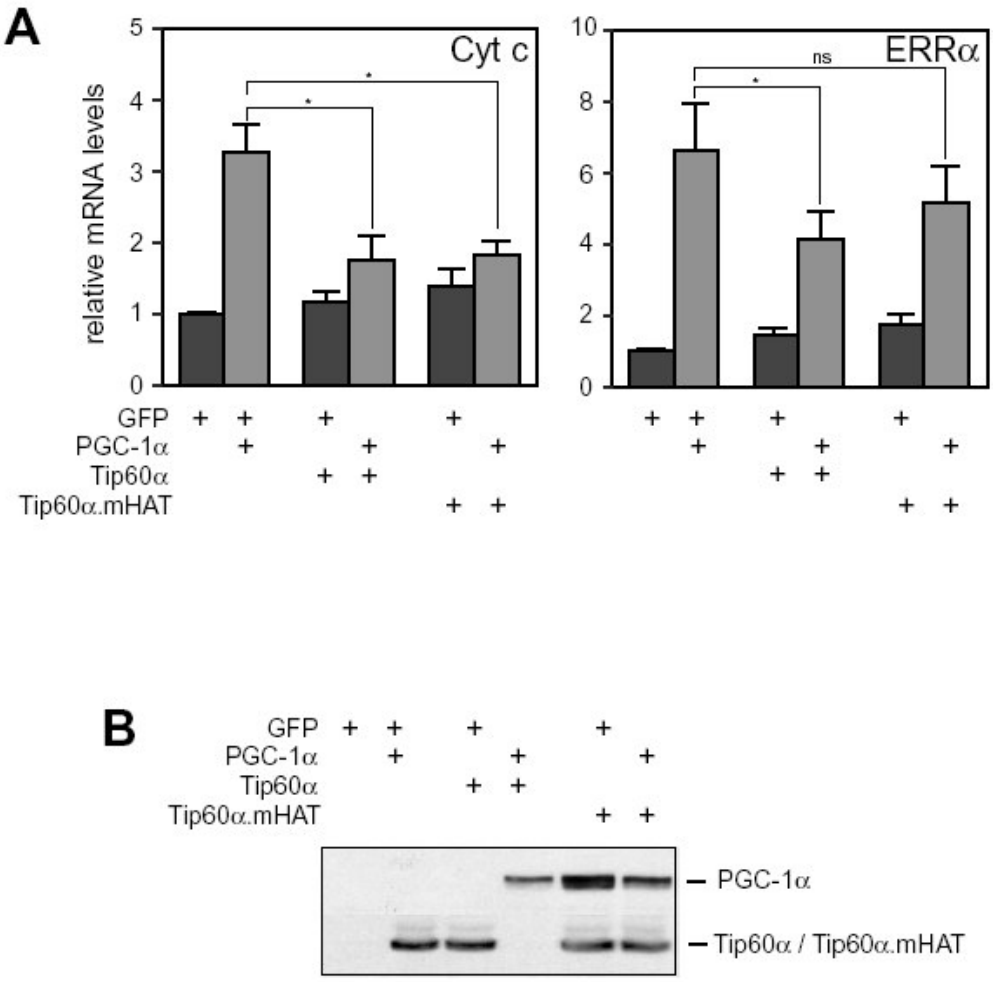

Figure 4.4. Tip60α and Tip60α.mHAT repress PGC-1α dependent gene expression.
(A) SAOS2-GR(+) cells were infected with adenoviruses expressing either GFP, 2xFlag-Tip60α or 2xFlag-Tip60α.mHAT at an moi of 50. 24 hours later, cells were infected with adenoviruses expressing GFP or HA-PGC-1α at an moi of 30. Another 24 hours later, RNA was isolated, and mRNA levels of Cyt c and ERRα were analysed by quantitative PCR, normalized to GAPDH and expressed relative to levels in cells infected with GFP alone. (* : p-value < 0.05, ns: non significant)
(B) SAOS2-GR(+) cells were infected as described in A. Protein extracts were prepared 24 hours after the infection with PGC-1α adenovirus and subjected to western blot analysis.

PGC-1α induces the expression of endogenous Tip60-regulated genes

Tip60 has been shown to enhance the expression of p21 through p53 after gamma-irradiation (Doyon et al., 2004). This effect was shown to depend on the acetyltransferase activity of Tip60. To test if PGC-1α can enhance the expression of p21, we transfected U2OS cells with adenoviruses expressing GFP or PGC-1α. 24 after infection, the cells were irradiated with 10 Gy for 17 minutes to induce the p53-dependent expression of p21. As shown in figure 4.5A, PGC-1α enhanced the expression of p21. The induction of the p21 expression by PGC-1α was more pronounced after cells had been irradiated with 10 Gy for 17 min.

We then wanted to know whether PGC-1α and Tip60α act synergistically in the enhancement of p21 expression. To this end, U2OS cells were infected with adenoviruses expressing PGC-1α and Tip60α wildtype or the Tip60α variant lacking acetyltransferase activity. Like PGC-1α, also Tip60α induced the expression of p21 after irradiation of the cells (Fig. 4.5B). Surprisingly, also the Tip60 acetyltransferase mutant induced p21 expression. Coexpression of Tip60 and PGC-1α did not lead to a higher expression of 21 that expression of either protein alone. Interestingly, overexpression of the Tip60 acetyltransferase mutant almost completely blocked the PGC-1α-dependent p21 expression, suggesting a requirement for the Tip60 acetyltransferase activity in the induction of p21 by PGC-1α (Fig. 4.5B).

Endogenous Tip60 is required for PGC-1α-dependent expression of p21

To determine the role of endogenous Tip60 in PGC-1α-dependent expression of p21, we used adenoviruses expressing siRNA to reduce Tip60 mRNA levels in U2OS cells (Fig. 4.5C). Two days later, the siRNA-treated or untreated U2OS cells were subsequently infected with adenovirus vectors encoding GFP or PGC-1α. As described above, PGC-1α expression led to increases in the levels of the endogenous p21 mRNAs. However, this induction by PGC-1α was dramatically inhibited in the cells treated with siRNA against Tip60, compared with the cells that were not exposed to the siRNA (Fig. 4.5C). Thus, endogenous levels of Tip60 contribute to the ability of PGC-1α to induce the expression of p21.

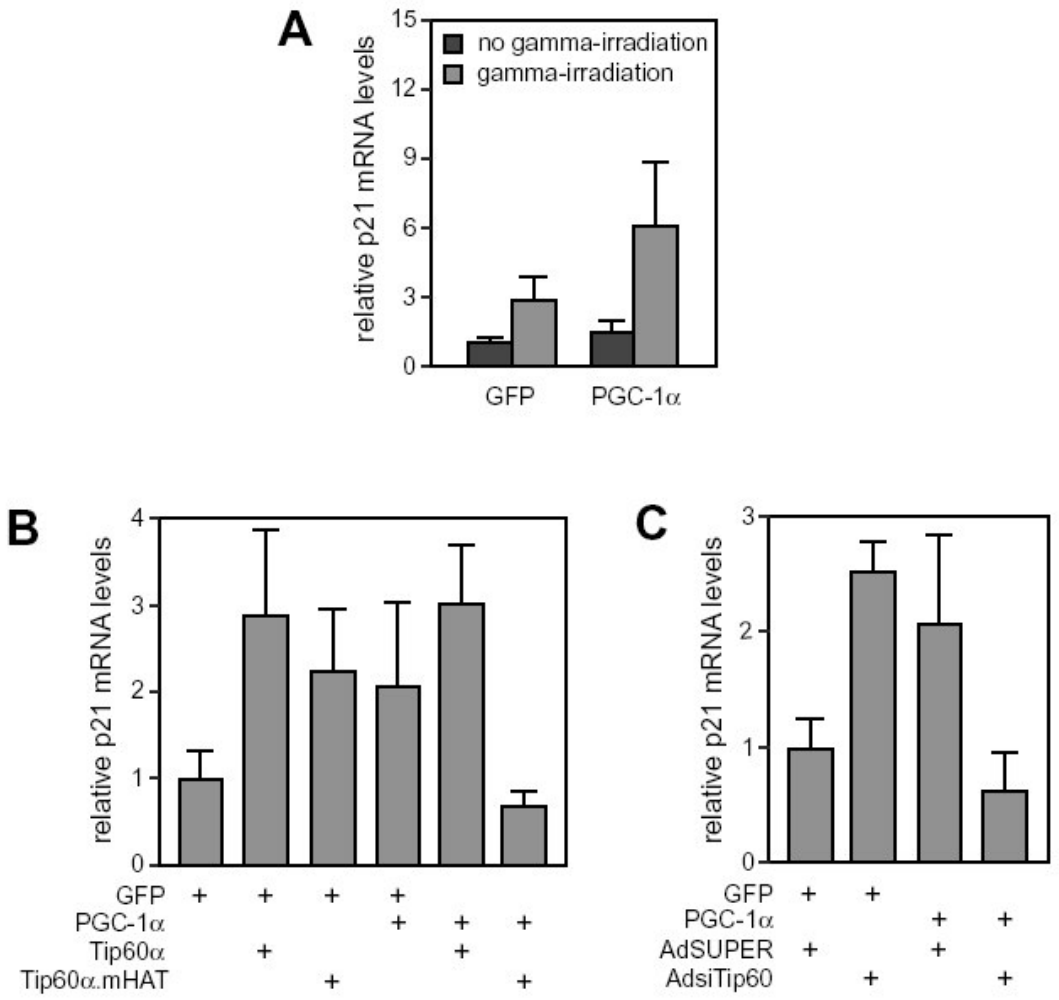

Figure 4.5. Tip60 is required for PGC-1α-mediated p21 expression.
(A) U2OS cells in 12-well plates were infected with adenoviruses expressing GFP or PGC-1α. The next day, GFP- and PGC-1α-infected cells were irradiated with 10 Gy for 17 min. 5 h later, cells were harvested and the mRNA levels for the p21gene were determined by quantitative PCR and normalized to the levels of GAPDH mRNA.
(B) U2OS cells were infected with adenoviruses expressing GFP, PGC-1α, Tip60α wild type, or Tip60α acetyltransferase mutant as shown in the figure. After the infection, cells were treated as described in (A). The mRNA levels for p21 was determined by quantitative PCR and normalized to the GAPDH mRNA levels.
(C) U2OScells were infected with adenoviruses expressing siRNA against Tip60 (AdsiTip60) or an adenovorus expressing no siRNA (AdSUPER). Two day later, cells were infected with adenoviruses expressing GFP or PGC-1α. 24 h later, cells were irradiated as described in (A) and mRNA levels for p21 was determined by quantitative PCR and normalized to the GAPDH mRNA levels.

Discussion

Gene transcription is in many cases regulated by the amounts of transcription factors that bind to regulatory sequences in the promoter region of specific genes. Identification and study of the nuclear receptor coactivator PGC-1α, a major regulator of metabolic functions in several tissues, demonstrates that the expression level of a single coactivator can lead to dramatic changes in gene expression and induce complete cellular programs. Expression levels of PGC-1α are induced by cold in brown fat and skeletal muscle, where it activates mitochondrial biogenesis and respiration. In the liver, PGC-1α is induced by fasting. This leads to increased mitochondrial β-oxidation of fatty acids and gluconeogenesis. Although the transcription activity of PGC-1α is regulated at the transcriptional level, recent work in our lab and by others has provided evidence for posttranslational regulation of this coactivator (Ichida et al., 2002; Knutti et al., 2001; Puigserver et al., 2001). Despite the the knowledge about PGC-1α that has accumulated over the past years, not much is known about the mechanisms that are used by PGC-1α to activate transcription.

In this study we describe the identification of Tip60 as a PGC-1α-interacting protein in a yeast two-hybrid screen using amino acids 294-798 of human PGC-1α as bait. We then showed that PGC-1α interacts with both Tip60α and Tip60β. Using a yeast two-hybrid assay, we were able to narrow down the Tip60α binding surface to amino acids 482-565 of PGC-1α. This region of PGC-1α doesn't contain any predicted motifs. The only function that has been assigned to this region is that it is important for the interaction of PGC-1α with the muscle specific transcription factor MEF2C (Michael et al., 2001). Overexpression of Tip60 using transient transfection or adenoviral vectors repressed PGC-1α-dependent transcription. Tip60 was first described as coactivator of several transcription factors including nuclear receptors. Besides having positive effects on transcription, Tip60 has also been shown to act as a corepressor. This repressive effect has been attributed to the recruitment of histone deacetylases by Tip60 to promoters (Lee et al., 2001; Xiao et al., 2003). We tried to block the inhibitory effect of Tip60 on the PGC-1α transcriptional activity by treating the cells with the deacetylase inhibitors TSA and nicotinamide. In the presence of those drugs Tip60 still repressed the PGC-1α activity, arguing against a involvement of histone deacetylases (data not shown). Tip60 has been

shown to be able to acetylate histones and other proteins like the androgen receptor. We constructed a Tip60 mutant (377E, 380E) that has lost its acetyltransferase activity. We found that this Tip60 mutant had in one case no effect on the PGC-1α transcriptional activity, in other cases a weaker effect that wild type Tip60, and in some experiments the same effect as wild type Tip60, depending on the experimental setup we used to measure the transcriptional activity of PGC-1α. Despite an obvious role for the acetyltransferase activity of Tip60 in the repression of PGC-1α-dependent transcription, we were not able to show conclusively that PGC-1α is acetylated by Tip60 (data not shown).

We not only observed negative effects of Tip60 on the PGC-1α transcriptional activity. Our results suggest that PGC-1α is able to induce the expression of the p53 target p21. In contrast to our other results, Tip60 does not repress the PGC-1α-dependent expression of p21, instead, Tip60 seemed to be required for the induction of the p21 expression by PGC-1α. Not only depletion of endogenous Tip60 by RNA interference blocked PGC-1α-dependent expression of p21. A similar effect was achieved by the overexpression of a Tip60 variant lacking acetyltransferase activity. Since this Tip60 mutant might act as a dominant negative protein, our results suggest that the acetyltransferase activity of Tip60 is required for the expression of p21 by PGC-1α. Further experiments will be necessary to find out how PGC-1α enhances the expression of p21. Since Tip60 is able to induce the expression of p21 in the absence of PGC-1α, it is possible that PGC-1α is recruited to the p21 promoter by direct interaction with Tip60. In a second model, PGC-1α interacts directly with transcription factors on the promoter and then recruits Tip60 for the activation of p21. In such a scenario, Tip60 would be able to enhance the expression of p21 in a PGC-1α-dependent and a PGC-1α-independent manner.

A functional interaction between PGC-1α and Tip60 in the regulation of gene expression might be a valuable target for the treatment of metabolic disorders, like diabetes, caused by an aberrant activity of PGC-1α. We have shown, that the expression levels of Tip60 can have big effects on PGC-1α-dependent gene expression. It has been published that phosphorylation of Tip60 increases its acetyltransferase activity (Lemercier et al., 2003). It is therefore possible that posttranslational modifications of Tip60 change the degree, by which it influences the PGC-1α transcriptional activity. Furthermore, two reports describe

an interaction of Tip60 with the integral membrane proteins IL9R and the endothelin receptor (Lee et al., 2001; Sliva et al., 1999). Therefore, activation of membrane associated hormone receptors may regulate the activity of PGC-1α, where Tip60 plays the messenger between the plasma membrane and the nucleus.

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CHAPTER IV SUPPLEMENTARY DATA

The C-terminal part of Tip60 interacts with PGC-1α

To determine the domain of Tip60α that mediates the interaction with PGC-1α, we tested the N-terminal domain and the C-terminal domain of Tip60α for their ability to interact with PGC-1α in a GST-pulldown experiment. The N-terminal domain of Tip60 harbors a CHROMO-domain, while the C-terminal part contains the MYST-domain with the acetyl-CoA binding motif (Fig. 1.1). As shown in figure S4.1B, *in vitro*-translated [^{35}S]methionine-labeled PGC-1α interacted with GST-fusions of Tip60α and Tip60β, while it did not interact with control GST alone. Thus, consistent with our findings from the yeast two-hybrid assay in figure 4.1A, presence of the amino acids encoded in the alternatively-spliced exon 5, present only in Tip60α, was not required or did not significantly affect the interaction with PGC-1α. Amino acids 1-147 of Tip60α fused to GST were not able to pull down PGC-1α, while amino acids 148-513 of Tip60α interacted with PGC-1α to a similar extent as full length Tip60α or Tip60β (Fig. S4.1B). This lead us to the conclusion that the MYST domain or the region between the exon 5-encoded amino acids and the MYST-domain mediates the interaction with PGC-1α. Using the same assay, we also demonstrated that the two point-mutations in the acetyl-CoA binding motif that destroy the acetyltransferase activity do not significantly affect the interaction with PGC-1α (Fig. S4.1B).

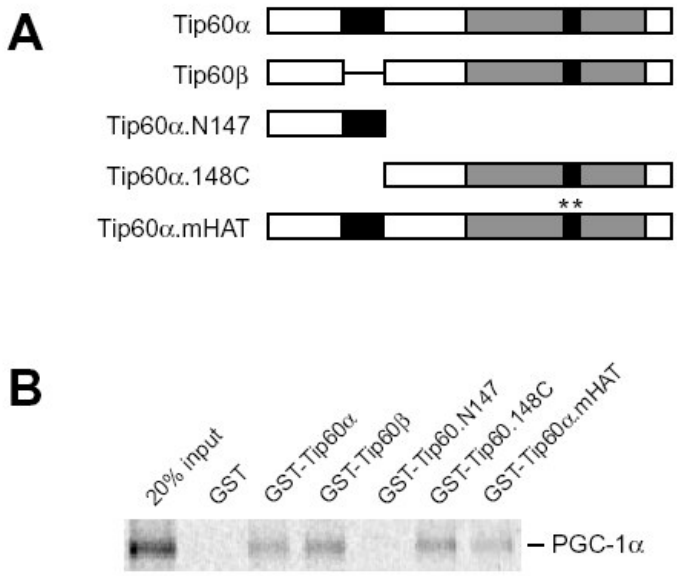

Figure S4.1. Mapping of PGC-1α-interaction domain in Tip60.
(A) Schematic presentation Tip60 variants.
Dark boxes indicate the Exon 5-encoded region and the MYST-domain with the acetyl-CoA binding motif. Asterixs indicate the Q377E and G380E mutations in the Tip60 acetyltransferase mutant.
(B) PGC-1α interacts with the C-terminal part of Tip60. Control GST protein alone or GST-fusions of the Tip60 variants shown in A were bound to glutathione agarose and incubated for 1 hour with in vitro-translated [^{35}S]-methionine-labeled PGC-1α. After the beads were washed, bound proteins were resolved by SDS-PAGE and visualized by autoradiography.

Effects of the Tip60 overexpression on PGC-1α expression

We proposed that Tip60 can be a negative regulator of the transcriptional activity of PGC-1α (chapter IV). To test the possibility that overexpression of Tip60 reduces PGC-1α-dependent transcription just by reducing the expression levels of PGC-1α, we analyzed the protein levels of PGC-1α in the presence and absence of overexpressed Tip60 variants. COS7 cells were transfected with expression vectors for PGC-1α alone or together with different Tip60 variants. 40 hours after transfection, total cells extracts were prepared by boiling the cells in SDS-lysis buffer buffer. Western blot analysis revealed that the PGC-1α protein levels were dramatically increased in the cells that overexpressed Tip60 (Fig. S4.2A). Only the Tip60 construct N147, which lacks the whole C-terminal part including the MYST domain, did not increase the PGC-1α levels. Since N147 was the only construct that did not interact with PGC-1α *in vitro*, this suggests that a direct interaction between PGC-1α and Tip60 is necessary for the Tip60-dependent increase in the PGC-1α protein levels. We also observed that the elevated levels of PGC-1α are accompanied by a appearance of a faster migrating PGC-1α species, which seems to be more abundant than the slower migrating form.

To determine whether the increased PGC-1α levels caused by overexpressed Tip60 are due to an enhanced synthesis rate of PGC-1α or due to a prolonged half-life of PGC-1α, We performed a pulse-chase experiment in COS7 cells. As shown in figure S4.2B, the half-life of PGC-1α in the absence of overexpressed Tip60 is in the range of 1.5 hours, in agreement with a previous report (Puigserver et al., 2001). Overexpression of Tip60α did not significantly increase the levels of radioactively labeled PGC-1α at timepoint zero, arguing against an enhanced synthesis rate of PGC-1α. However, overexpression of Tip60α dramatically prolonged the half-life of PGC-1α to more than 4 hours. In this experiment, it was also apparent that Tip60α overexpression induces a faster migrating PGC-1α species.

Analysis of PGC-1α protein in earlier studies (data not shown), did not show clear differences in the levels of PGC-1α in extracts from cells not overexpressing Tip60 and from cells overexpressing Tip60. The major difference of those experiments with the

experiments shown in figure S4.2A is that the cell extracts were prepared using an NP-40 lysis buffer and that only the soluble fraction was analyzed. The fact that the elevated PGC-1α levels caused by the overexpression of Tip60 could only been seen in extracts with complete extraction of all nuclear proteins suggested that overexpression of Tip60 leads to an accumulation of PGC-1α protein in an NP-40-insoluble cellular/nuclear compartment. Therefore, we next determined the ratios of the PGC-1α levels in the NP-40-soluble and insoluble fraction. To this end, COS7 cells were transfected with PGC-1α alone or cotransfected with PGC-1α and various Tip60 variants. Cells were then lysed with NP40 lysis buffer. Both the NP-40-soluble and insoluble fractions of the lysates were boiled in SDS-PAGE sample buffer and analyzed by western blot. As shown figure S4.2C, the steady state levels of the NP-40-soluble PGC-1α did not change dramatically upon overexpression of Tip60, but there was a dramatic increase in the amounts of PGC-1α protein in the NP-40-insoluble fraction. Most interestingly, the majority of the insoluble PGC-1α consisted of the faster migrating PGC-1α species. The appearance of a new PGC-1α form caused by the overexpression of Tip60 could be the result of a posttranslational modification of PGC-1α. These results suggest that this posttranslational modification leads to the accumulation of PGC-1α in the insoluble fraction. However, it is also possible that the posttranslational modification is a consequence of the accumulation of PGC-1α in a new, insoluble compartment.

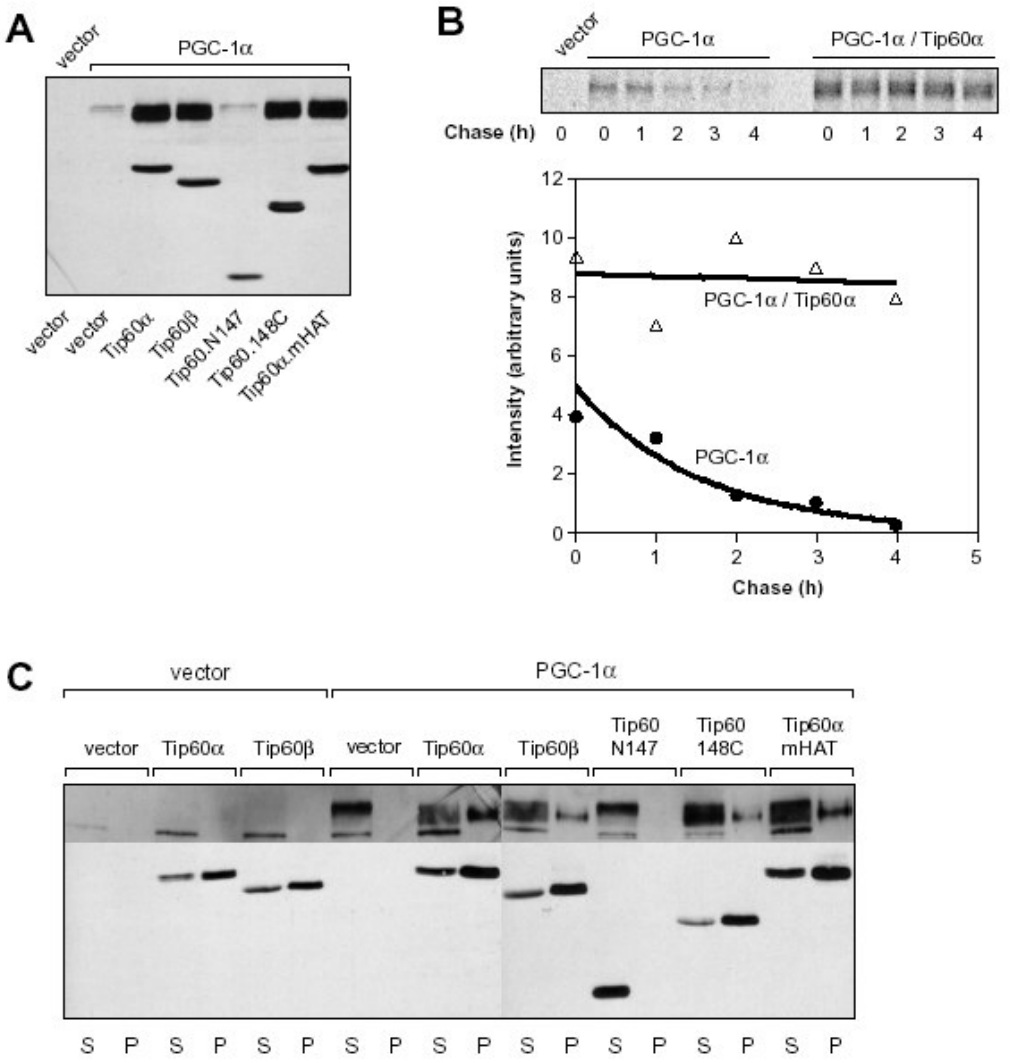

Figure S4.2. Tip60 stabilizes PGC-1α protein.
(A) Overexpression of Tip60 increases the steady-state protein levels of PGC-1α.
COS7 cells were cotransfected with expression vectors for PGC-1α and various Tip60 variants. Total protein extracts were prepared by boiling the cells in SDS-lysis buffer. Expression levels of PGC-1α and the Tip60 constructs was determined by western blot analysis.
(B) The half-life of PGC-1α is prolonged by Tip60 overexpression.
COS7 cells were transfected with a plasmid expressing PGC-1α, alone, or together with a plasmid expressing Tip60α. A pulse-chase experiment was used to determine the half-life of PGC-1α in the absence and presence of overexpressed Tip60α.
(C) Tip60 overexpression leads to the accumulation of PGC-1α in an NP-40 insoluble cellular compartment.
COS7 cells were transfected with expression vectors for PGC-1α and various Tip60 constructs. 40 h post transfection cells were lysed in NP-40 lysis buffer. Both NP-40 and NP-40 insoluble cell material was subjected to western blot anylysis.

Can Tip60 acetylate PGC-1α *in vitro* ?

The repressive effect of Tip60 on the PGC-1α transcriptional activity depends in some cases entirely, in some cases partially on the acetyltransferase activity of Tip60. These results prompted us to test whether PGC-1α could be a substrate for the acetyltransferase activity of Tip60. In a first experiment, COS7 cells were transformed with the PGC-1α expression construct alone or in combination with a plasmid expressing Tip60α or the Tip60α acetyltransferase mutant. 40 h post-transfection, cell lysates were prepared and immunoprecipitated with an anti-PGC-1α antibody. Immunocomplexes were resolved by SDS-PAGE and subjected to western blot analysis using an anti acetyl-lysine antibody. No band corresponding to the size of PGC-1α could be detected (data not shown). The reactivity of antibodies raised against acetylated-lysine residues can be strongly influenced by the amino acids surrounding an acetylated lysine. In can happen that an acetylated lysine residue is not recognized. Thus, the lack of detection of acetylation in this assay was not conclusive.

We therefore chose an in vitro approach to detect a possible acetylation of PGC-1α by Tip60. Fusions of three different PGC-1α constructs to MBP, and fusions of Tip60α and the Tip60α acetyltransferase mutant to GST were expressed in E. coli and purified using amylose and glutathione beads, respectively. MBP and the MBP-PGC-1α constructs were then incubated with GST-Tip60α or GST-Tip60α.mHAT in the presence of tritium labeled acetyl-coenzyme A, which served as acetyl donor in this reaction, thereby allowing an easy detection of acetylated proteins by autoradiography. As shown in figure S4.3, Tip60α strongly acetylated histones, which are well established substrates of Tip60 (Creaven et al., 1999; Kimura and Horikoshi, 1998; Lemercier et al., 2003; Yamamoto and Horikoshi, 1997). As expected, the Tip60 acetyltransferase mutant did not acetylate histones. No acetylation of control MBP or full length PGC-1α could be detected. A very faint band, which may correspond to the PGC-1α N565 construct, was visible. This band could not be detected, when the Tip60 acetyltransferase was used.

These results suggest that PGC-1α is not a good substrate for the Tip60 acetyltransferase activity *in vitro*. Further experiments *in vivo* with other detection methods than anti-

acetyllysine antibodies will be necessary to show a possible acetylation of PGC-1α by Tip60. An efficient acetylation of PGC-1α may require the whole Tip60 complex, as shown for the acetylation of chromatin templates by Tip60 (Ikura et al., 2000).

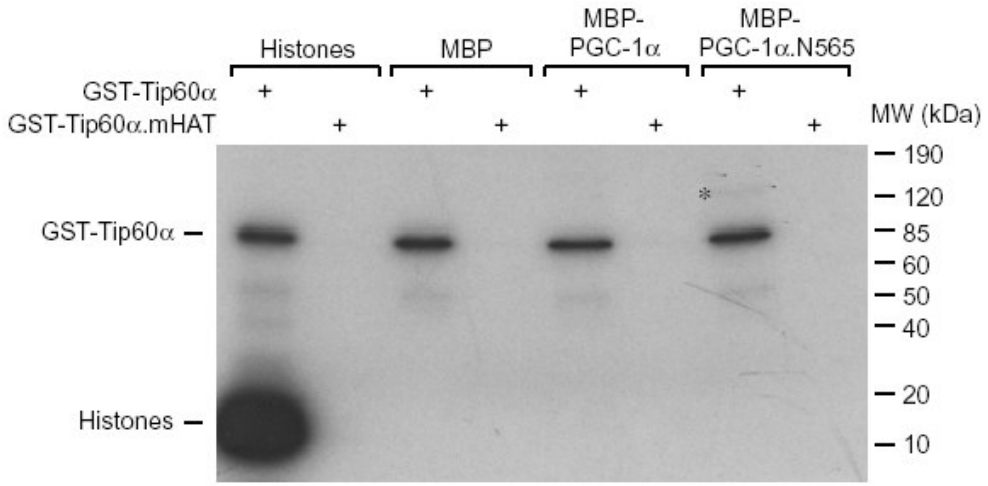

Figure S4.3. Acetylation of PGC-1α by Tip60α *in vitro*.
Histone proteins, maltose binding protein (MBP), alone, or fused to full length PGC-1α, or amino acids 1-565 of PGC-1α (N565) were incubated with GST-fusions of Tip60α wild type or acetyltransferase mutant and [^{3}H]-labeled acetyl-CoA. After reactions had been stopped, proteins were resolved by SDS-PAGE and acetylated proteins were detected by autoradiography. Asteriks indicates a band that may correspond to acetylated MBP-PGC-1α.N565.

Regulation of other non-NR transcription factors by PGC-1α and Tip60

Our results have shown that overexpression of Tip60 reduces PGC-1α-dependent gene expression in several experiments. However, we also found that PGC-1α enhances the expression of p21, which requires Tip60. Since Tip60 has been shown to be a coactivator for several transcription factors, we wanted to know whether PGC-1a can enhance the expression of genes other than p21 that have been previously shown to be activated by Tip60.

Tip60 has also been reported to be in a ternary transcriptionally active complex together with the intracellular domain of the amyloid precursor protein (APP) and Fe65. The very low transcriptional activity of a GAL4-DBD fusion of Tip60 was shown to be dramatically enhanced by the simultaneous overexpression of APP and Fe65 but not by either of the two proteins alone (Cao and Sudhof, 2001). We were able to reproduce the results of Cao and Sudhof, but no further activation of the Tip60/APP/Fe65-complex by PGC-1α was observed. PGC-1α rather seemed to have a negative, if any role, in the activity of the Tip60/APP/Fe65-complex (Fig. S4.4.A)

Two reports suggest that Tip60 is a coactivator for homodimers of the NFκB-family transcription factor p52, where Bcl-3 is thought to mediate the interaction of p52 and Tip60 (Baek et al., 2002; Dechend et al., 1999). To test whether PGC-1α acts synergistically with Tip60 and Bcl-3 in the activation of a p52-regulated promoter, we transformed COS7 cells with a luciferase reporter under control of the P-selectin promoter, an expression vector for p52, and plasmids expressing Tip60α, Bcl-3, and PGC-1α. As shown in Figure S4.4B, PGC-1α, either alone or in combination with Tip60α and Bcl-3, did not coactivate p52.

A recent report suggested that Tip60 is actively involved in the activation of the 45S ribosomal pre-RNA through acetylation of the transcription factor UBF1, as well as through the direct recruitment of Tip60 to the human ribosomal gene promoter (Halkidou et al., 2004). Recruitment of Tip60 to the ribosomal RNA promoter after release of cells from starvation was followed by acetylation of histone H4 at this promoter, suggesting that Tip60 not only acetylated UBF but also histone H4. In support of these findings, the yeast

homologue of Tip60 has been shown to play a role in the regulation of the transcription of ribosomal RNA. PGC-1α has been shown to induce cellular programs that allow the cells to adapt to limited energy availability. We therefore considered a model, in which PGC-1α represses ribosomal RNA synthesis by downregulating the Tip60 activity. To test this model, we serum-starved SAOS2 cells overnight to shut off the ribosomal RNA synthesis and to induce the release of Tip60 from the promoter. Occupancy of the rRNA promoter by Tip60 and acetylated histone H4 in cells expressing no PGC-1α and cells overexpressing PGC-1α was measured before and after incubation of the cells in serum containing medium using chromatin immunoprecipitation assays. We were not able to show Tip60 binding to the rRNA promoter under any conditions. We detected acetylated histone H4 associated with the rRNA promoter, but the levels did not differ significantly under no-serum and serum conditions. Treatment of cells with rapamycin, a strong inhibitor of rRNA synthesis (Mayer et al., 2004), also did not reduce the acetylation of histone H4. In addition, no reproducible effect on the level of histone H4 was observed (data not shown). In conclusion, our experiments did not provide any support for a role of Tip60 or PGC-1α in RNA polymerase I-regulated transcription. However, not all possible optimizations of the experimental setup have been tried, and it may still be worth running these experiments under different conditions.

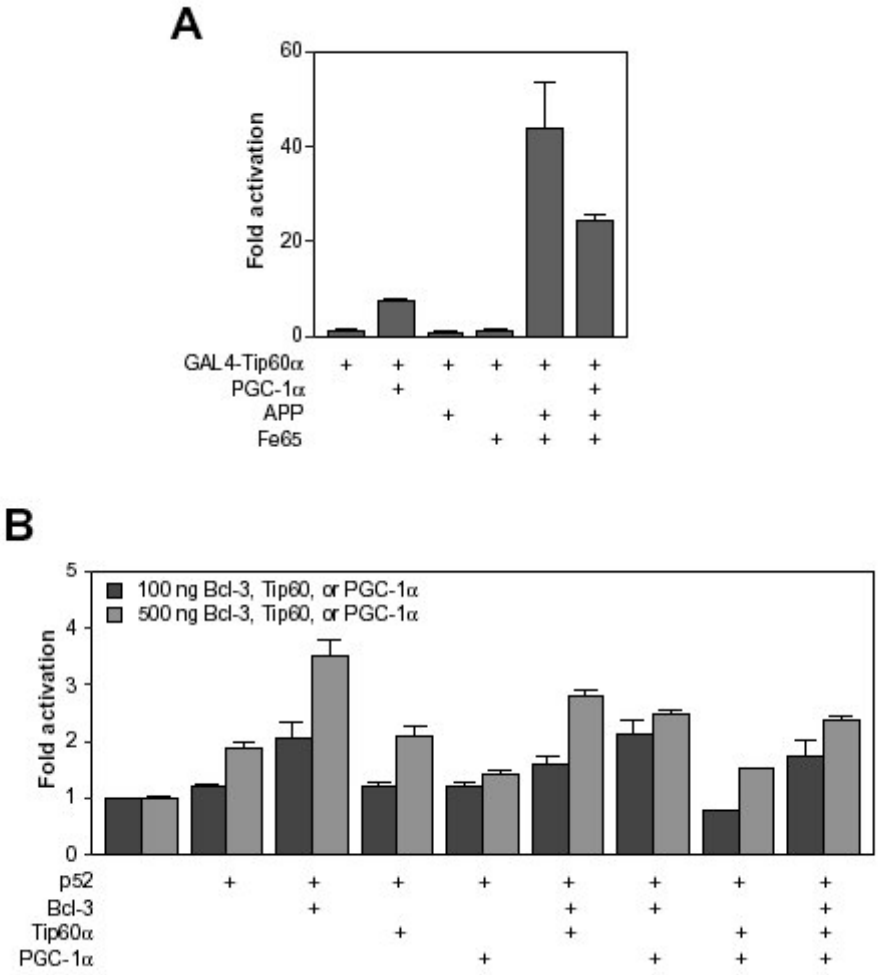

Figure S4.4. Regulation of Tip60-mediated transcription by PGC-1α.
(A) PGC-1a does not further enhance the transcriptional activity of the Tip60, APP, Fe65 complex. COS7 cell were transfected with a p52 regulated luciferase reporter construct and expression vectors for GAL4-Tip60α, PGC-1α, APP, and Fe65 as indicated. 40 h post transfection, luciferase activity was determined. The Results show the enhancement of the luciferase activity. Results in the presence of GAL4-Tip60α was set equal to 1.
(B) PGC-1α is not able to coactivate the NFκB transcription factor p52.
COS7 cells were transfected with a GAL4-regulated luciferase reporter plasmid and expression vectors for the proteins shown in the figure. 40 h after transfection, cells were assayed for luciferase and β-galactosidase activity. Results are expressed as fold enhancement of the expression of the reporter construct. Results in the presence of p52 alone was set equal to 1.

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CHAPTER V: ACTIVATION OF NUCLEAR RECEPTOR COACTIVATOR PGC-1α BY ARGININE METHYLATION

(Submitted Manuscript)

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Abstract

Peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) is a tissue-specific and inducible transcriptional coactivator for several members of the nuclear receptor family and plays a key role in energy metabolism. PRMT1, the major mammalian protein arginine methyltransferase, is also known to be a nuclear receptor coactivator. We report here that the activity of PGC-1α is potentiated by PRMT1, in a manner dependent on the specific methyltransferase activity of PRMT1. A glutamate-rich region upstream from the RNA binding motif in the C-terminal domain of PGC-1α is methylated by PRMT1 in vitro. Mutations of three substrate arginines in this region of PGC-1α abolish the cooperative function of PGC-1α and PRMT1, and compromise the ability of PGC-1α to induce endogenous target genes. Finally, we show that endogenous PRMT1 contributes to PGC-1α coactivator activity, and to the induction of genes important for mitochondrial biogenesis. Thus, the function of PGC-1α is regulated by arginine methylation.

Keywords: arginine methylation/ coactivator/ mitochondrial biogenesis/ nuclear receptor/ transcription

Running title: Regulation of PGC-1α by arginine methylation

Subject Categories: *Chromatin & Transcription/ Proteins*

Introduction

Peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) is a transcriptional coactivator for many members of the nuclear hormone receptors (NRs) superfamily, including peroxisome proliferator-activated receptor γ (PPARγ), the thyroid hormone receptors (TR), retinoid acid receptors, glucocorticoid receptor (GR), and estrogen receptor (ER), as well as other types of DNA-binding transcription factors such as nuclear respiratory factor 1 (NRF-1) (Knutti & Kralli 2001; Puigserver & Spiegelman 2003). PGC-1α harbors leucine-rich motifs (also called LXXLL motifs or NR boxes), which bind to the hormone-activated NRs. The N-terminal region contains a strong autonomous activation domain, which binds to other well-known coactivators such as SRC-1 and CBP and is required for the coactivator function of PGC-1α (Puigserver *et al,* 1999). The C-terminal region of PGC-1α contains an RNA recognition motif (RRM) and an RS domain (rich in Arg and Ser residues), both of which are characteristic of proteins involved in splicing, such as hnRNP and SR proteins. Indeed, PGC-1α not only functions as a transcriptional coactivator, but can also influence splicing patterns of specific transcripts (Monsalve *et al,* 2000). Deletions within the C-terminal domain disrupt the ability of PGC-1α to associate with RNA polymerase II, RNA processing factors and the Mediator, and to induce target genes, suggesting that this region contains both transcriptional coactivator and splicing regulator functions (Monsalve *et al,* 2000; Wallberg *et al,* 2003).

PGC-1α mRNA is predominantly expressed in heart, brown adipose tissue, kidney, skeletal muscle, and liver (Puigserver *et al,* 1998; Knutti *et al,* 2000), and its expression is induced in response to stimuli known to regulate metabolic activity. For example, PGC-1α expression is induced in brown adipose tissue and skeletal muscle by exposure to cold (Puigserver *et al,* 1998; Boss *et al,* 1999), in muscle in response to physical exercise (Goto *et al,* 2000), and in the liver and heart in response to short-term fasting (Lehman *et al,* 2000; Herzig *et al,* 2001; Yoon *et al,* 2001). In accordance with the nature of the stimuli that induce its expression, PGC-1α regulates a broad range of metabolic processes, including mitochondrial biogenesis, respiration, and gluconeogenesis, and thus enables physiologic adaptation to the energy needs imposed by the stimuli (Knutti & Kralli 2001; Puigserver & Spiegelman 2003; Kelly & Scarpulla 2004). These effects of PGC-1α are

mediated by several DNA-binding transcription factors. Mitochondrial biogenesis and the stimulation of oxidative phosphorylation in particular require the cooperation of PGC-1α with the nuclear respiratory factors, NRF-1 and NRF-2, and the orphan nuclear receptor ERRα (estrogen-related receptor α) (Wu *et al,* 1999; Schreiber *et al,* 2004; Mootha *et al,* 2004). PGC-1α is an essential coactivator for ERRα, enabling the induction of the ERRα gene, which is auto-regulated in a positive feedback loop, as well as the induction of other ERRα target genes that encode proteins important for mitochondrial biogenesis and function (Huss *et al,* 2002; Schreiber *et al,* 2003; Schreiber *et al,* 2004; Laganiere *et al,* 2004; Mootha *et al,* 2004).

As the major protein arginine methyltransferase (PRMT) in mammalian cells (Tang *et al,* 2000a), PRMT1 methylates many RNA-binding proteins (Rajpurohit *et al,* 1994; Liu & Dreyfuss 1995; Li *et al,* 2002; Cote *et al,* 2003), as well as histone H4 (Chen *et al,* 1999; Strahl *et al,* 2001; Wang *et al,* 2001). Arginine methylation has been implicated in the regulation of many cellular processes (Lee *et al,* 2004), including signal transduction (Bedford *et al,* 2000; Tang *et al,* 2000b; Mowen *et al,* 2001), subcellular localization of proteins (McBride *et al,* 2000; Yun & Fu 2000), protein-protein interactions (Bedford *et al,* 2000; Friesen *et al,* 2001), and transcriptional regulation (Wang *et al,* 2001; Xu *et al,* 2001; Ma *et al,* 2001; An *et al,* 2004). Indeed, PRMT1 serves as a coactivator for NRs (Koh *et al,* 2001) and other DNA-binding transcription factors, such as p53 (An *et al,* 2004) and YY1 (Rezai-Zadeh *et al,* 2003). Once recruited to the target gene promoter region through direct or indirect association with the DNA-bound transcription factor, PRMT1 cooperates with other histone-modifying enzymes to lead to chromatin remodeling and transcriptional activation (Koh *et al,* 2001; Metivier *et al,* 2003; An *et al,* 2004).

Since PGC-1α contains an RNA-binding domain and many RNA-binding proteins are substrates for PRMT enzymes, we tested whether PGC-1α could be methylated on arginine residues by PRMT1 or the related PRMT family member CARM1 (Chen *et al,* 1999). Furthermore, because some PRMTs act as coactivators for NRs and other transcription factors, we investigated whether PGC-1α could cooperate with PRMTs as coactivators.

Materials and Methods

Plasmids

The following plasmids were previously described: pcDNA3-HA-PGC-1α encoding full-length human PGC-1α (Knutti *et al,* 2000); pSG5.HA-PRMT1 encoding HA epitope-tagged PRMT1 (Koh *et al,* 2001); GST-PRMT1, encoding a GST-PRMT1 fusion protein (Lin *et al,* 1996); pSG5.HA-CARM1 encoding HA-CARM1 and pGEX4T1.CARM1 encoding GST-CARM1 (Chen *et al,* 1999); pHE0, encoding human estrogen receptor α (Green *et al,* 1988); and the luciferase reporter plasmid MMTV(ERE)-LUC (Umesono & Evans 1989).

Construction of plasmids encoding fragments of PGC-1α fused to GST: PCR amplified fragments, representing the following PGC-1α fragments aa 1-306; aa 306-532; aa 532-640 and aa 640-798, were inserted into the BamHI and XhoI sites of pGEX4T1; pGEX4T3-RS encoding PGC-1α(566-632), pGEX4T3-E encoding PGC-1α(632-676), and pGEX4T3-RRM encoding PGC-1α(676-755) were constructed by inserting the appropriate DNA fragments, amplified from pcDNA3-HA-PGC-1α as a template and with primers containing BamHI and SalI sites, into the BamHI and SalI sites of pGEX4T3. The following mutations were generated with the Quickchange site-directed mutagenesis kit (Stratagene), using the corresponding wild type plasmids as templates: pSG5.HA-PRMT1(E153Q); pGEX4T3-E(R4K), containing R644K, R647K, R651K, and R652K mutations; pGEX4T3-E(R3K), containing R665K, R667K, and R669K mutations; pcDNA3-HA-PGC-1α(R3K). Construct pcDNA3-HA-PGC-1α.ΔC (Δ566-755), has been generated by fusion PCR and verified by sequencing. To construct the luciferase reporter pERRα-Luc, the ERRα-responsive sequences of the ESRRA promoter (nt –537 to –829 nt, relative to transcription initiation site) were amplified by PCR and subcloned upstream of the minimal alcohol dehydrogenase promoter of pΔLuc (Iñiguez-Lluhí *et al,* 1997).

Cell Culture and Transfection

CV-1 and COS7 cells (Gluzman 1981) were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and seeded in 12-well dishes for transient transfections as described previously (Ma *et al,* 1999). pSG5.HA and pcDNA3.1 empty vectors were added to all transfections to balance the total amount of

DNA used for each transfection. After transfection, cells were grown in medium supplemented with 5% charcoal-stripped fetal bovine serum for 40 h before harvest; where indicated, medium was supplemented with 20 nM estradiol (E2) during the last 30 h of growth. Luciferase assays were performed by using the Promega Luciferase Assay Kit according to manufacturer's protocols. Luciferase activity is shown as the mean and range of variation of two transfected cell cultures. Because some coactivators enhance the activities of so-called constitutive promoters, constitutive reporter plasmids (e.g. β-galactosidase) were not used to normalize the luciferase activities. Instead, such internal controls were used strategically to monitor transfection efficiency.

Adenovirus infections and RNA analysis

Adenoviral vectors expressing GFP and PGC-1α were used to infect SAOS2 cells as described previously (Schreiber *et al,* 2003). Adenoviruses expressing PGC-1α R3K were constructed by using the insert of pcDNA3-HA-PGC-1α R3K. Total RNA was isolated using the TRIzol reagent (Invitrogen), cDNA was synthesized with the SuperScript Choice system (Invitrogen), and specific transcripts were quantified by quantitative real-time PCR using the SYBR Green PCR system (Applied Biosystems) and the Chromophore detection system (MJ Research) (Fig. 5A) or the ABI PRISM® 7700 Detection System (Fig. 5B). Gene-specific primers were described previously for human ERRα (Schreiber *et al,* 2003) and for human cytochrome c (Schreiber *et al,* 2004). For human GAPDH the sequences of the PCR primers used were 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-GAAGATGGTGATGGGATTTC-3'. ERRα and Cytc mRNA levels have been normalized to GAPDH mRNA levels, and estimated using standard curves (Schreiber *et al,* 2004) (Fig. 5A) or the □□Ct method cited in ABI User Bulletin #2 (Fig. 5B).

Methylation assays

Substrates and enzymes were prepared as recombinant GST fusion proteins and eluted from glutathione-agarose beads (Sigma) with 20 mM glutathione (Koh *et al,* 2001). The methylation assays were performed in HMT Buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 0.4 mM EDTA) as follows: 1 μg of GST fused to PGC-1α full-length or fragments was incubated with 1 μg of GST-PRMT1 or GST-CARM1 and 7 μM S-adenosyl-L-[^{3}H-methyl]methionine ([^{3}H]AdoMet: specific activity 14.7 Ci/mmol) in 30 μl of HMT buffer

for 1 h at 30 °C. Reactions were stopped by addition of SDS sample buffer and analyzed by 12% SDS-PAGE and fluorography.

RNA interference and immunoblots

The following siRNA oligonucleotides for human PRMT1 were designed using the Target Finder program (Ambion), and chemically synthesized by the USC Norris Comprehensive Cancer Center Microchemical Core: siRNA#1 sense: 5'-AGAUUACUACUUUGACUCCdTdT-3'; siRNA#1 antisense: 5'-GGAGUCAAAGUAGUAAUCUdTdT-3'; siRNA#2 sense: 5'-CUUAUGUUUUUAUAUGGUUdTdT-3'; siRNA#2 antisense: 5'-AACCAUAUAAAAACAUAAGdTdT-3'. Annealed siRNAs were transfected with lipofectamine 2000 (Invitrogen), according to manufacturer's protocol, into COS7 cells seeded in 24-well plates, 48 h before DNA transfection. Luciferase assays were performed 48 h after DNA transfection. Parallel cell extracts were prepared at the same time in SDS sample buffer, separated by 12% SDS-PAGE and transferred on PVDF membrane. Immunoblots were then performed with the anti-PRMT1 antibody (Abcam), and after stripping, with antibody against β-actin (Santa Cruz). For SAOS2 cells, siRNA#1 was transfected with lipofectamine 2000 24 h before infection with virus at a multiplicity of infection of 10. Total RNA was isolated 24 h after virus infection and analyzed by quantitative real-time PCR as described above.

Results

PRMT1 and its enzymatic activity stimulate PGC-1α coactivator activity

As demonstrated previously (Knutti *et al,* 2000; Tcherepanova *et al,* 2000), PGC-1α can enhance the activity of ERα on a reporter plasmid controlled by estrogen responsive enhancer elements (Fig. 5.1). A 2-fold enhancement of reporter gene expression was observed when PGC-1α was co-expressed with ERα. Co-expression of PRMT1 with PGC-1α dramatically increased the reporter gene activity observed with PGC-1α alone or PRMT1 alone. In contrast, co-expression of another coactivator in the arginine methyltransferase class CARM1 (Chen *et al,* 1999; An *et al,* 2004), had little or no effect on PGC-1α activity. The functional synergy between PGC-1α and PRMT1 was completely dependent on the integrity of the LXXLL motifs of PGC-1α, which are responsible for binding hormone-activated ERα, and the N-terminal activation domain of PGC-1α (Supplement Fig. 1). Thus, the effect of PRMT1 in this case depends entirely on the integrity of the coactivator functions of PGC-1α, suggesting that PRMT1 is amplifying the coactivator function of PGC-1α. The fact that PGC-1α and PRMT1 cooperated to enhance the transcriptional activity of other NRs as well, such as GR and TR (Supplement Fig. 2), suggests that PRMT1 stimulates the coactivator function of PGC-1α in a variety of transcriptional complexes.

To test whether the methyltransferase activity of PRMT1 is required for its synergistic coactivator function with PGC-1α, we used an enzymatically deficient mutant of PRMT1. X-ray crystal structures of PRMT1 and PRMT3 (Zhang *et al,* 2000; Zhang & Cheng 2003) indicated that two Glu residues that are highly conserved in the PRMT family form salt bridges with the two terminal guanidino group nitrogen atoms of the substrate arginine residue. In PRMT1, mutation of either of these conserved residues, Glu144 and Glu153, to Gln completely or nearly abolishes enzymatic activity (Zhang & Cheng 2003) (Supplement Fig. 3). The PRMT1 E153Q mutant failed to stimulate PGC-1α coactivator activity (Fig. 1), although it was expressed at levels comparable to that of wild type PRMT1 (Supplement Fig. 3). Thus, the specific protein arginine methyltransferase activity of PRMT1 (but not CARM1) is necessary for the enhancement of coactivator function of PGC-1α. The requisite substrate that is methylated by PRMT1 to cause the enhancement

of PGC-1α activity could be histone H4 (Chen *et al,* 1999; Wang *et al,* 2001) or a component of the transcription machinery that has not been previously characterized as a PRMT1 substrate, including PGC-1α itself.

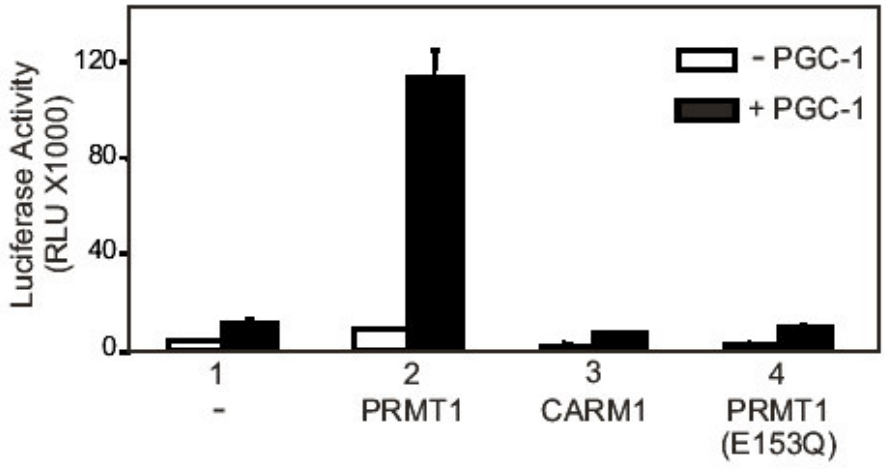

Figure 5.1. Specific and cooperative coactivator function between PGC-1α and PRMT1.
CV-1 cells were transiently transfected with MMTV(ERE)-LUC reporter plasmid (250 ng) and expression vectors encoding ER (0.1 ng), PGC-1α (50 ng) and PRMT1 wild type, PRMT1 E153Q mutant or CARM1 (250 ng). Transfected cells were grown in culture medium with 20 nM estradiol, and extracts of the harvested cells were tested for luciferase activity. The results presented are from a single experiment representative of four independent experiments.

PRMT1 methylates PGC-1α

Since PGC-1α contains an RNA binding domain and many RNA binding proteins are known to be substrates for arginine methylation, we tested whether PGC-1α could be methylated by PRMT1. For this, recombinant PGC-1α fused to GST was incubated with bacterially-expressed GST-PRMT1 in the presence of [^{3}H]AdoMet. As seen in Fig. 2A, PRMT1 led to the methylation of PGC-1α. In contrast, the methyltransferase CARM1 was not able to methylate PGC-1α (Fig. 2A), even though it was active on other CARM1 substrates (data not shown). The differing abilities of the two methyltransferases to modify specifically PGC-1α is in agreement with the finding that PRMT1, but not CARM1, enhances PGC-1α activity.

To map the region that becomes methylated, recombinant GST fusion proteins representing various fragments of PGC-1α were incubated with GST-PRMT1 in the presence of [^{3}H]AdoMet (Fig. 2B). While N-terminal fragments containing amino acids 1 to 306 or 306 to 532 were not methylated, PRMT1 methylated two different C-terminal fragments of PGC-1α (amino acids 532-640 and a subfragment of amino acids 640-798). Note that the full length fragment 640-798 was not methylated but a truncated fragment was (asterisk in last lane of Fig. 2B, left panels). Since the GST protein is fused to the N-terminal part of the PGC-1α fragments and these GST fusion proteins were isolated by binding to glutathione-agarose beads, the methylated PGC-1α fragment apparently represents only the N-terminal portion (corresponding to the E or Glu-rich region) of amino acids 640-798. The lack of methylation of the full-length PGC-1α (640-798) fragment suggests that folding of the fragment may make the methylation site in the N-terminal portion of this fragment inaccessible. The C-terminal portion of PGC-1α was further subdivided into three fragments representing discreet domains of PGC-1α. The acidic E region and the RS region were both substrates for PRMT1, but the RNA recognition motif (RRM) by itself was not (Fig. 2B, right panels).

We focused on identifying the methylated residues of the E domain, because deletion of this small region abolished the enhancement of PGC-1α coactivator activity by PRMT1, whereas deletion of the RS region did not eliminate the functional synergy between these two coactivators (Supplement Fig. 4); these results suggest that the E region plays an important role in the stimulation of PGC-1α activity by PRMT1. Two groups of Arg residues within the E region were modified to Lys. A mutant GST-E(R4K) fragment with Lys substitutions at Arg residues 644, 647, 651 and 652 was methylated efficiently by PRMT1 (Fig. 2C), at levels indistinguishable to the wild type GST-E fragment (data not shown). However, conversion of Arg residues 665, 667 and 669 to lysines in the GST-E(R3K) mutant fragment completely abolished methylation by PRMT1 (Fig. 2C), showing that these residues are important for the methylation and presumably contain the methylation site(s). Individual lysine substitutions at Arg residues 665, 667, and 669 failed to eliminate the methylation of GST-E, suggesting that more than one of these three residues are methylation sites for PRMT1 (data not shown).

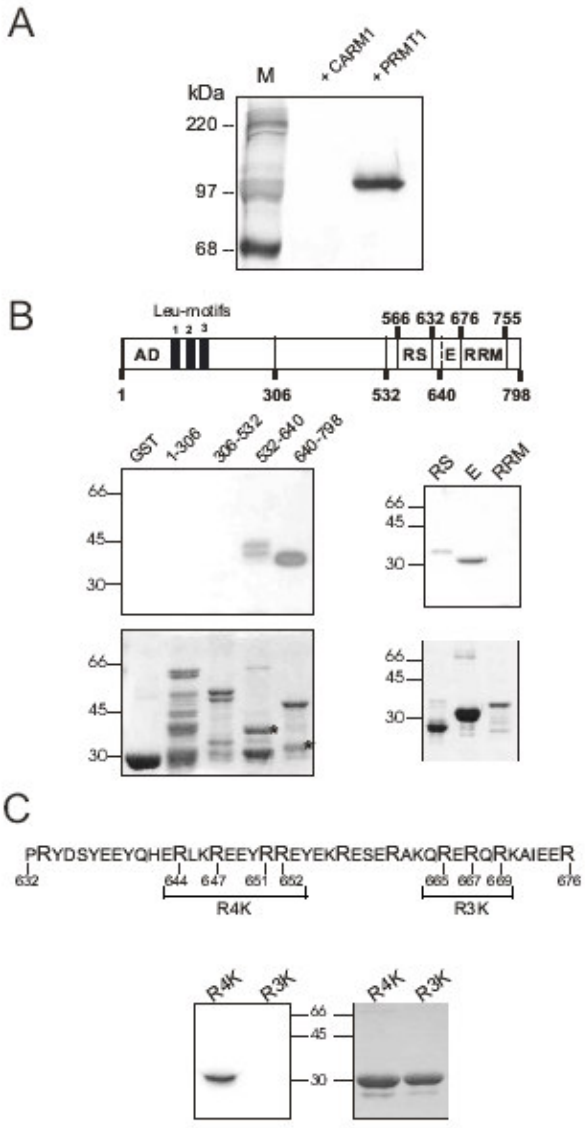

Figure 5.2. Methylation of PGC-1α by PRMT1.
(A) Full-length PGC-1α fused to GST was incubated with GST-CARM1 or GST-PRMT1 in presence of [^{3}H]AdoMet for 1 h at 30°C. The products were analyzed by SDS-PAGE followed by fluorography.
(B) PGC-1α fragments fused to GST were incubated with GST-PRMT1 and [^{3}H]AdoMet, and the products were analyzed by SDS-PAGE followed by fluorography (upper panels) and Coomassie blue staining (lower panels). Amino acid numbers of the PGC-1α fragments are shown below the diagram and above the panels. The asterisks show the methylated fragments, and positions of molecular weight are indicated beside the panels.
(C) GST fusion proteins representing mutated forms of the PGC-1α E fragment (sequence shown) were incubated with GST-PRMT1 and [^{3}H]AdoMet and analyzed as in A, by fluorography (left panel) and Coomassie blue staining (right panel). In the R4K and R3K mutants, the numbered and bracketed Arg residues (R) were changed to Lys. The results presented are from a single experiment representative of three independent experiments.

Importance of Arg methylation in PGC-1α coactivator function

To investigate the role of the C-terminal region and the three apparent Arg methylation sites in the stimulation of PGC-1α activity by PRMT1, we tested mutants of PGC-1α (Fig. 3A) for their ability to induce the expression of the ERRα promoter, which is an important regulatory target of PGC-1α in the process of mitochondrial biogenesis (Schreiber et al, 2004; Mootha et al, 2004). ERRα binds to and stimulates transcription from its own gene promoter, and this stimulation is highly dependent on the coactivator function of PGC-1α (Laganiere et al, 2004; Mootha et al, 2004). As observed previously (Laganiere et al, 2004; Mootha et al, 2004), over-expression of wild type PGC-1α dramatically enhanced the expression of a transiently transfected reporter gene controlled by the upstream regulatory region of the ERRα promoter (Fig. 3B). Deletion of a C-terminal part of PGC-1α (ΔC) or mutation of the three apparent Arg methylation sites (Arg665, Arg667, and Arg669) to Lys (R3K mutant) reduced, but did not abolish the coactivator function of PGC-1α; the mutant proteins still caused substantial enhancement of ERRα promoter activity (Fig. 3B) and were expressed at wild type levels (data not shown). The observation that the C-terminal deletion mutant was more active than the R3K mutant suggests that both inhibitory and stimulatory activities reside within this C-terminal region. Importantly, over-expression of PRMT1 enhanced the ability of wild type PGC-1α to stimulate transcription from the ERRα promoter, but failed to stimulate the activity of the two mutant PGC-1α proteins (Fig. 3C). Similarly, PRMT1 failed to cooperate with the R3K PGC-1α mutant in the context of another NR, ERα, and its respective target (Supplement Fig. 5). Thus, the inherent activity of PGC-1α as well as its ability to be stimulated by over-expression of PRMT1 depends on the three Arg residues that are substrates for methylation by PRMT1. These results strongly suggest that the methylation of PGC-1α by PRMT1 plays a key role in its activity and ability to function cooperatively with PRMT1.

The dramatically reduced activity of the R3K mutant in the absence of over-expressed PRMT1 suggests that the activity of the wild type PGC-1α protein depends on methylation by endogenous PRMT1, which is present in these cells (data not shown). To test this, we determined the effect of reducing endogenous levels of PRMT1 on the ability of over-expressed PGC-1α to stimulate expression of the transiently transfected ERRα-Luc reporter. Two different siRNAs targeting the human PRMT1 sequence reduced efficiently

the level of PRMT1 protein, but not β-actin protein, in COS7 cells in a dose-dependent manner (Fig. 4, lower panel). The siRNA-treated or untreated cells were subsequently transfected with a firefly luciferase gene reporter plasmid controlled by the ERRα promoter, a PGC-1α expression plasmid, and a control Renilla luciferase reporter plasmid driven by a constitutive cytomegalovirus promoter. Both siRNAs against PRMT1 caused a dose-dependent decrease in expression of the ERRα promoter-driven firefly luciferase reporter gene, which paralleled the decrease in PRMT1 protein levels (Fig. 4). Since the firefly luciferase data is normalized against the Renilla luciferase activity, these results demonstrate that reduction in endogenous PRMT1 levels causes a gene-specific decrease in the ability of PGC-1α to stimulate expression from the transiently transfected ERRα promoter.

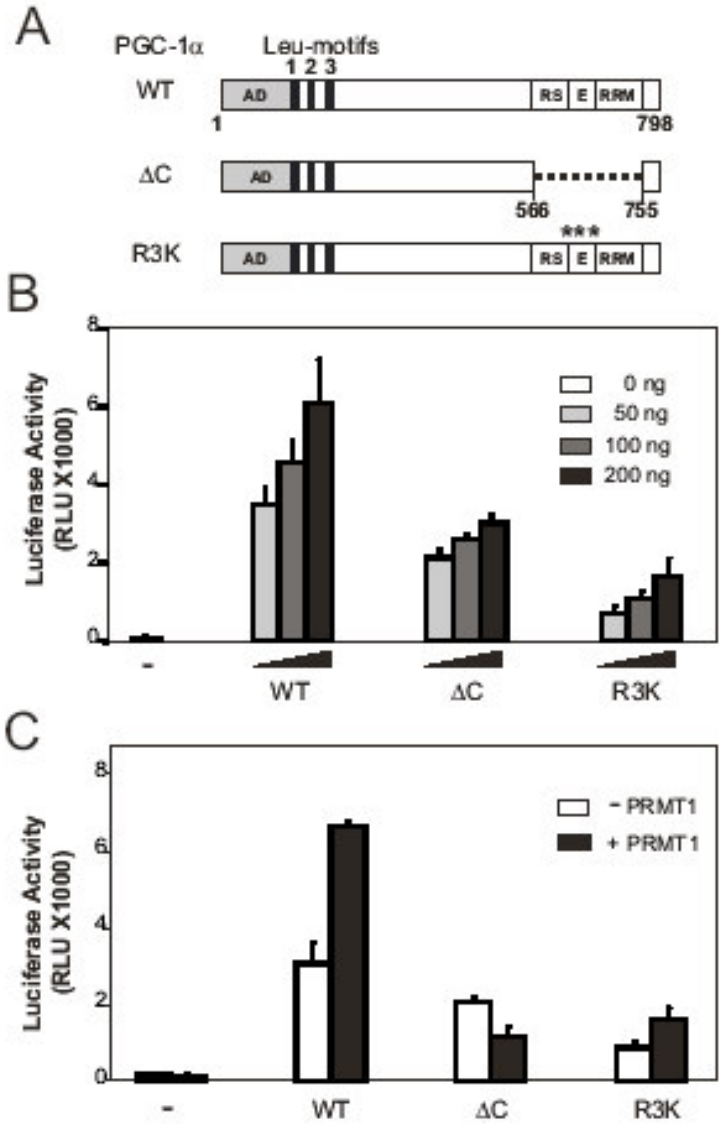

Figure 5.3. Role of the methylated Arg residues of the PGC-1α E region in coactivator function.

(A) Functional domains of PGC-1α and mutants examined for coactivator function. AD, activation domain, Leu-motif, LXXLL motifs; asterisks represent three Arg-to-Lys mutations in the E region (see Fig. 2C).

(B) COS7 cells were transiently transfected with pERRα-LUC reporter plasmid (250 ng) and the indicated amounts of expression vectors encoding PGC-1α wild type or mutant proteins. Extracts of the harvested cells were tested for luciferase activity.

(C) COS7 cells were transiently transfected with pERRα-LUC reporter plasmid (250 ng) and expression vectors encoding PRMT1 (250 ng) and PGC-1α wild type or mutants (50 ng), as indicated. Extracts of the harvested cells were tested for luciferase activity. The results presented are from a single experiment representative of three independent experiments.

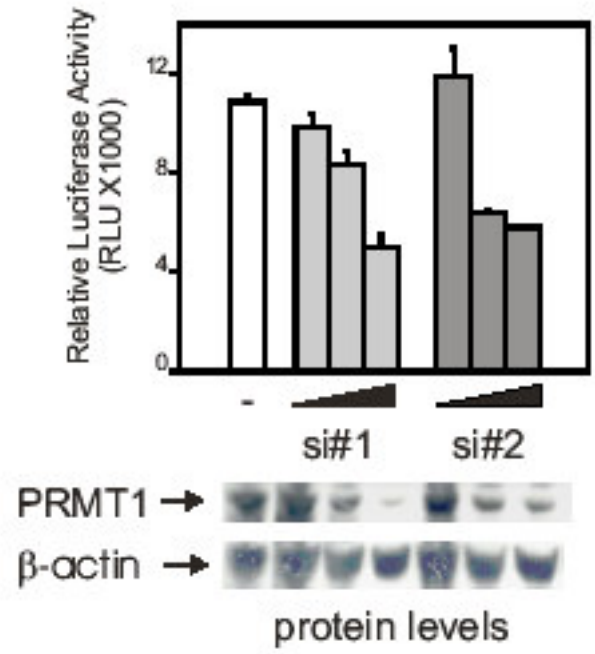

Figure 5.4. Role of endogenous PRMT1 in PGC-1α coactivator function.
COS7 cells, plated in 24-well plates, were transfected with increasing amounts (20 pmol; 40 pmol; 60 pmol) of two different siRNAs (si#1 and si#2) against PRMT1. Two days later, cells were transfected with a firefly luciferase reporter plasmid driven by the ERRα promoter (pERRα-Luc, 125 ng), a PGC-1α expression vector (25 ng), and a Renilla luciferase reporter plasmid driven by a cytomegalovirus promoter (12.5 ng). Luciferase activities were quantified 48 h later, and firefly luciferase activity was normalized by Renilla luciferase activity. Data shown are from a single experiment representative of three independent experiments, each performed in triplicate. Protein levels were determined by immunoblots from cell extracts of a fourth siRNA transfected well.

Arg methylation of PGC-1α and endogenous PRMT1 contribute to the induction of endogenous PGC-1α target genes

Wild type PGC-1α and the R3K mutant were expressed in SAOS2 osteosarcoma cells by infection with adenovirus vectors, to test their ability to stimulate expression of the endogenous ERRα and cytochrome c (Cyt c) genes, which are targets of PGC-1α and important for mitochondrial biogenesis (Schreiber *et al,* 2003; Schreiber *et al,* 2004; Mootha *et al,* 2004). A viral vector expressing green fluorescent protein (GFP) was used as a control. Although equivalent expression of mutant and wild type proteins was observed (Fig. 5A), the PGC-1α R3K mutant was substancially less efficient than the wild type protein in inducing the expression of ERRα and Cyt c mRNA (Fig. 5A), suggesting that arginine methylation is important for PGC-1α function at endogenous target genes in their native chromatin state.

To determine the role of endogenous PRMT1 in PGC-1α-dependent transcriptional programs, we next used the siRNA#1 to reduce PRMT1 mRNA levels in SAOS2 cells (Fig. 5B). The siRNA-treated or untreated SAOS2 cells were subsequently infected with adenovirus vectors encoding GFP or PGC-1α. PGC-1α expression led to increases in the levels of the endogenous Cyt c and ERRα mRNAs; however, this induction by PGC-1α was partially inhibited in the cells treated with siRNA against PRMT1, compared with the cells that were not exposed to the siRNA (Fig. 5B). The effects of PGC-1α and the siRNA against PRMT1 were gene specific, since the data shown are normalized against the expression of GAPDH mRNA. Thus, endogenous levels of PRMT1 contribute to the ability of PGC-1α to induce the expression of target genes that are important for mitochondrial biogenesis.

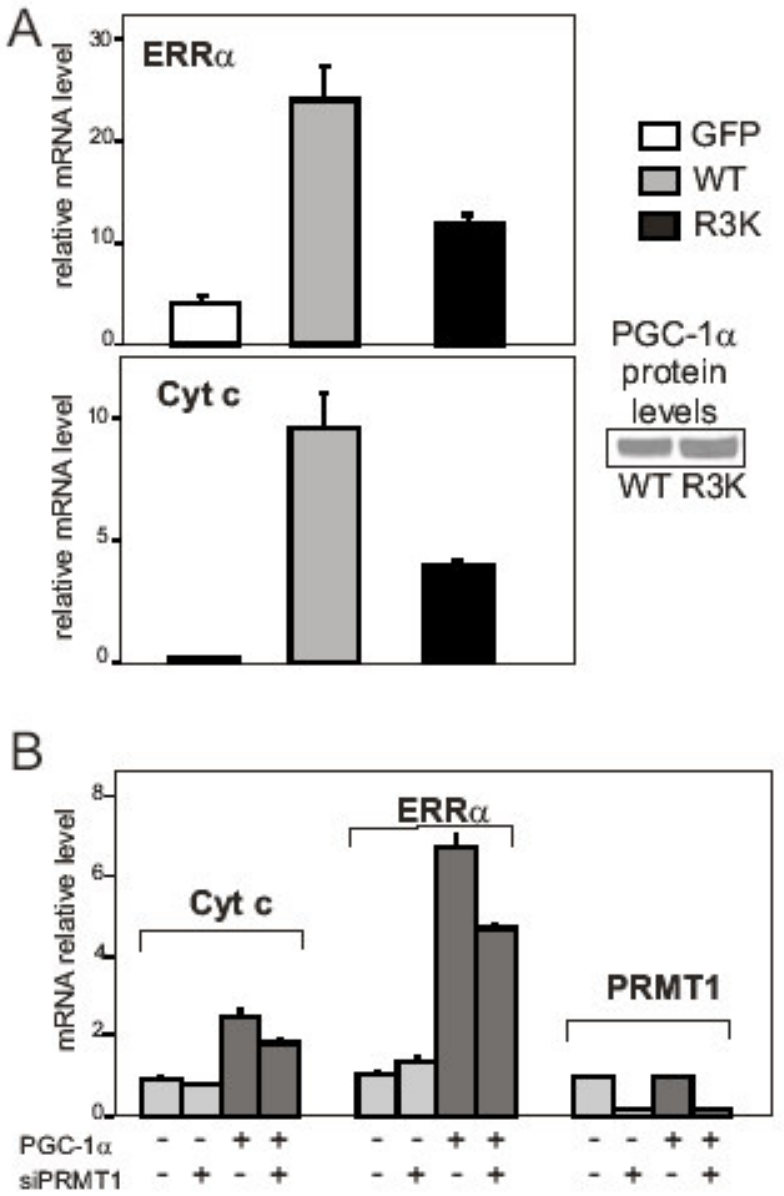

Figure. 5.5. Role of Arg-methylation in the induction of endogenous genes important for the biogenesis or function of mitochondria.
(A) SAOS2 cells in 6-well plates were infected with adenoviruses expressing GFP, PGC-1α wild type or PGC-1α R3K mutant (moi 40) and harvested 24 h later. mRNA levels for the ERRα and Cyt c genes were determined by quantitative PCR and normalized to the level of GAPDH mRNA.
(B) SAOS2 cells in 6-well plates were transfected with siRNA#1 against PRMT1 (100 pmol/well). One day later, cells were infected with adenoviruses expressing GFP or PGC-1α (moi 10) and harvested 24 h later. mRNA levels for the indicated genes were determined by quantitative PCR and normalized to the GAPDH mRNA level. Data shown are from a single experiment representative of three independent experiments, each performed in duplicate.

Discussion

Post-translational regulation of PGC-1α coactivator function

PGC-1α controls a number of metabolic processes that are important for energy homeostasis. Accordingly, PGC-1α expression is highly regulated at the transcription level, in a tissue specific manner and in response to several physiologic signals (Knutti & Kralli 2001; Puigserver & Spiegelman 2003; Kelly & Scarpulla 2004). In addition, the important roles of PGC-1α in metabolic regulation also appear to be modulated by a variety of post-translational modifications and interactions with other proteins. The docking of PPARγ to PGC-1α induces an apparent conformational change that allows binding of coactivators SRC-1 and CBP/p300 to the N-terminal activation domain of PGC-1α, thereby increasing the transcriptional activation potential of PGC-1α (Puigserver *et al,* 1999). Moreover, there is evidence that an unknown repressor protein competes with NRs for binding to PGC-1α, and this competition appears to be regulated by MAPK p38 signaling (Knutti *et al,* 2001). p38 MAPK directly phosphorylates sites within this suppression domain of PGC-1α and thereby enhances its transcriptional activity (Puigserver *et al,* 2001), by inhibiting the binding of the repressor p160 myb binding protein to PGC-1α (Fan *et al,* 2004). Our data add arginine methylation to the list of post-translational regulatory mechanisms that govern PGC-1α activity and, in addition, implicate PRMT1 as an important component cooperating with PGC-1α in metabolic processes such as mitochondrial biogenesis. Mitochondrial dysfunction contributes to several inherited and acquired human diseases, including insulin resistance and type 2 diabetes (Bjorntorp *et al,* 1967; Wallace 1999; Petersen *et al,* 2003). Decreases in PGC-1α in particular, have been suggested to contribute to the development of diabetes in humans (Patti *et al,* 2003; Mootha *et al,* 2003; Mootha *et al,* 2004). Understanding the signals and mechanisms that enable PRMT1 to modify PGC-1α may lead to ways to enhance PGC-1α activity and intervene in diseases associated with reduced mitochondrial function, such as diabetes.

Methylation of PGC-1α by PRMT1

We have provided evidence that PGC-1α coactivator function is stimulated by arginine methylation by the major mammalian methyltransferase PRMT1. This enzyme, which serves as a coactivator for many different types of DNA-binding transcription factors,

enhances PGC-1α activity, and this effect is dependent on and specific for the PRMT1 enzymatic activity. Our evidence indicates that two or three of the Arg residues in an RERQR sequence, found within the Glu-rich E region located between the RS domain and the RRM, are methylated by PRMT1 in vitro; furthermore, methylation of these Arg residues plays a critical role in the coactivator function of PGC-1α and its ability to function synergistically with PRMT1. PRMT1 has previously been shown to methylate other proteins at RXR sequences (Smith *et al,* 1999). We also observed methylation of the RS domain of PGC-1α but currently have no evidence that methylation of the RS domain is important for PGC-1α function.

Detecting methylation of specific proteins in vivo is still a developing art, as witnessed by a variety of recent methodological papers demonstrating success in specific cases (Rappsilber *et al,* 2003; Boisvert *et al,* 2003). We have been unable to demonstrate methylation of the PGC-1α E region in vivo by mass spectrometry, labeling of cells with methyl-labeled methionine, or antibodies with supposedly general specificity towards proteins containing methyl-arginine. Our experience suggests that success with each of these approaches depends on the specific protein under investigation. It is possible that methylation of PGC-1α by PRMT1 is highly regulated and occurs in vivo only under specific conditions, e.g. when they are both recruited into a transcription complex on a target gene promoter and PGC-1α adopts the appropriate conformation to allow methylation by PRMT1. In fact, as discussed above, the conformation of PGC-1α is regulated by protein-protein interactions (Puigserver *et al,* 1999), and our data demonstrated that certain fragments of PGC-1α that contain the E region are not methylated and must be partially degraded to allow methylation of the E region to occur (Fig. 2, PGC-1α (640-798)).

In spite of our lack of direct evidence for in vivo methylation, our data provide strong indirect evidence that methylation of PGC-1α by PRMT1 does occur in vivo and plays an important role in facilitating PGC-1α coactivator function: 1) PGC-1α and its E region are good substrates for PRMT1 in vitro. 2) PRMT1 and PGC-1α act synergistically as coactivators and this synergy depends on the methyltransferase activity of PRMT1. 3) CARM1, another protein arginine methyltransferase which also acts as a coactivator for

NRs and other types of transcription factors, does not methylate PGC-1α or cooperate functionally with PGC-1α as a coactivator. 4) The Arg residues that are methylated by PRMT1 are very important for the synergy between PGC-1α and PRMT1 and for the ability of PGC-1α to stimulate expression of transiently transfected and endogenous target genes. 5) Endogenous PRMT1 is important for the coactivator function of PGC-1α in the induction of transiently transfected and endogenous target genes.

PGC-1α has both methylation-dependent and methylation-independent coactivator activities

While PRMT1 and its methyltransferase activity clearly affect the coactivator function of PGC-1α, considerable PGC-1α coactivator activity remains when PRMT1 levels are severely reduced (Fig. 5B), when the Arg methylation sites in the E region are mutated (Figs. 3 & 5A), or even when the entire C-terminal region (RS, E, and RRM domains) is deleted (Fig. 3). These data indicate that there are two components of PGC-1α coactivator function, one independent of PRMT1 and one dependent on PRMT1. The strong N-terminal activation domain appears to be important for both of these activities (Supplement Fig. 1), and the specific downstream mechanisms that mediate these two components of PGC-1α activity remain to be defined.

How does PGC-1α methylation affect its function?

PGC-1α arginine methylation may influence PGC-1α activity at various levels. Arginine methylation of PGC-1α occurs in the E region, which contains a subnuclear localization signal (Monsalve *et al,* 2000), and thus could modulate the subcellular distribution of PGC-1α, as it has been shown for the methylation of some RNA-binding proteins (Shen *et al,* 1998; Nichols *et al,* 2000; Cote *et al,* 2003). A PGC-1α mutant lacking the C-terminal region exhibited reduced coactivator function (Fig. 3) and was deficient in binding to RNA polymerase II and some SR-type splicing factors (Monsalve *et al,* 2000), suggesting that this region is required for both transcriptional activation and regulation of mRNA splicing by PGC-1α. Methylation of arginine residues within this domain could enhance or inhibit specific protein-protein interactions (intramolecular or intermolecular) involving PGC-1α. The positive effect of PRMT1-mediated methylation suggests that methylation may cause an activating conformational change in PGC-1α, enhance binding of positively acting

proteins, such as TRAPP220 (Wallberg *et al,* 2003) and/or inhibit binding of repressor proteins. Further investigations will be required to elucidate the downstream effects of PGC-1α methylation and how they contribute to enhanced transcriptional activation.

Acknowledgments

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CHAPTER V SUPPLEMENTARY FIGURES

Supplementary figures

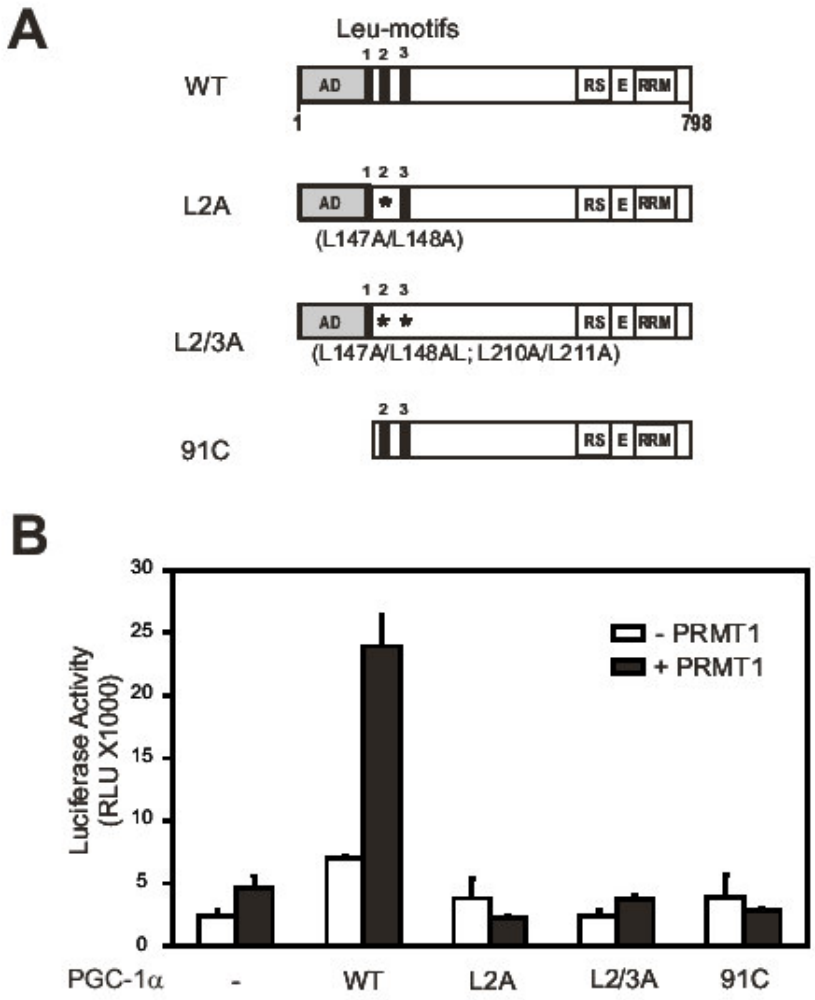

Figure 5S.1 Functional synergy between PRMT1 and PGC-1α requires the coactivator functions of PGC-1α.

(A) Schematic representation of PGC-1α mutants used in B. The plasmids expressing PGC-1α.L2A, PGC-1α.L2/3A (PGC-1α bearing point mutations within the Leu-rich motifs, as indicated) and PGC-1α.91C (PGC-1α lacking the N-terminal activation domain AD) have been described (Knutti *et al,* 2000).

(B) CV-1 cells were transiently transfected with MMTV(ERE)-LUC reporter plasmid (250 ng) and expression vectors encoding ER (0.1 ng), PGC-1α wild type or mutants as indicated (50 ng) in absence (white boxes) or in presence (black boxes) of PRMT1 (200 ng). Transfected cells were grown in culture medium with 20 nM estradiol, and extracts of the harvested cells were tested for luciferase activity. The results presented are from a single experiment representative of three independent experiments.

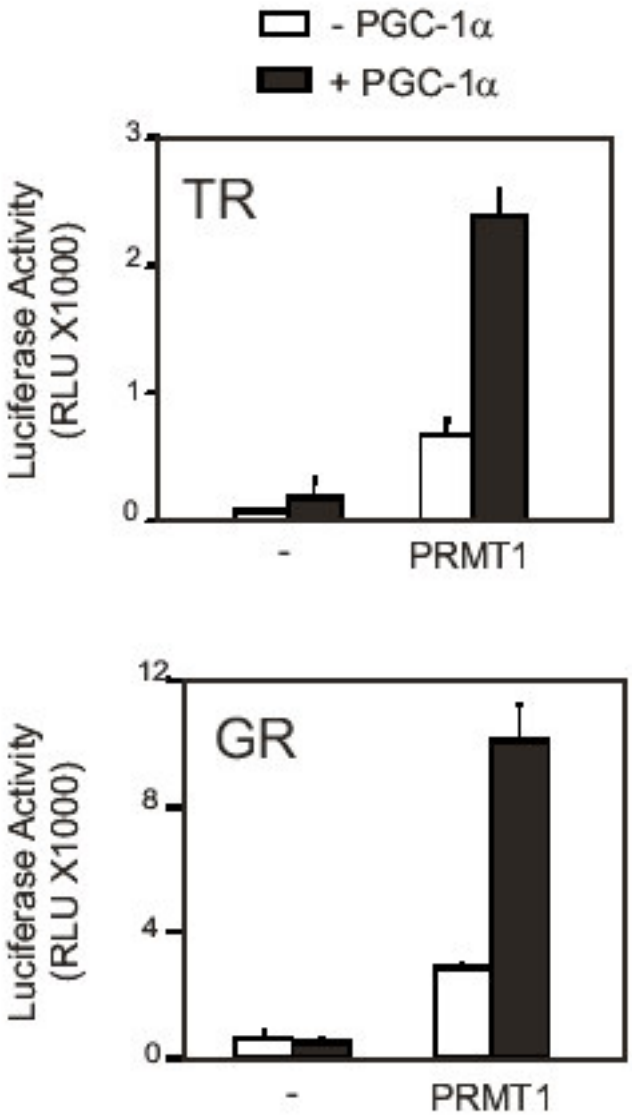

Figure 5S.2. PGC-1α and PRMT1 cooperate to enhance TR- and GR-mediated transcription. CV-1 cells were transiently transfected with MMTV(TRE)-LUC reporter plasmid (250 ng) (Umesono & Evans 1989) and expression vector pCMX.hTRβ1 encoding the human thyroid hormone receptor (TR) β1 (Huang & Stallcup 2000) (1 ng) (upper panel) or with MMTV-LUC reporter plasmid and expression vector pKSX encoding the mouse glucocorticoid receptor (GR) (Huang & Stallcup 2000) (1 ng) (bottom panel), and PRMT1 (200 ng) in absence (white boxes) or in presence (black boxes) of PGC-1α wild type (50 ng). Transfected cells were grown in culture medium with 20 nM thyroid hormone (upper panel) or dexamethasone (lower panel), and extracts of the harvested cells were tested for luciferase activity. The results presented are from a single experiment representative of three independent experiments.

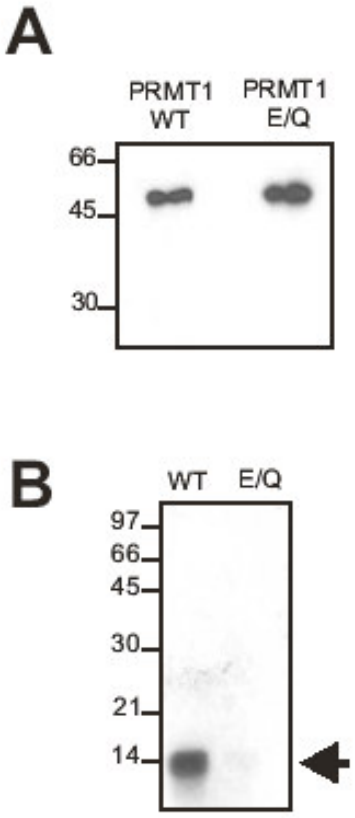

Figure 5S.3. Expression levels and methyltransferase activity of PRMT1 wild type and E153Q mutant.

(A) COS7 cells were transfected with vectors encoding HA-PRMT1 wild type or E153Q mutant and the enzymes were immunoprecipitated from cell extracts with anti-HA antibodies, as previously described (Teyssier *et al,* 2002). The expression level of the proteins was determined by immunoblot using anti-HA tag antibodies (monoclonal antibody 3F10, Roche Molecular Biochemicals).

(B) 1µg of histone H4 was incubated for 1 h at 30 °C with the immunoprecipitated methyltransferases and 7 µM [^{3}H]AdoMet (specific activity 14.7 Ci/mmol) in HMT buffer. Reactions were stopped by addition of SDS loading buffer and analyzed by 15% SDS-PAGE and fluorography. Methylated histone H4 is indicated by the arrow.

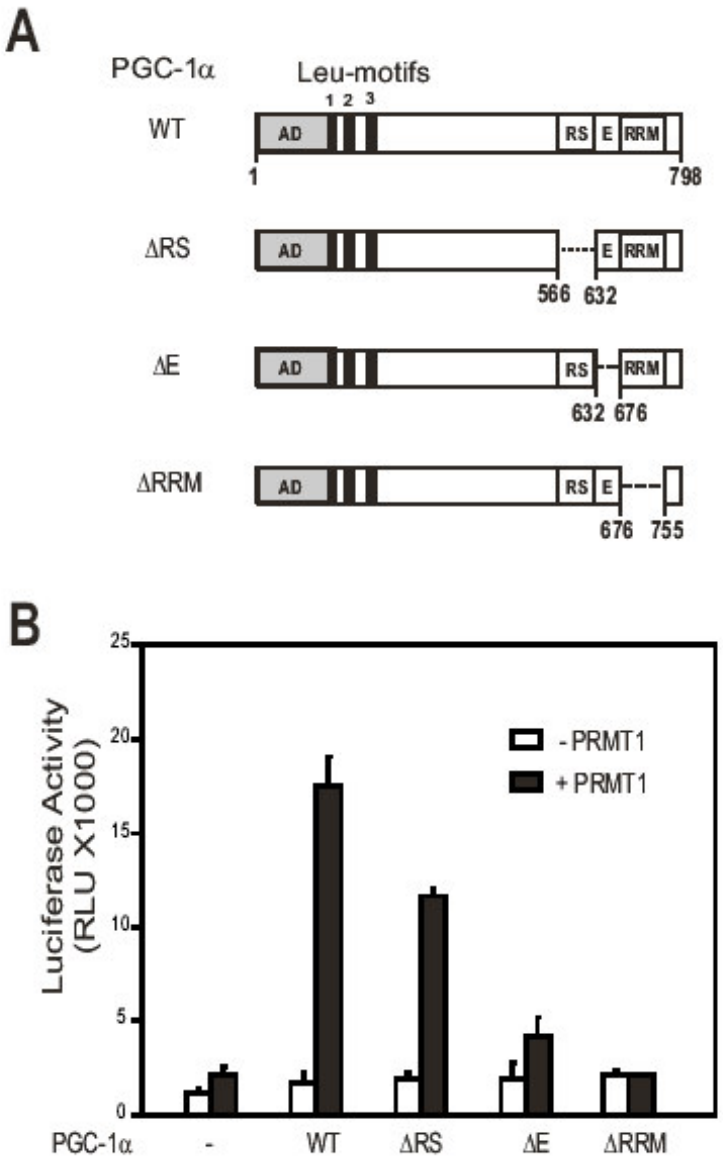

Figure 5S.4 Role of PGC-1α C-terminal domains in synergy with PRMT1.
(A) Schematic representation of PGC-1α deletion mutants used in B. Expression constructs pcDNA3-HA-PGC-1α.ΔRS (Δ566-632), pcDNA3-HA-PGC-1α.ΔE (Δ632-676), pcDNA3-HA-PGC-1α.ΔRRM (Δ676-755) were generated by fusion PCR and verified by sequencing.
(B) CV-1 cells were transiently transfected with MMTV(ERE)-LUC reporter plasmid (250 ng) and expression vectors encoding ER (0.1 ng), PGC-1α wild type or mutants as indicated (50 ng), in absence (white boxes) or in presence (black boxes) of PRMT1 (200 ng). Transfected cells were grown in culture medium with 20 nM estradiol, and extracts of the harvested cells were tested for luciferase activity. The results presented are from a single experiment representative of three independent experiments.

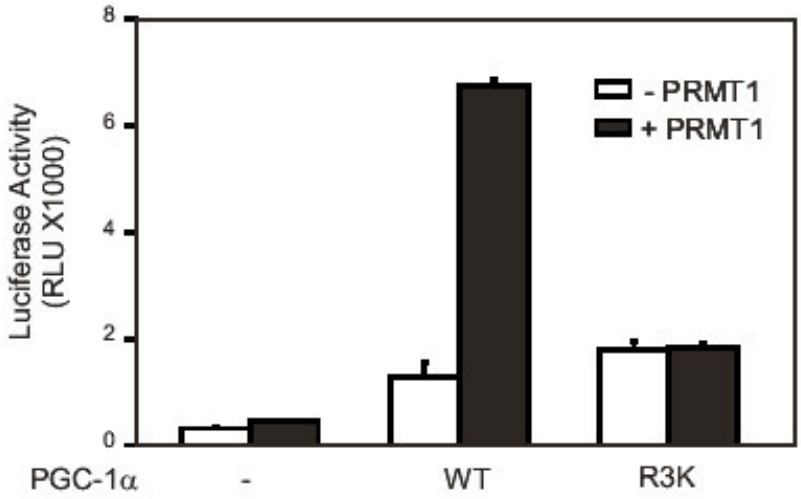

Figure 5S.5. Role of the methylated Arg residues in the synergy between PGC-1α and PRMT1 in CV-1 cells.
CV-1 cells were transiently transfected with MMTV(ERE)-LUC reporter plasmid (250 ng) and expression vectors encoding ER (0.1 ng), PGC-1α wild type or R3K mutant as indicated (50 ng), in absence (white boxes) or in presence (black boxes) of PRMT1 (200 ng). Transfected cells were grown in culture medium with 20 nM estradiol, and extracts of the harvested cells were tested for luciferase activity. The results presented are from a single experiment representative of five independent experiments.

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CHAPTER VI: DISCUSSION

Aim

Before this study started, results from our laboratory suggested that the C-terminal half of PGC-1α plays an important role in the transcriptional activation of PGC-1α target genes (Knutti et al., 2000). The presence of two SR-rich domains and a putative RNA-recognition motif in the C-terminal half of PGC-1α pointed to function of PGC-1α in pre-mRNA processing. Besides the SR-rich domains and the RRM, no other protein motifs could be identified that would have allowed to predict possible functions of the PGC-1α C-terminal domains. The goal of this thesis was to identify the role of the PGC-1α C-terminal domains in the regulation of gene expression.

A yeast two-hybrid screen identified new PGC-1α interacting proteins

We first tried to narrow down the region in the C-terminal part of PGC-1α that contributes to the transcriptional activity of PGC-1α in our transient transfection assays. Deletion of a glutamate-rich region between the SR-rich domain and the RRM turned out to be the only domain that would reduce the transcriptional activity of PGC-1α. We knew that PGC-1α can dimerize via its C-terminal half, but we did not know which domains contribute to this dimerization. To test the possibility that the glutamate-rich region contributes to the transcriptional activity of PGC-1α by mediating the dimerization of PGC-1α, we mapped the domains of PGC-1α that are required for dimerization. We found that a region encompassing the SR-rich, the glutamate-rich, and the RRM are required for dimerization of PGC-1α. These results suggest that dimerization is not essential for full activity of PGC-1α in our assays and that the glutamate-rich region contains another function.

To identify the function of the glutamate-rich region, but also of the other domains in the C-terminal part of PGC-1α, we wanted to identify PGC-1α-interacting protein by using a yeast two-hybrid screen. A human skeletal muscle cDNA library was screened and a number of interesting PGC-1α-interacting proteins were identified. We have grouped the identified proteins according to their function as far as possible (Table 3.1). One group

consists of proteins that have a clear or possible function in the regulation of transcription. Among them are the two transcription factors ERRα and NRF-1, both of which have been shown to interact with PGC-1α in other studies (Schreiber et al., 2003; Wu et al., 1999). ERRα and NRF-1 were isolated with the bait construct 294C, which contains part the region that has been proposed to interact with NRF-1. However, The isolation of ERRα was surprising, since the two L-motifs L2 and L3 of PGC-1α, which have been shown to mediate the interaction with ERRα, were not present in the bait constructs (Schreiber et al., 2003). This result suggests that regions of PGC-1α other than the L-motifs contribute to the interaction with ERRα. Importantly, the identification of two proteins already known to be important for PGC-1α function shows that the screen is identifying proteins relevant to the PGC-1α pathway.

We found that the acetyltransferase Tip60 forms a stable complex with PGC-1α in mammalian cells as demonstrated by co-immunoprecipitation assays. We therefore analyzed function of Tip60 in the PGC-1α signaling pathway in detail. The results of these studies are discussed in chapter IV and in a later section of this chapter.

Skip is known to interact with the coactivators p300 and SRC-1 (Leong et al., 2004; Zhang et al., 2001), both of which are also recruited by PGC-1α (Puigserver et al., 1999). Another interesting connection between PGC-1α and Skip is that both proteins seem to be involved in the processing of pre-mRNA (Figueroa and Hayman, 2004; Monsalve et al., 2000; Zhang et al., 2003). Thus, studies on the functional consequence of the PGC-1α-Skip interaction could involve assays that determine the ability of proteins to regulate RNA splicing.

Not much is known about the remaining proteins of this group, TSPYL (Vogel et al., 1998) and HPIP (Abramovich et al., 2002; Abramovich et al., 2000). If a direct interaction of the two proteins with PGC-1α can be demonstrated in mammalian cells, it will be interesting to study the effect of overexpression or knock-down by siRNA of TSPYL and HPIP on PGC-1α-dependent gene expression.

A second group of PGC-1α-interacting proteins is formed by proteins that have been shown to be involved in the processing of pre-mRNA (Table 3.1). Identification of these proteins supports the proposed role of PGC-1α in the regulation of pre-mRNA processing. Monsalve et al. have shown that PGC-1α can influence the alternative splicing of an exogenous reporter construct. There are no reports on whether PGC-1α can change the ratio of the splice variants of a specific endogenous gene. Identification and study of PGC-1α-induced genes that can also be alternatively spliced will be important to show that PGC-1α is indeed a regulator of RNA splicing and to determine the effects of the identified splice regulators in a physiologic context.

The three related kinases Clk1, Clk3, and Clk4 have been implicated in the regulation of alternative splicing, because they phosphorylate splicing factors and thereby regulate their subnuclear localization (Colwill et al., 1996; Duncan et al., 1997; Duncan et al., 1998; Hartmann et al., 2001; Prasad et al., 1999). It would be interesting and relatively easy to test if PGC-1α is phosphorylated by one of the Clk kinases. Possibly, such phosphorylation could regulate PGC-1α function not only in pre-mRNA processing but also in transcription regulation.

The third group is composed of proteins with a connection to the small protein modifiers ubiquitin or the related SUMO. Both ubiquitination and SUMOylation have established roles in the regulation of transcription factors and cofactors (Gill, 2003; Herrera and Triezenberg, 2004). Ubiquitin is a conserved 76 amino acid protein that has been discovered as macromolecular tag that can be covalently attached to certain target proteins to mark them for degradation by the proteasome, what is now called the classic ubiquitin-dependent proteolysis pathway (Pickart, 2001). The activity of a number of transcription regulators is known to be determined by their steady-state protein level. Therefore, regulation of the ubiquitination of a specific transcription factor by signaling pathways can determine its expression levels (Muratani and Tansey, 2003). A variety of evidence suggests that ubiquitin can also play more direct roles in transcription activation. Several studies suggest that ubiquitination of transcription factors not only targets them for destruction, but is also contributing to the activation of transcription. It could be observed that the half-lives of a set of model transcription factors correlated inversely with the potencies of their transcription activation domains suggesting that certain transcription

factors are degraded during or after they have activated transcription (Molinari et al., 1999). In addition, ligand-dependent transcription activation by the estrogen receptor was found to depend on an intact ubiquitin-dependent proteolysis pathway (Lonard et al., 2000), suggesting a necessary turnover of certain components of the transcription machinery. A very recent study presents a model, where ubiquitin has a function in transcription, which does not involve the degradation of proteins. The authors propose that ubiquitination of the VP16 activation domain increases its interaction with a transcription elongation factor (Kurosu and Peterlin, 2004). The proteins identified in the yeast two-hybrid screen that have a connection to ubiquitination may therefore have important function in the regulation of transcription by PGC-1α. The PGC-1α-interacting protein hHR23A and its isoform hHR23B are the two human homologues of the yeast protein Rad23, which has been shown to have a function in nucleotide excision DNA repair (Game, 2000). They belong to a class of proteins that contain ubiquitin-like (UbL) and ubiquitin-associated (UBA) domains. UbL domains resemble ubiquitin and allow interaction with the proteasome (Mueller and Feigon, 2003), while UBA domains bind to polyubiquitin chains (Raasi et al., 2004). This is consistent with their role as adapters for substrate-proteasome recognition. It has been reported that hHR23 can enhance, but also reduce the degradation of the transcription factor p53, possibly by mediating the interaction of p53 with the proteasome in one case (Glockzin et al., 2003) or by blocking this interaction in the other case (Brignone et al., 2004; Raasi and Pickart, 2003). Such a function of hRR23A could also be possible in the regulation of PGC-1α protein levels. It is known that PGC-1α is stabilized by phosphorylation by p38 kinase (Puigserver et al., 2001), but the mechanism for this is not known. It is also worth to mention that hHR23A can interact with the coactivator p300/CBP and thereby downregulate p53-dependent transcription (Zhu et al., 2001).

Ubiquitin-specific protease 25 is a recently identified protein that shares high homology with members of the ubiquitin protease (UBP) family. UBPs are a family of deubiquitinating enzymes that cleave ubiquitin conjugates on a variety of substrates (Valero et al., 2001; Valero et al., 1999). USP25 could theoretically increase the transcriptional activity of PGC-1α by cleaving ubiquitin tags that would otherwise target PGC-1α for degradation.

The involvement of PGC-1α ubiquitination in PGC-1α-dependent transcription requires that PGC-1α is a ubiquitinated protein. We have shown that PGC-1α can indeed be ubiquitinated (data not shown).

Ubc9 and PIASx have been shown to contain SUMO conjugating (Johnson and Blobel, 1997; Schwarz et al., 1998) and SUMO ligase activities (Kotaja et al., 2002), respectively. SUMO, like ubiquitin, is a small polypeptide that comprises 97 amino acids and is attached to lysine residues in substrate proteins. Some transcriptional regulator proteins have been shown to be modified by SUMO. Similar to other post-translational modifications, SUMO-modification can increase or repress the activity of transcriptional activators. These effects may be through alteration of protein-protein interactions that are important for transcriptional activation (Gill, 2003). Since lysine residues are also targets for other modifications, such as ubiquitination, acetylation, and methylation, SUMO may block other modifications that contribute to the activity of a specific protein (Gill, 2003). A consensus SUMO acceptor site has been identified, consisting of the sequence ΨKXE, where Ψ is a large hydrophobic amino acid and where K is the site of SUMO conjugation (Rodriguez et al., 2001). Two such motifs exist in PGC-1α. Mutation of each motif individually has however no effect on the activity of PGC-1α as a transcriptional coactivator (see appendix A). No evidence exists yet on whether PGC-1α becomes SUMOylated, but the identification of Ubc9 and PIASx suggest that it would be worth pursuing this possibility.

The yeast-two hybrid screen also identified several structural proteins that do not have an obvious functional relation to PGC-1α. Although a biological significance of these interactions can not be excluded, the identification of these proteins is probably due to the high abundance of the their cDNAs in the skeletal muscle library used for the yeast two-hybrid screen.

The last group of PGC-1α interacting proteins also contains proteins, whose functions are not related to PGC-1α. Further experiments will be necessary to get more evidence for an interaction of these proteins with PGC-1α. Confirmation of such an interaction may lead to the identification of unknown functions of PGC-1α.

Functional interaction between PGC-1α and Tip60

The interaction of PGC-1α with the acetyltransferase Tip60 identified in the yeast two-hybrid screen could be confirmed in other experiments, such as GST-pulldowns and coimmunoprecipitations in mammalian cells (FIG. 3.3 and 4.1D). The functional interaction between PGC-1α and Tip60 was therefore studied in more detail during this work. In several experiments, we found that overexpression of Tip60 inhibited PGC-1α-dependent gene expression. Interestingly, the repressive effect of Tip60 depended in one case entirely, in some experiments partly, and in other experiment not on the acetyltransferase activity of Tip60. We then found that the expression of p21, a gene that can be induced by the transcription factor p53 and Tip60, was enhanced by the overexpression of PGC-1α. In contrast to the previous results, overexpression of Tip60 did not affect the PGC-1α-dependent expression of p21. Instead, ectopic expression of a Tip60 variant lacking acetyltransferase activity or knock-down of endogenous Tip60 strongly inhibited PGC-1α-dependent p21 expression (FIG. 4.5). These results suggest that Tip60 is required for the induction of p21 by PGC-1α. The acetyltransferase mutant of Tip60 seems to act as a dominant negative protein. This suggests that the acetyltransferase activity of Tip60 is necessary for PGC-1α-dependent expression of p21.

Why does Tip60 on one side repress PGC-1α-dependent gene expression and why is it on the other hand required for PGC-1α-dependent gene expression? There may be at least two types of PGC-1α induced genes; (i) genes for which Tip60 is required and (ii) genes for which Tip60 is not required. The repressive effect of Tip60 overexpression on PGC-1α-dependent gene expression may be explained by a recruitment of PGC-1α away from promoters that do not require Tip60 to promoters that are coregulated by PGC-1α and Tip60. Further experiments will be necessary to determine if and how PGC-1α is recruited to the p21 promoter. Is it through Tip60 alone or does PGC-1α also bind to transcription factors, such as p53?

Western blot analysis and pulse-chase experiments revealed that overexpression of Tip60 leads to a dramatic increase in the steady-state PGC-1α protein levels by increasing the half-life of PGC-1α. Analysis of the NP-40-soluble and insoluble cell fractions showed that overexpression of Tip60 led to small reduction of PGC-1α protein levels in the NP-40-

soluble cellular fraction (FIG. S4.3). While in the absence of overexpressed Tip60 no PGC-1α could be found in the NP-40-insoluble cell fraction, Tip60 overexpression led to a dramatic accumulation of PGC-1α in the NP-40-insoluble fraction. Interestingly, the PGC-1α protein in the insoluble fraction and about half of the NP40-soluble PGC-1α displayed a slightly smaller apparent molecular weight than PGC-1α in the absence of overexpressed Tip60 (FIG. S4.3). This change in migration may be a consequence of a posttranslational modification that is required for the relocalization or that is a consequence of the relocalization of PGC-1α to the NP-40- insoluble cellular compartment. What could this NP-40-insoluble compartment be, and why does Tip60 recruit PGC-1α into this compartment? Nuclear bodies, also called PML-bodies, are subnuclear structures formed by PML and other proteins and have been shown to be important for the regulation of several cellular processes (Borden, 2002; Takahashi et al., 2004). Previous studies have demonstrated that proteins localized in PML-bodies are insoluble in NP-40 lysis buffer (Muller et al., 1998), suggesting that PGC-1α may be translocated to PML-bodies upon overexpression of Tip60. Both p53 and Tip60 have been shown to be partly localized in PML-bodies. Although the functions of PML-bodies are not well understood, they have been suggested to serve as platforms, where interaction of p53 with specific cofactors is favored (Gostissa et al., 2003). Further experiments will be necessary to determine if PGC-1α is really directed to PML-bodies by the overexpression of Tip60 and what modification of PGC-1α is associated with this subcellular relocalization.

Despite an obvious role for the acetyltransferase activity of Tip60 in PGC-1α-dependent transcription, we were not able to show conclusively that PGC-1α is acetylated by Tip60. We immunoprecipitated PGC-1α from mammalian cells and tried to show that PGC-1α is acetylated by western blot analysis using antibodies that are able to recognize acetylated lysine residues. These antibodies are not able to detect any acetylated protein, maybe due to the nature of the amino acids surrounding the acetylated lysine. It cannot be excluded that the amino acid sequence of PGC-1α does not allow the detection of one or several acetylated lysines by these antibodies. In a second approach, we performed an *in vitro* acetylation assay with purified, recombinant Tip60 and PGC-1α. Although Tip60 nicely acetylated a mix of histone proteins, there was no detectable acetylation of full length PGC-1α and only a very weak band that may correspond to a shorter PGC-1α fragment (FIG. S4.3). Not every acetylation reaction happening *in vivo* also works efficiently *in*

vitro. Tip60 itself is a good example for this, since only the purified Tip60 complex, but not recombinant Tip60 is able to acetylate chromatin templates (Ikura et al., 2000). Therefore, additional proteins may be required for Tip60 to be able to acetylate PGC-1α efficiently.

Synergy between PGC-1α and PRMT1

In collaboration with the group of Michael Stallcup, we have shown that the coactivator function of PGC-1α is enhanced by methylation by the methyltransferase PRMT1. Three arginines in the E-rich region of PGC-1α are methylated by PRMT1. Methylation of these arginine residues plays an important role in the coactivator function of PGC-1α and its ability to function synergistically with PRMT1. The mechanism by which methylation affects the function of PGC-1α is not known. Methylation may change the interaction of PGC-1α with other proteins that either regulate its function or that act as downstream effectors for the activation of transcription or for other functions, such as pre-mRNA processing. PGC-1α is a highly regulated protein at the transcriptional level and seems to be regulated also at the post-translational level. Several signaling pathways may converge upon PGC-1α and regulate its activity positively or negatively to result in an appropriate response. Methylation of PGC-1α may be a signal that allows another activating posttranslational modification to take place, for example, by creating a new interaction surface for another enzyme. In such a model, only the simultaneous action of two independent signaling pathways would result in an increase of the PGC-1α activity. On the other hand, methylation may block the attachment of a posttranslational modification that would otherwise inhibit the PGC-1α activity. Identification of the kinases Clk 1, 3, and 4, the SUMO-conjugating enzyme Ubc9, and the SUMO ligase PIASx as potential PGC-1α-interacting proteins suggests that PGC-1α is regulated by a number of posttranslational modifications, some of which might have synergistic and some of which might have antagonistic effects. How can posttranslational modifications influence the activity of PGC-1α? MAP kinase p38 has been shown to directly phosphorylate PGC-1α and thereby enhances its transcriptional activity, by inhibiting the binding of the repressor $p160^{MBP}$ binding protein to PGC-1α (Fan et al., 2004). PRMT1, like p38, may regulate the interaction of proteins with PGC-1α, most likely with its C-terminal part. It will be

interesting to determine whether the interaction of PGC-1α with some of its possible downstream effectors are regulated by PRMT1. Previous reports and especially this study have identified several proteins with either a role in transcription activation or pre-mRNA processing that could be tested for their ability to distinguish methylated or non-methylated variants of PGC-1α.

Summary and conclusions

Many different coactivators have been identified and studied in the last years and their number is still increasing. Some of them were found to be components of large multiprotein complexes that serve to recruit the basal transcription machinery to DNA-bound transcription factors or to remodel chromatin structures for easier access of the transcription machinery to specific promoter regions.

The coactivator PGC-1α seems to be highly regulated, both at the transcriptional and post-translational level. Due to the central role of PGC-1α in energy metabolism, missregulation of this protein has been suggested to contribute to the development of diabetes and obesity. PGC-1α has not been shown to contain any enzymatic activities and despite a lot of research, its mechanisms regulating gene expression are far from being understood. To better understand the mechanism of gene regulation by PGC-1α or to identify new posttranslational regulatory mechanisms for PGC-1α, we used a yeast two-hybrid screen to find new PGC-1α-interacting proteins. Of the identified proteins, the acetyltransfease Tip60 was chosen for more detailed analysis. Tip60 appears to negatively regulate the transcriptional activity of PGC-1α in several biological contexts, but we also found evidence that PGC-1α and Tip60 collaborate in the expression of p21. We have also provided evidence that PGC-1α coactivator function is stimulated by arginine methylation by the major mammalian methyltransferase PRMT1. Future work will have to address how methylation affects the activity of PGC-1α as a coactivator.

The identification of novel PGC-1α-interacting proteins may provide new targets for the development of drugs for the treatment of metabolic diseases caused by missregulation of PGC-1α or PGC-1α-regulated genes.

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APPENDIX A: MUTATION OF THE PUTATIVE SUMOYLATION MOTIFS DOES NOT AFFECT THE PGC-1α ACTIVITY

Results and discussion

The yeast two-hybrid screen identified the proteins PIASx and UBC9 as PGC-1α–interacting proteins, both of which have been suggested to play important roles in the modification of proteins with the small ubiquitin-like modifier (SUMO) (Johnson and Blobel, 1997; Kotaja et al., 2002; Schwarz et al., 1998) . We didn't try or weren't able to confirm the interactions between those two proteins and PGC-1α in experiments other than yeast two-hybrid assays. Despite a lack of evidence for an interaction of PGC-1α with Ubc9 or PIASx in mammalian cell, we searched the PGC-1α protein sequence for putative SUMOylation motifs. A consensus SUMOylation motif has been identified consisting of the protein sequence ΨKXE, where Ψ is a large hydrophobic amino acid and K the residue where SUMO is attached (Rodriguez et al., 2001). The PGC-1α protein sequence contains two putative SUMOylation sites ranging from amino acids 183-186 and amino acids 645-648, respectively. To determine whether those SUMOylation motifs play a role in the regulation of transcription by PGC-1α, we introduced changes in the nucleotide sequence of the PGC-1α cDNA leading to the insertion of arginines instead of a lysines at amino acid positions 184 and 646 in the putative SUMOylation motifs. COS7 cells were transfected with a PGC-1α-inducible luciferase reporter under control of the ERRα-promoter and expression vectors for either wild type PGC-1α or PGC-1α variants having lysine to arginine substitutions in either of the two SUMOylation motifs. As shown in FIG. A1.1, transfection of wild type PGC-1α induces the expression of the reporter construct approximately 27 fold. A similar induction of the luciferase reporter was observed, when the two PGC-1α variants harboring lysine to arginine substitution in the putative SUMOylation motifs were transfected. This result suggests that the predicted SUMOylation of the lysine residues at positions 184 and 646 does not positively or negatively affect the PGC-1α transcriptional activity under those conditions. This results does not exclude that SUMOylation of PGC-1α can be important in a different biological context or that PGC-1α gets SUMOylated on other amino acids residues. PGC-1α contains 40 lysines, which all are potential sites for SUMO attachment. Even if the mutated lysine

residues are indeed targets for SUMO modification, mutation of a single lysine may not always be a clear indication of the effect of loss of SUMO- modification, since multiple signaling pathways may converge upon critical lysine residues to determine transcription factor activity. Further experiments will be necessary to determine if PGC-1α can be modified by SUMO and how such a modification would influence the function of PGC-1α.

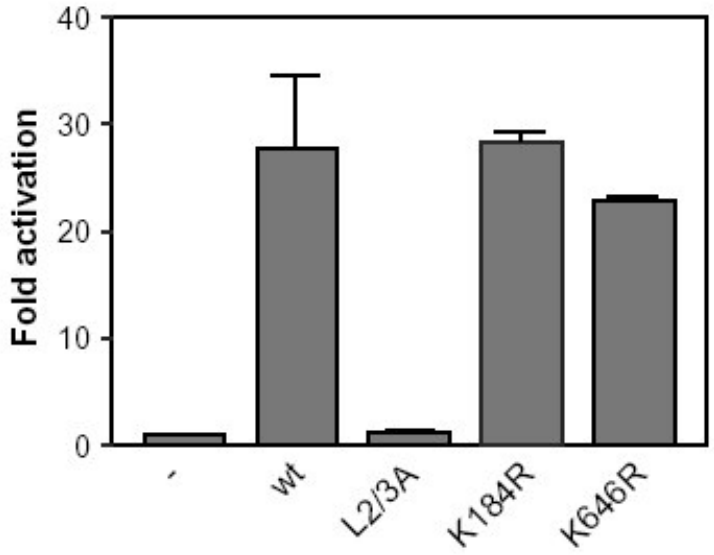

Figure A.1. Mutation of two putative SUMOylation motifs does not affect the activity of PGC-1α as a coactivator.
COS7 cells were transfected with a luciferase reporter under control of the ERRα gene promoter (pERRα-luc) and plasmids expressing wildtype or mutant PGC-1α. Cells were assayed for luciferase activty 40 h after transfection. Results show the enhancement of the luciferase activty by the different PGC-1α variants.

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The estrogen-related receptor α (ERRα) functions in PPARγ coactivator 1α (PGC-1α)-induced mitochondrial biogenesis

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Estrogen-related receptor α (ERRα) is one of the first orphan nuclear receptors to be identified, yet its physiological functions are still unclear. We show here that ERRα is an effector of the transcriptional coactivator PGC-1α [peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α], and that it regulates the expression of genes involved in oxidative phosphorylation and mitochondrial biogenesis. Inhibition of ERRα compromises the ability of PGC-1α to induce the expression of genes encoding mitochondrial proteins and to increase mitochondrial DNA content. A constitutively active form of ERRα is sufficient to elicit both responses. ERRα binding sites are present in the transcriptional control regions of ERRα/PGC-1α-induced genes and contribute to the transcriptional response to PGC-1α. The ERRα-regulated genes described here have been reported to be expressed at reduced levels in humans that are insulin-resistant. Thus, changes in ERRα activity could be linked to pathological changes in metabolic disease, such as diabetes.

Estrogen-related receptor α (ERRα, NR3B1) was identified on the basis of its sequence similarity to classical, hormone-regulated steroid receptors (1). Based on its ability to recognize similar DNA sequences as the estrogen receptors, ERRα has been proposed to modulate estrogen signaling (2–5). ERRα may also regulate bone formation, given that it is highly expressed at ossification sites, promotes osteoblast differentiation *in vitro*, and activates the promoter of the bone matrix protein osteopontin (6, 7). Finally, ERRα may regulate fatty acid oxidation. Consistent with this function, ERRα is prominently expressed in tissues with high capacity for β-oxidation of fatty acids, such as brown fat, heart, muscle, and kidney, and induces the expression of the medium-chain acyl-CoA dehydrogenase gene (8, 9).

A better understanding of the transcriptional programs and cellular pathways that depend on ERRα has been hampered by the lack of tools to regulate the activity of this receptor. Despite the high similarity between ERRα and other ligand-dependent nuclear receptors, it is not clear whether ERRα activity is regulated by small lipophilic ligands. Compounds that inhibit ERRα-dependent transcription, such as toxaphene, chlordane, and diethylstilbestrol, have been described (10, 11). However, these compounds are not specific enough for ERRα to facilitate studies of its cellular function. Recently, we demonstrated that the transcriptional coactivator peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α (PGC-1α) regulates ERRα function (12). PGC-1α induces the expression of ERRα and interacts physically with ERRα, enabling it to activate transcription (12, 13). These findings suggest that PGC-1α can be used as a protein "ligand" to regulate ERRα-dependent transcription and study ERRα function.

PGC-1α has been identified as a tissue-specific coactivator of nuclear receptors (14–16). The expression of PGC-1α is most prominent in tissues with high energy demands, similar to the expression pattern of ERRα (12, 16). PGC-1α mRNA levels are induced in response to signals that relay metabolic needs, such as exposure to cold, fasting, and physical exercise (reviewed in refs. 15 and 16). Strikingly, increases in PGC-1α levels seem sufficient to induce cellular pathways important for energy metabolism, including adaptive thermogenesis, mitochondrial biogenesis, and fatty acid oxidation (14, 17–19). This is accomplished by the interaction of PGC-1α with transcription factors, which recruit PGC-1α to target DNA regulatory sequences and enable the induction of genes important in energy metabolism pathways. Transcription factors that guide PGC-1α action to specific genes include nuclear receptors, members of other transcription factor families, such as nuclear respiratory factor (NRF)-1, which controls the expression of mitochondrial proteins, and myocyte enhancer factors 2C/2D (15, 16).

The recent identification of ERRα as a protein that is coexpressed with and induced and activated by PGC-1α suggests that ERRα plays a role in some of the known PGC-1α-regulated pathways. Consistent with this hypothesis, we show here that ERRα and PGC-1α cooperate to induce mitochondrial biogenesis.

Materials and Methods

Adenoviruses and Plasmids. Adenoviral vectors expressing GFP, PGC-1α, small interfering RNA (siRNA) for ERRα, and control AdSUPER have been described (12). Adenoviruses expressing ERRα or ERRα fused to the VP16 activation domain (VP16-ERRα) were constructed by using the insert of pSG5-mERRα or pSG5-ΔA/BmERRα, respectively (6). For the reporter plasmids, human genomic DNA and gene-specific oligonucleotides (Table 1, which is published as supporting information on the PNAS web site) were used to amplify the sequences -385 to $+90$ and -686 to $+55$ (relative to transcription initiation site) of the ATP synthase β (ATPsynβ) and cytochrome *c*, somatic (Cyt *c*) genes, respectively. The PCR products were cloned upstream of the luciferase coding sequences of pGL3-Basic (Promega). Mutations and deletions were introduced by fusion PCR (20). The ERR response elements (ERREs) at ATPsynβ/-338 (CCAAGGACA), Cyt *c*/-596 (ACAAGGTCA), and Cyt *c*/-9 (CCAAGGACA) were changed to CCAgatctt, ACAgatctA and CCAgatctA, respectively. The NRF-2 binding sites of ATPsynβ were deleted by removing sequences -300 to -270; the NRF-1

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Abbreviations: ERRα, estrogen-related receptor α; ERRE, ERR response element; PGC-1α, peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α; NRF, nuclear respiratory factor; siRNA, small interfering RNA; L2/3, leucine motifs 2 and 3; Cyt *c*, cytochrome *c*, somatic; ATPsynβ, ATP synthase β; IDH3A, isocitrate dehydrogenase α; TR, thyroid hormone receptor; moi, multiplicity of infection; mtTFA, mitochondrial transcription factor A.

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binding site of Cyt *c* (CCAGCATGCGCG) was changed to CCAGgATcCaac.

Cell Culture and Transfections. Cells were cultured in DMEM supplemented with 9% charcoal-stripped FCS. SAOS2 (SAOS2-GR(+) in ref. 12) cells were infected with adenoviruses at a multiplicity of infection (moi) of 20–100. COS7 cells were transfected by calcium phosphate precipitation and analyzed as described (21). The amounts of plasmids per transfection were 100 ng of the reporters pCytc/−686Luc or pATPsynβ/−385Luc, 100 ng pcDNA3/HA-PGC-1α (21), and 50 ng pcDNA3/ERRα (22).

cRNA Preparation and Array Hybridization. Total RNA (10 μg) was reverse transcribed with the SuperScript Choice system (Invitrogen). The cDNA (1 μg) was *in vitro* transcribed by using the Enzo BioArray High Yield RNA system (Enzo Diagnostics). The cRNA (10 μg) was fragmented and hybridized to a HG-U133A GeneChip (Affymetrix, Santa Clara, CA) by using standard procedure (45°C, 16 h). Washing and staining were performed in a Fluidics Station 400 (Affymetrix) by using the protocol EukGE-WS2v4 and scanned in an Affymetrix GeneChip 2500 scanner.

Microarray Analysis. Data from three experiments were analyzed with MICROARRAY SUITE 5 software (Affymetrix) and GENESPRING 5.1 (Silicon Genetics, Redwood City, CA). Changes in gene expression were assessed by looking for concordant changes between replicates by using a signed Wilcoxon rank test. The "change" *P*-value threshold was <0.003. Genes whose detection *P*-value was >0.05 in all experimental conditions were excluded from the analysis. Genes that reproducibly changed in the same direction were subjected to a one-way ANOVA test ($P < 0.05$) with a Benjamini and Hochberg multiple testing correction. Classification into genes encoding mitochondrial proteins was based on annotations of the Affymetrix NetAffx Analysis Center, SOURCE and the National Center for Biotechnology Information PubMed, and the OXPHOS and human_mitoDB_6_2002 lists curated at the Whitehead Institute Center for Genome Research (23).

DNA Isolation and Quantification. Total DNA was prepared according to standard procedures and digested with 100 μg/ml RNase A for 30 min at 37°C. The relative copy numbers of mitochondrial and nuclear DNA were determined by real-time PCR with primers specific to the COX2 (mitochondrial) and β actin (nuclear) genes (Table 1), 2 ng DNA, and the Light Cycler system (Roche Diagnostics). Serial dilutions of DNA from uninfected cells were analyzed in parallel to establish a standard curve. Quantification was as described (24).

RNA Analysis. Isolation of RNA, conversion to cDNA, and quantification of transcripts by real-time PCR by using the Light Cycler system (Roche Diagnostics) and gene-specific primers (Table 1) have been described (24).

Western Analysis. Cell lysates were subjected to Western analysis by using antibodies against PGC-1α (12) and ERRα (3).

Labeling of Mitochondria and Flow Cytometry. Cells were incubated, first with 500 nM CM-H_2XRos or 500 nM MitoFluor Red 594 (Molecular Probes) in culture medium for 30 min and, second, in fresh, dye-free medium for 30 min at 37°C. CM-H_2XRos-labeled mitochondria were visualized by fluorescence microscopy. MitoFluor Red 594-labeled cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson), by using the software WINMDI 2.8.

***In Silico* Analysis for ERREs.** Thirty-five sequences reported to bind ERRα (3, 4, 8, 25) were aligned by using CLUSTALW and used to compile a position-weighted nucleotide distribution matrix. Crossvalidation of the matrix revealed a mean and median score for the 35 sequences of 0.915 and 0.946, with a maximum at 0.994 and a minimum of 0.695, the best possible score being 1. For candidate genes, 5 kb of 5′ upstream region sequence were searched for matches to the matrix, by using a variant of the NUBISCAN algorithm (26).

Electrophoretic Mobility Shift Assay. *In vitro* translated ERRα (0.5 μl) [T7 Coupled Reticulocyte system (Promega)] or unprogrammed lysate was incubated in 20 μl of buffer (10 mM Hepes, pH 7.5/2.5 mM $MgCl_2$/50 mM EDTA/1 mM DTT/6% glycerol) with 1 ng of ^{32}P-end-labeled oligonucleotide probe and 1 μg of poly dI:dC, in the absence or presence of 100 ng of unlabeled oligonucleotide competitor (Table 1). Complexes were resolved in 6% native polyacrylamide gels.

Results

PGC-1α Induces Mitochondrial Biogenesis in SAOS2 Cells Through a Pathway That Requires Interaction with Nuclear Receptors. To identify the cellular programs that are regulated by PGC-1α in SAOS2 cells and where ERRα could play a role, we used high-density oligonucleotide arrays and compared the RNA profiles of cells expressing PGC-1α to those of control cells. Seventeen hours after infection with a PGC-1α expressing adenovirus, 151 of the up-regulated transcripts were classified as nuclear genes encoding mitochondrial proteins (Table 2, which is published as supporting information on the PNAS web site). These genes define "mitochondrial functions" that are up-regulated in the early phase of the PGC-1α-induced response (≈12 h after PGC-1α protein becomes detectable), and encode proteins with roles in many facets of mitochondrial biogenesis and function, including mitochondrial protein synthesis (20 genes), transport across the mitochondrial membrane (17 genes), fatty acid oxidation (8 genes), the tricarboxylic acid cycle (17 genes), and oxidative phosphorylation (55 genes) (Table 2). An additional 23 of the up-regulated transcripts represent genes that do not encode known mitochondrial proteins but have been reported as coregulated with "mitochondrial genes" and proposed to carry functions relevant to mitochondrial biology (27) (Table 2). PGC-1α also induced the expression of mitochondrial transcription and translation factor A (mtTFA). Interestingly, PGC-1α did not affect the expression of NRF-1 or NRF-2 (Table 2), the transcription factors that regulate the expression of many nuclear genes encoding mitochondrial proteins and that are induced by PGC-1α in C2C12 cells (17). We concluded that PGC-1α induces the gene expression program of mitochondrial biogenesis in SAOS2 cells in a manner that differs from the NRF-1 pathway described in C2C12 cells (17).

To determine whether the PGC-1α-mediated induction of mitochondrial proteins led to an increase in mitochondrial content, SAOS2 cells were stained with MitoTracker, a dye that accumulates specifically in respiring mitochondria. Mitochondria in control cells infected with a GFP-expressing adenovirus had a characteristic tubular appearance and were concentrated around the nuclei, similar to mitochondria in noninfected cells (Fig. 1*A* and data not shown). Expression of PGC-1α led to a distinct mitochondrial reticulum, which filled the cytoplasm. The increased mean fluorescence intensity in PGC-1α expressing cells (geometric mean = 151) compared with control cells (geometric mean = 90) was consistent with an increase in mitochondrial content (Fig. 1*B*). To measure mitochondrial DNA directly, we isolated total DNA and determined the relative copy number of mitochondrial DNA by quantitative PCR. PGC-1α expression led to an increase in mitochondrial

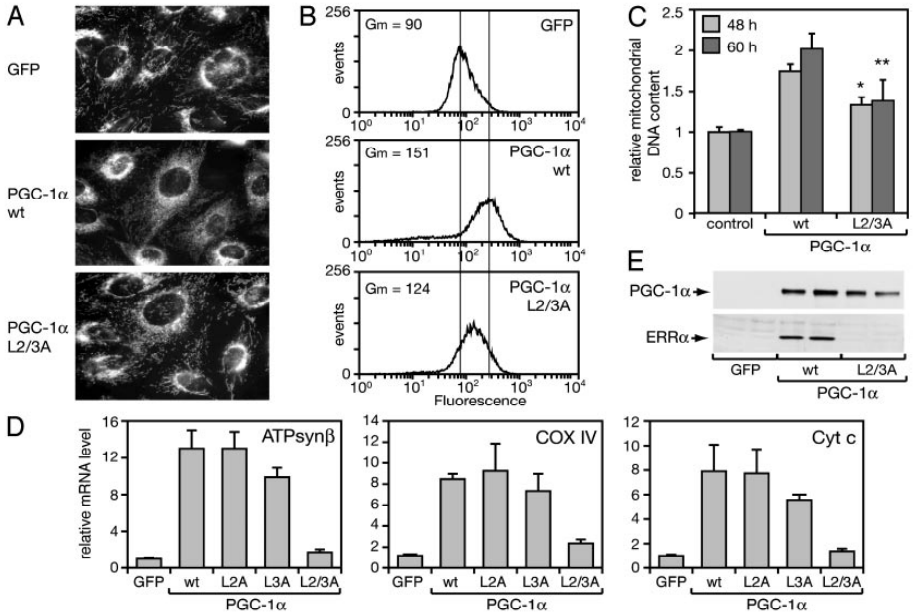

Fig. 1. PGC-1α induces mitochondrial biogenesis in SAOS2 cells, dependent on interaction with nuclear receptors. Cells were infected with GFP- or (WT or mutant L2/3A) PGC-1α-expressing adenoviruses at an moi of 40. (*A*) Mitochondria in cells labeled with CM-H_2Xros were imaged 48 h after infection. (*B*) Accumulation of MitoFluor Red 594 in cells was measured by flow cytometry 48 h after infection. Gm represents the geometric mean fluorescence intensity of 20,000 cells. (*C*) Mitochondrial (COX2) DNA levels normalized to nuclear (β actin) DNA levels are expressed relative to levels in control cells expressing GFP, which were set to 1, at 48 and 60 h after infection. Data are the mean ± SEM of three experiments performed in duplicates. *, $P < 0.0001$ versus WT PGC-1α at 48 h; **, $P < 0.001$ versus WT PGC-1α at 60 h, as determined by the Student's *t* test. (*D*) mRNA levels of ATPsynβ, Cyt c, and COX4 at 48 h after infection were determined by quantitative RT-PCR, normalized to the mRNA levels of 36B4, and expressed relative to levels in GFP-infected cells. (*E*) Protein levels of PGC-1α and ERRα were determined by Western analysis at 48 h after infection.

DNA content per cell, by 1.7- and 2-fold, at 48 and 60 h, respectively (Fig. 1*C*).

PGC-1α interacts with nuclear receptors through two leucine-rich motifs. Leucine motif 2 (L2) mediates interaction with most nuclear receptors, including ERRα, whereas motif 3 (L3) recognizes specifically ERRα and the related receptors ERRβ and ERRγ (12, 13, 28). Mutation of L2 and L3 (mutation L2/3A) disrupts interactions with nuclear receptors, without affecting the interaction domains for other factors, like NRF-1 and myocyte enhancer factor 2C (15, 16). To determine the role of nuclear receptors in PGC-1α-induced mitochondrial biogenesis, we tested the effect of the L2/3A mutation. As seen in Fig. 1*A*–*C*, the PGC-1α variant L2/3A showed a reduced ability to induce mitochondria when compared with WT PGC-1α. The L2/3A PGC-1α was also deficient in inducing the expression of nuclear genes encoding mitochondrial proteins and of ERRα (Fig. 1 *D* and *E*). The single L2A mutant, which is defective for interactions with peroxisome proliferator-activated receptors, the glucocorticoid receptor, and thyroid hormone receptors (TR), but retains interactions with ERRα (12, 13, 18, 29, 30), was as active as WT PGC-1α in inducing the expression of target genes (Fig. 1*D* and data not shown). We concluded that interactions of PGC-1α with nuclear receptors and potentially ERRα are important for PGC-1α to induce the program of mitochondrial biogenesis.

ERRα Expression Is Required for the PGC-1α-Induced Mitochondrial Biogenesis. To address the involvement of ERRα specifically, we compared the ability of PGC-1α to induce genes encoding mitochondrial proteins in cells that express endogenous ERRα and in cells in which ERRα expression was inhibited by siRNA. As seen in Fig. 2*A*, siRNA specific for ERRα abolished the basal expression of ERRα in the absence of PGC-1α and reduced strongly the induction of ERRα by PGC-1α. Under these conditions, we determined the mRNA levels of PGC-1α up-regulated genes that carry important roles in different aspects of mitochondrial biogenesis and function: mtTFA (mitochondrial DNA replication and transcription), Tim22 (protein import into mitochondria), isocitrate dehydrogenase α (IDH3A; tricarboxylic acid cycle), carnitine/acylcarnitine translocase (fatty acid oxidation), and Cyt *c* and ATPsynβ (oxidative phosphorylation). For all six genes, PGC-1α expression led to increases in their mRNA levels when endogenous ERRα levels were not perturbed, confirming results from the arrays. Inhibition of ERRα expression by siRNA reduced significantly the ability of PGC-1α to induce these genes, without affecting basal levels in the absence of PGC-1α (Fig. 2*B*). Because the siRNA diminished but did not abolish ERRα expression, the remaining induction by PGC-1α could still be mediated by the low levels of ERRα (Fig. 2*A*) as well as by other pathways. Inhibition of ERRα did not prevent PGC-1α from inducing glucocorticoid receptor targets, such as p21 (data not shown), or affect the mRNA levels of the transcription factors NRF-1 and NRF-2 (Fig. 2*B*).

The requirement of ERRα for the induction of genes, such as mtTFA and Tim22, suggests that ERRα is required for PGC-1α-dependent mitochondrial biogenesis. Indeed, inhibition of ERRα expression significantly diminished the ability of PGC-1α to increase mitochondrial DNA content (Fig. 2*C*). Inhibition of ERRα had no effect on mitochondrial DNA in the absence of PGC-1α, leading us to conclude that ERRα

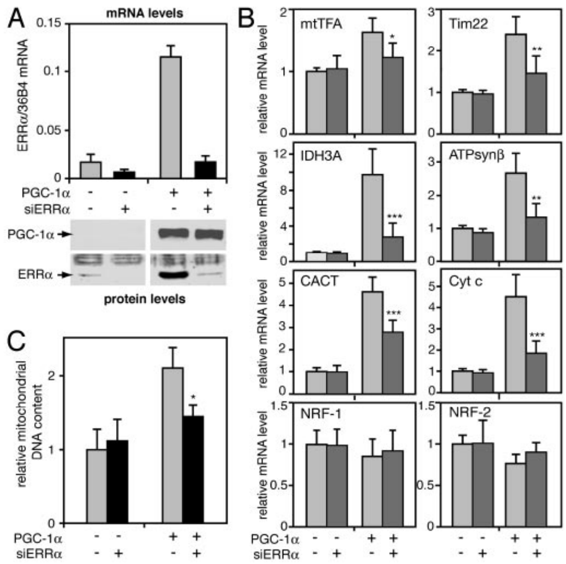

Fig. 2. Inhibition of ERRα expression impairs the induction of mitochondrial biogenesis by PGC-1α. SAOS2 cells were infected with control- (AdSUPER) or an adenovirus-expressing siRNA for ERRα (siERRα) at a moi of 100. Two days later, cells were infected with GFP- or PGC-1α-expressing adenoviruses at a moi of 20 (*A* and *B*) or 40 (*C*). Cells were harvested 24 h (*A* and *B*) or 48 h (*C*) later. (*A*) ERRα mRNA levels were determined by quantitative RT-PCR and normalized to 36B4 levels. Data shown are the mean ± SEM of three experiments performed in duplicates. (*B*) mRNA levels for mtTFA, Tim22, IDH3A, ATPsynβ, carnitine/acylcarnitine translocase (CACT), Cyt *c*, NRF-1, and NRF-2, were determined by quantitative RT-PCR, normalized to the mRNA levels of 36B4, and expressed relative to levels in AdSUPER/GFP infected cells. Data are the mean ± SEM of three experiments performed in duplicates. *, $P < 0.02$; **, $P < 0.003$; ***, $P < 0.0005$ versus PGC-1α-expressing cells in the absence of siERRα. (*C*) Mitochondrial (COX2) DNA levels were normalized to nuclear (β actin) DNA levels and expressed relative to levels in control- infected (AdSUPER and GFP) cells, which were set to 1. Data are the mean ± SEM of two experiments performed in duplicates. *, $P < 0.008$ versus PGC-1α-expressing cells in the absence of siERRα.

contributes to the PGC-1α-mediated induction but not the basal expression of genes important in mitochondrial biogenesis.

A Constitutive Form of ERRα Induces Mitochondrial Biogenesis in the Absence of PGC-1α. The lack of effect of ERRα on the basal expression of genes encoding mitochondrial proteins could reflect the low levels of ERRα in the absence of PGC-1α (Fig. 2*A*), the low transcriptional activity of ERRα in the absence of PGC-1α, and/or the requirement for other PGC-1α-dependent pathways that enable the induction of these genes. To address these possibilities, we determined the effect of overexpression of ERRα or of ERRα endowed with a heterologous strong transcriptional activation domain in the absence of PGC-1α. ERRα, VP16-ERRα, and, as control, the VP16 activation domain alone, were expressed in SAOS2 cells by using adenoviral vectors. As seen in Fig. 3, neither ERRα nor VP16 by itself induced the expression of mtTFA, ATPsynβ, Cyt *c*, or IDH3A. In contrast, VP16-ERRα induced all four genes to ≈50% of the PGC-1α-induced levels (Fig. 3*A*). VP16-ERRα also led to a significant increase in the amount of cellular mitochondrial DNA (Fig. 3*B*), indicating that ERRα is capable of inducing mitochondrial biogenesis in the absence of PGC-1α, if activated by other means.

ERRα Binds to Regulatory Sites in the Promoters of ATPsynβ and Cyt *c*. We next asked whether ERRα acts directly at the promoters of genes encoding mitochondrial proteins. ERRα binds to DNA sites with the consensus sequence TCAAGGTCA, termed ERREs (3, 4, 6, 8). Analysis of the promoter and upstream regulatory sequences of 18 genes that are induced by PGC-1α indicated the presence of putative ERREs in most of them (data not shown). We focused on ATPsynβ and Cyt *c*, whose promoters have been studied (31–33) and where the putative ERREs are within 1 kb of the characterized transcription initiation sites (Fig. 4*A*). First, we tested whether ERRα binds to these sites in a gel mobility shift assay. *In vitro*-translated ERRα formed a specific complex with an oligonucleotide representing the putative ERRE at −596 bp of the Cyt *c* promoter (Fig. 4*B*). The complex was inhibited by a 100-fold excess of an oligonucleotide bearing a known ERRE from the TRα promoter (25) and oligonucleotides representing the candidate ERREs from the Cyt *c*/−9, ATPsynβ/−338, and IDH3A/−4,023 but not by oligonucleotides harboring a mutated TRα ERRE (M4), a random sequence, or another site of the IDH3A gene.

To test the significance of the ERRα binding sites for the induction of ATPsynβ and Cyt *c*, we measured the response of these two promoters to PGC-1α/ERRα in COS7 cells. PGC-1α induced the ATPsynβ and Cyt *c* promoters, driving the expression of luciferase by 2.7- and 2.6-fold (Fig. 5). Coexpression of ERRα enhanced further the induction, to 7.6- and 7.3-fold.

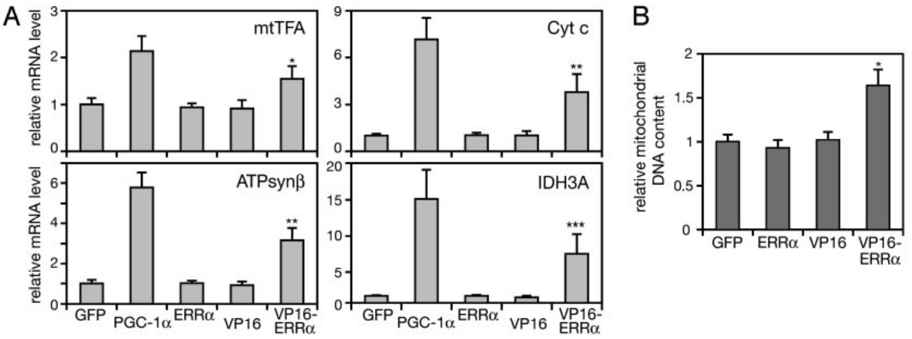

Fig. 3. A constitutively active ERRα induces mitochondrial biogenesis. SAOS2 cells were infected with adenoviruses expressing GFP, PGC-1α, ERRα, VP16, or VP16-ERRα (moi 40) and analyzed 24 h (*A*) or 60 h (*B*) later. (*A*) mRNA levels for the indicated genes were determined by quantitative RT-PCR, as in Fig. 1*D*. Data are the mean ± SEM of three experiments performed in duplicates. *, $P < 0.002$; **, $P \leq 0.0001$; ***, $P < 0.0004$ versus GFP-infected cells. (*B*) Mitochondrial DNA content was determined as in Fig. 1*C*. Data are the mean ± SEM of two experiments performed in triplicates. *, $P < 0.0001$ versus GFP-infected cells.

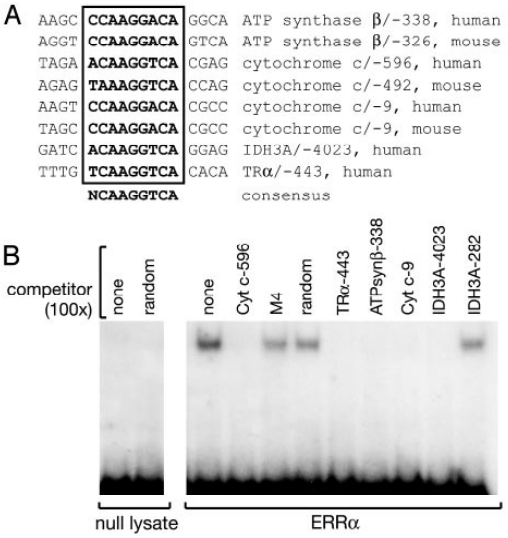

Fig. 4. ERRα recognizes sites in ATPsynβ and Cyt *c* regulatory sequences. (*A*) Sequences of candidate ERREs identified by *in silico* analysis. The TRα/−443 ERRE has been described (25). (*B*) Electrophoretic mobility shift assay. ERRα was incubated with a ^{32}P-labeled oligonucleotide containing the ERRE of Cyt *c*/−596 in the presence of unlabeled oligonucleotides as indicated. M4 oligonucleotide has the TRα/−443 sequence with a 2-bp substitution in the core ERRE (25).

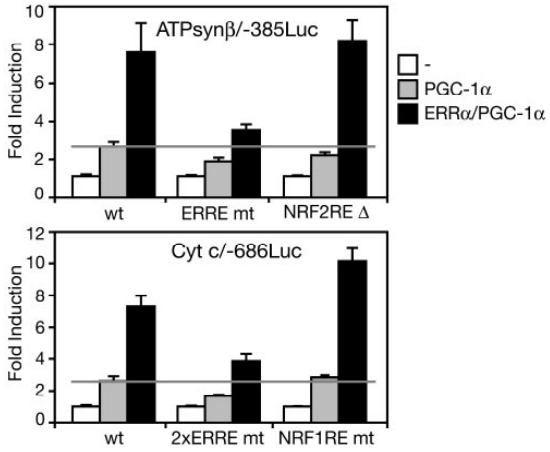

Fig. 5. The ERREs of ATPsynβ and Cyt *c* contribute to the transcriptional response to PGC-1α. COS7 cells were transfected with reporters pATPsynβ/−385Luc or pCytc/−686Luc, [WT and bearing mutations (mt) or deletions (Δ) at the ERREs and NRF-1/NRF-2 binding sites] and control vector (−), PGC-1α-, and/or ERRα-expressing plasmids as indicated. Data are expressed as fold activation by PGC-1α or PGC-1α/ERRα, with the basal activity of each construct (white bars) set to 1, and are the mean ± SEM of at least three experiments performed in duplicates.

Mutations in the ERREs decreased the response to PGC-1α and PGC-1α/ERRα by 40–50% (Fig. 4*C*) without affecting the basal levels of expression in the absence of PGC-1α (data not shown). The ATPsynβ and Cyt *c* promoters harbor also binding sites for NRF-2 and NRF-1, respectively (32, 33). Deletion of the NRF-2 site in the ATPsynβ promoter caused a drop in basal expression levels (by 40%) and a small decrease in the response to PGC-1α but did not affect the response to ERRα. Mutations in the NRF-1 site of Cyt *c* also reduced basal levels of expression by 40% but did not decrease the response to PGC-1α or PGC-1α/ERRα (Fig. 5). Taken together, our results indicate that the promoters of the two genes are responsive to ERRα and that the identified ERREs contribute to, but are not solely responsible for, the induction by PGC-1α and ERRα.

Discussion

PGC-1α has been shown previously to induce mitochondrial biogenesis and oxidative metabolism in muscle cells, adipocytes, and cardiomyocytes (14, 17, 19). These studies also provided evidence that the transcription factors NRF-1 and NRF-2 mediate the effects of PGC-1α on the expression of nuclear genes encoding mitochondrial proteins (17). We now show that PGC-1α expression in SAOS2 cells, osteoblast progenitors with adipocyte differentiation capacity (34), also induces mitochondrial biogenesis. Interestingly, this PGC-1α-driven program depends on the induction and activation of the orphan nuclear receptor ERRα. Moreover, in the absence of PGC-1α, a constitutively active ERRα induces mitochondrial biogenesis and the expression of genes essential for oxidative phosphorylation. Our findings demonstrate a role for ERRα in the control of mitochondrial biogenesis and function and suggest that, depending on the cell type, ERRα activity is necessary and sufficient to induce mitochondrial biogenesis. Consistent with these findings, RNA profiling studies have recently shown a tight correlation of the expression of ERRα with that of genes encoding mitochondrial proteins (27).

Mitochondrial abundance and oxidative capacity are cell type-specific and regulated by energy demand. For example, physical exercise and chronic exposure to cold lead to the biogenesis of mitochondria in muscle and brown fat, respectively (35, 36). This adaptive response requires the coordinated induction of a large set of nuclear genes, accomplished, at least in part, by PGC-1α and the transcription factors NRF-1 and NRF-2 (35, 37). Because not all genes encoding mitochondrial proteins have binding sites for NRF-1 and NRF-2, additional factors must contribute to the response (37, 38). Possibly, the different factors contribute selectively to mitochondrial biogenesis in different cellular contexts; e.g., the levels of NRF-1 are induced during PGC-1α-mediated mitochondrial biogenesis in muscle but decreased when PGC-1α and mitochondria levels rise during brown fat development (39, 40). NRF-1, NRF-2, and ERRα may act synergistically in some cell types and operate independently in others. The presence of multiple factors may serve to integrate diverse signals into mitochondrial biogenesis. Furthermore, the different factors may enhance differentially the expression of specific genes, thereby enabling the newly made mitochondria to be selectively endowed with cell type- or signal-specific functions. Interestingly, ERRα alone (i.e., in the absence of PGC-1α or other activating signals) had no effect on "mitochondrial genes," suggesting a function in the tissue-specific or signal-dependent regulation, rather than basal expression of genes encoding mitochondrial proteins.

Consistent with our findings, expression of Cyt *c* is downregulated in mice that carry a targeted null mutation in the ERRα gene (41). Further studies will be necessary to define mitochondrial defects and to determine whether other factors may partially compensate for the loss of ERRα function in these mice. One such candidate factor is the related receptor ERRγ, which is not expressed in the SAOS2 cells used in our study (data not shown). The ERRα-null mice display also altered expression of many genes involved in lipid metabolism (41). Together with our findings that ERRα is important for the PGC-1α-driven

induction of the carnitine/acylcarnitine translocase and medium-chain acyl-CoA dehydrogenase genes (12), these observations suggest that ERRα function contributes to other PGC-1α-induced pathways, such as fatty acid β-oxidation (8, 9, 18). Finally, while our study demonstrates a role for ERRα as an important effector of PGC-1α, it is still possible that ERRα carries additional roles in regulating PGC-1α activity, as previously suggested (28).

Mitochondrial dysfunction and, in particular, decreases in oxidative capacity have been linked to insulin resistance and type 2 diabetes (42, 43). Recent studies also suggest that decreases in the levels of PGC-1α and the related coactivator PGC-1β contribute to the reduced oxidative capacity in diabetic subjects (23, 44). Supporting this notion, polymorphisms in the PGC-1α gene have been associated to an increased risk of diabetes (45, 46), whereas mice overexpressing PGC-1β show increased levels of ERRα and resistance to high-fat induced obesity (47). Strategies aimed at enhancing ERRα activity may thus have therapeutic applications in diseases associated with reduced mitochondrial function, such as diabetes.

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Supporting tables

Table 1. Oligonucleotides used in the study

A. Oligonucleotides used in quantitative real-time PCR

Gene	Forward primer 5'-3	Reverse primer 5'-3'	NCBI no.	exons
ATPsynβ	GCAAGGCAGGGAGACCAGA	CCCAAAGTCTCAGGACCAACA	NM_001686	2/3
β actin	TCACCCACACTGTGCCCATCTACGA	CAGCGGAACCGCTCATTGCCAATGG	NM_001101	3
CACT	CTGGAGAACGGATCAAGTGCT	CCCTTTGTAGATGCCTCGGAT	NM_000387	4/5
COX2	CCTGCGACTCCTTGACGTTG	AGCGGTGAAAGTGGTTTGGTT	NM_173705	---
COX4i1	CAAGCGAGCAATTTCCACCT	GGTCACGCCGATCCATATAAG	NM_001861	2/3
Cyt c	CCAGTGCCACACCGTTGAA	TCCCCAGATGATGCCTTTGTT	NM_018947	2/3
ERRα	AAGACAGCAGCCCCAGTGAA	ACACCCAGCACCAGCACCT	NM_004451	4/6
IDH3A	ATTGATCGGAGGTCTCGGTGT	CAGGAGGGCTGTGGGATTC	NM_005530	9/10
mtTFA	GATGCTTATAGGGCGGAGTGG	GCTGAACGAGGTCTTTTTGGT	NM_003201	5/6
NRF-1	CAGCAGGTCCATGTGGCTACT	GCCGTTTCCGTTTCTTTCC	NM_005011	3/4
NRF-2	CAAGGCAACAGATGAAACGG	GACTTGCTGACCCCCTGAACT	NM_005254	7/9
36B4	CTGTGCCAGCCCAGAACACT	TGACCAGCCCAAAGGAGAAG	NM_001002	3/4
Tim22	CCAAGTCCAGCCAAGAGTGAG	CAGCGGTAAACACCCCAAAT	NM_013337	1/2

B. Oligonucleotides used in gel mobility shift assays
(the core sequence of an ERRα recognition site is in bold)

Gene/position	Sequence 5'-3
TRα/-443	GCGATTTG**TCAAGGTCA**CACAGCGC
TRα M4	GCGATTTG**TCAAG**tg**CA**CACAGCGC
random	GCGCTAGACTCGGGCTTGCGGACGC
ATPsynβ/-338	GCGAAAGC**CCAAGGACA**GGCAACGC
Cyt c/-596	CGCCTAGA**ACAAGGTCA**CGAGCCGC
Cytc/-9	GCGTAAGT**CCAAGGACA**CGCCGCGC
IDH3A/-4023	GCGAGATC**ACAAGGTCA**GGAGGCGC
IDH3A/-282	GCGCGCTG**TTAAGGTAA**GACGTCGC

C. Oligonucleotides used to clone the ATPsynβ and Cyt c promoter sequences
(gene-specific sequences are in bold)

Gene	Forward primer 5'-3	Reverse primer 5'-3'
ATPsynβ (-385/+90)	GGACTCGAG**GCCCCTATGGCTGTCACCTAG**	GCCAAGCTT**GCGACGCTGAAGGGGTGAGT**
Cyt c (-686/+55)	GGAGTCGA**CAAATGCAGCACCTTCCTCAGT**	GCCAAGCT**TCGCTGGCACAACGAACACT**

Table 2. Gene lists

A. List of genes that encode mitochondrial proteins and that were significantly upregulated by PGC-1α in all three experiments

For genes that are represented by more than one probe set on the array, data for all representing probe sets are shown
Expression indicates the mean ± SEM of values from three experiments
The significance of the increase in PGC-1α expressing cells compared to control (β-gal-expressing cells) was calculated using the one-way ANOVA test

Gene	Probe set	Expression , control	Expression , +PGC-1α	Significance	Classification	Description	RefSeq
SUPV3L1	212894_at	237 ± 34.5	534 ± 61.2	0.046	replication and transcription	suppressor of var1, 3-like 1 (S. cerevisiae)	NM_003171.1
ENDOG	204824_at	262 ± 24.1	562 ± 77.0	0.041	replication/apoptosis	endonuclease G	NM_004435.1
LARS2	204016_at	152 ± 13.8	347 ± 18.8	0.006	protein synthesis	leucyl-tRNA synthetase, mitochondrial	NM_015340.1
	34764_at	107 ± 13.9	373 ± 24.3	0.008	protein synthesis	leucyl-tRNA synthetase, mitochondrial	D21851
MTIF2	203095_at	463 ± 49.2	846 ± 53.9	0.023	protein synthesis	mitochondrial translational initiation factor 2	NM_002453.1
MRPL2	218887_at	218 ± 14.9	398 ± 19.8	0.006	protein synthesis	mitochondrial ribosomal protein L2	NM_015950.1
MRPL11	219162_s_at	382 ± 24.6	530 ± 28.6	0.018	protein synthesis	mitochondrial ribosomal protein L11	NM_016050.1
MRPL12	203931_s_at	1408 ± 99.0	1990 ± 123.5	0.030	protein synthesis	mitochondrial ribosomal protein L12	NM_002949.1
MRPL15	218027_at	498 ± 38.5	1072 ± 75.8	0.008	protein synthesis	mitochondrial ribosomal protein L15	NM_014175.1
MRPL34	221692_s_at	520 ± 42.6	1078 ± 48.9	0.004	protein synthesis	mitochondrial ribosomal protein L34	AB049652.1
MRPL35	218890_x_at	421 ± 32.9	848 ± 55.4	0.006	protein synthesis	mitochondrial ribosomal protein L35	NM_016622.1
MRPL46	219244_s_at	240 ± 18.7	617 ± 61.0	0.011	protein synthesis	mitochondrial ribosomal protein L46	NM_022163.1
MRPS7	217932_at	1303 ± 80.4	1792 ± 116.0	0.028	protein synthesis	mitochondrial ribosomal protein S7	NM_015971.1
MRPS10	218106_s_at	187 ± 14.6	264 ± 15.3	0.031	protein synthesis	mitochondrial ribosomal protein S10	NM_018141.1
MRPS12	210008_s_at	140 ± 12.1	327 ± 32.1	0.017	protein synthesis	mitochondrial ribosomal protein S12	AA513737
	204331_s_at	549 ± 49.5	1070 ± 105.3	0.029	protein synthesis	mitochondrial ribosomal protein S12	NM_021107.1
MRPS15	221437_s_at	282 ± 22.0	478 ± 38.6	0.019	protein synthesis	mitochondrial ribosomal protein S15	NM_031280.1
MRPS22	219220_x_at	542 ± 33.9	903 ± 39.5	0.005	protein synthesis	mitochondrial ribosomal protein S22	NM_020191.1
MRPS30	218398_at	330 ± 26.1	553 ± 34.1	0.015	protein synthesis	mitochondrial ribosomal protein S30	NM_016640.1
MRPS31	212603_at	272 ±25	447 ± 35.5	0.035	protein synthesis	mitochondrial ribosomal protein S31	NM_005830.1
	212604_at	191 ± 13.5	328 ± 18.3	0.007	protein synthesis	mitochondrial ribosomal protein S31	NM_005830.1
MTO1	222014_x_at	165 ± 9.6	244 ± 16.6	0.029	protein synthesis	MTO1 protein	AI249752
	218716_x_at	402 ± 21.8	574 ± 27.4	0.008	protein synthesis	MTO1 protein	NM_012123.1
HMGE	212434_at	361 ± 24.5	598 ± 29.3	0.011	protein transport	GrpE-like protein cochaperone	AL542571
MTX1	210386_s_at	740 ± 56.8	1373 ± 73.6	0.006	protein transport	metaxin 1	BC001906.1
MTX2	203517_at	238 ± 17.5	426 ± 51.2	0.041	protein transport	metaxin 2	NM_006554.1
TIMM17A	215171_s_at	853 ± 76.7	1616 ± 76.0	0.010	protein transport	translocase of inner mitochondrial membrane 17 homolog A (yeast)	AK023063.1
	201821_s_at	447 ± 34.7	906 ± 74.0	0.011	protein transport	translocase of inner mitochondrial membrane 17 homolog A (yeast)	BC004439.1
TIMM22	219184_x_at	342 ± 29.9	662 ± 31.5	0.006	protein transport	translocase of inner mitochondrial membrane 22 homolog (yeast), Tim22	NM_013337.1
TOMM40	202264_s_at	300 ± 26.7	571 ± 55.0	0.024	protein transport	translocase of outer mitochondrial membrane 40 homolog (yeast)	NM_006114.1
TOMM70A	201519_at	264 ± 19.3	482 ± 25.6	0.009	protein transport	translocase of outer mitochondrial membrane 70 homolog A (yeast)	NM_014820.1
	201512_s_at	226 ± 14.5	367 ± 21.1	0.011	protein transport	translocase of outer mitochondrial membrane 70 homolog A (yeast)	BC003633.1
FRDA	205565_s_at	116 ± 10.6	267 ± 23.5	0.012	small molecule transport	Friedreich ataxia	NM_000144.1
SLC25A3	200030_s_at	4734 ± 349.9	7009 ± 326.3	0.008	small molecule transport	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3	NM_002635.1
SLC25A4	202825_at	484 ± 41.0	1152 ± 49.4	0.003	small molecule transport	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4	NM_001151.1
SLC25A5	200657_at	3830 ± 252.4	6080 ± 372.5	0.012	small molecule transport	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5	NM_001152.1
SLC25A6	212826_s_at	3272 ± 201.4	5035 ± 297.2	0.008	small molecule transport	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6	AI961224
	212085_at	3413 ± 210.2	5277 ± 367.6	0.019	small molecule transport	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6	AA916851
SLC25A11	207088_s_at	499 ± 41.1	916 ± 79.0	0.021	small molecule transport	solute carrier family 25 (mitochondrial carrier; oxoglutarate carrier), member 11	NM_003562.1
	209003_at	412 ± 37.8	754 ± 58.0	0.021	small molecule transport	solute carrier family 25 (mitochondrial carrier; oxoglutarate carrier), member 11	AF070548.1
SLC25A12	203340_s_at	150 ± 15.8	426 ± 36.6	0.010	small molecule transport	solute carrier family 25 (mitochondrial carrier, Aralar), member 12	NM_003705.1
	203339_at	122 ± 7.7	244 ± 21.5	0.015	small molecule transport	solute carrier family 25 (mitochondrial carrier, Aralar), member 12	AI887457
SLC25A20	203658_at	186 ± 13.6	460 ± 21.9	0.005	small molecule transport/FAO	solute carrier family 25 (carnitine/acylcarnitine translocase), member 20 (CACT)	BC001689.1
ATP5F1	211755_s_at	2833 ± 195.3	4715 ± 204.8	0.006	oxphos	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1	BC005960.1
ATP5G3	207508_at	2479 ± 167.5	4402 ± 258.2	0.006	oxphos	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9) isoform 3	NM_001689.1
	207507_s_at	1612 ± 110.9	2855 ± 179.7	0.007	oxphos	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9) isoform 3	NM_001689.1
ATP5G1	208972_s_at	1084 ± 70.5	1554 ± 78.1	0.005	oxphos	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 1	AL080089.1
ATP5J	202325_s_at	1823 ± 171.3	2743 ± 168.4	0.046	oxphos	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit F6	NM_001685.1
ATP5L	208745_at	351 ± 20.9	519 ± 40.8	0.030	oxphos	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit g	AA917672
ATP5A1	213738_s_at	3462 ± 206.2	5193 ± 306.1	0.008	oxphos	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1, cardiac muscle	AI587323
ATP5B	201322_at	4345 ± 330.1	7369 ± 329.1	0.007	oxphos	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (ATPsynb)	NM_001686.1
ATP5C1	205711_x_at	1050 ± 67.1	1697 ± 83.0	0.005	oxphos	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	NM_005174.1
	213366_x_at	1013 ± 64.1	1690 ± 99.0	0.008	oxphos	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	AV711183
	208870_x_at	1028 ± 72.1	1704 ± 103.7	0.009	oxphos	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	BC000931.2
ATP5O	200818_at	2921 ± 185.5	4708 ± 232.0	0.006	oxphos	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit (oligomycin sensitivity confer	NM_001697.1

ATP6V1D	208898_at	1071 ± 61.7	1451 ± 76.1	0.009	oxphos	ATPase, H+ transporting, lysosomal 34kDa, V1 subunit D	AF077614.1
COQ3	221227_x_at	252 ± 17.0	458 ± 21.0	0.003	oxphos	coenzyme Q3 homolog, methyltransferase (yeast)	NM_017421.1
COQ7	210820_x_at	96 ± 6.0	151 ± 6.3	0.004	oxphos	coenzyme Q7 homolog, ubiquinone (yeast)	AL136647.1
	209746_s_at	123 ± 8.2	184 ± 12.7	0.011	oxphos	coenzyme Q7 homolog, ubiquinone (yeast)	AF032900.1
COX10	203858_s_at	268 ± 27.3	523 ± 25.9	0.010	oxphos	COX10 homolog, cytochrome c oxidase assembly protein, heme A: farnesyltransferase (yeast)	NM_001303.1
COX4I1	200086_s_at	1445 ± 87.8	2128 ± 135.9	0.018	oxphos	cytochrome c oxidase subunit IV isoform 1	AA854966
COX5A	203663_s_at	982 ± 67.9	1555 ± 102.4	0.015	oxphos	cytochrome c oxidase subunit Va	NM_004255.1
COX5B	202343_x_at	1752 ± 118.8	2916 ± 202.5	0.012	oxphos	cytochrome c oxidase subunit Vb	NM_001862.1
	213735_s_at	1844 ± 143.3	3049 ± 187.7	0.014	oxphos	cytochrome c oxidase subunit Vb	AI557312
	211025_x_at	1749 ± 116.6	2852 ± 206.9	0.015	oxphos	cytochrome c oxidase subunit Vb	BC006229.1
COX6A1	200925_at	3694 ± 294.5	5697 ± 385.7	0.028	oxphos	cytochrome c oxidase subunit VIa polypeptide 1	NM_004373.1
COX6B	201441_at	1447 ± 94.3	2365 ± 166.9	0.013	oxphos	cytochrome c oxidase subunit VIb	NM_001863.2
COX6C	201754_at	2390 ± 149.2	3447 ± 212.6	0.010	oxphos	cytochrome c oxidase subunit VIc	NM_004374.1
COX7B	202110_at	1245 ± 108.3	2068 ± 145.5	0.022	oxphos	cytochrome c oxidase subunit VIIb	NM_001866.1
COX7C	217491_x_at	1553 ± 85.5	2071 ± 135.9	0.030	oxphos	cytochrome c oxidase subunit VIIc	AF042165
CYCS	208905_at	3279 ± 239.8	5341 ± 323.8	0.012	oxphos	cytochrome c, somatic (Cyt c)	BC005299.1
CYC1	201066_at	2512 ± 226.1	4201 ± 255.3	0.022	oxphos	cytochrome c-1	NM_001916.1
ETFDH	33494_at	57 ± 6.5	196 ± 8.1	0.006	oxphos	electron-transferring-flavoprotein dehydrogenase	S69232
	205530_at	32 ± 4.2	127 ± 5.6	0.006	oxphos	electron-transferring-flavoprotein dehydrogenase	NM_004453.1
HCCS	203745_at	202 ± 19.9	474 ± 29.0	0.010	oxphos	holocytochrome c synthase (cytochrome c heme-lyase)	AI801013
	203746_s_at	612 ± 51.0	1132 ± 88.4	0.018	oxphos	holocytochrome c synthase (cytochrome c heme-lyase)	NM_005333.1
NDUFA2	209224_s_at	703 ± 40.0	973 ± 76.6	0.037	oxphos	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2, 8kDa	BC003674.1
NDUFA4	217773_s_at	2502 ± 162.7	3494 ± 265.9	0.042	oxphos	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4, 9kDa	NM_002489.1
NDUFA5	201304_at	248 ± 20.3	513 ± 32.6	0.008	oxphos	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5, 13kDa	NM_005000.2
NDUFA6	202001_s_at	995 ± 65.2	1552 ± 99.1	0.010	oxphos	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6, 14kDa	NM_002490.1
	202000_at	274 ± 18.9	520 ± 59.1	0.023	oxphos	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6, 14kDa	BC002772.1
NDUFA8	218160_at	618 ± 52.0	1428 ± 103.5	0.008	oxphos	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8, 19kDa	NM_014222.1
NDUFA9	208969_at	1077 ± 67.9	1711 ± 110.4	0.012	oxphos	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9, 39kDa	AF050641.1
NDUFA10	217860_at	723 ± 46.7	1154 ± 62.8	0.008	oxphos	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42kDa	NM_004544.1
NDUFB5	203621_at	776 ± 62.2	1538 ± 86.4	0.005	oxphos	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5, 16kDa	NM_002492.1
NDUFB6	203613_s_at	1038 ± 70.0	1361 ± 85.2	0.032	oxphos	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6, 17kDa	NM_002493.1
NDUFAB1	202077_at	2068 ± 151.8	3092 ± 163.0	0.009	oxphos	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1, 8kDa	NM_005003.1
NDUFS1	203039_s_at	524 ± 41.8	872 ± 61.2	0.022	oxphos	NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa (NADH-coenzyme Q reductase)	NM_005006.1
NDUFS2	201966_at	762 ± 50.9	1241 ± 92.3	0.014	oxphos	NADH dehydrogenase (ubiquinone) Fe-S protein 2, 49kDa (NADH-coenzyme Q reductase)	NM_004550.1
NDUFS3	201740_at	1009 ± 86.5	2563 ± 117.1	0.005	oxphos	NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa (NADH-coenzyme Q reductase)	NM_004551.1
NDUFS7	211752_s_at	365 ± 29.8	551 ± 26.7	0.018	oxphos	NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20kDa (NADH-coenzyme Q reductase)	BC005954.1
NDUFV1	208714_at	793 ± 71.9	1191 ± 71.3	0.033	oxphos	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa	AF092131.1
NNT	202784_s_at	119 ± 10.4	249 ± 24.2	0.011	oxphos	nicotinamide nucleotide transhydrogenase	NM_012343.1
	202783_at	72 ± 5.3	151 ± 15.3	0.012	oxphos	nicotinamide nucleotide transhydrogenase	U40490.1
SDHA	222021_x_at	1189 ± 73.3	1626 ± 82.1	0.006	oxphos	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	AI348006
	201093_x_at	976 ± 63.0	1370 ± 82.3	0.018	oxphos	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	NM_004168.1
SDHB	202675_at	751 ± 60.7	1639 ± 127.0	0.009	oxphos	succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	NM_003000.1
SDHD	202026_at	203 ± 16.3	540 ± 48.2	0.006	oxphos	succinate dehydrogenase complex, subunit D, integral membrane protein	NM_003002.1
UQCRB	205849_s_at	1945 ± 137.3	3068 ± 180.9	0.009	oxphos	ubiquinol-cytochrome c reductase binding protein	NM_006294.1
	209065_at	116 ± 8.7	212 ± 14.9	0.009	oxphos	ubiquinol-cytochrome c reductase binding protein	BC005230.1
	209066_x_at	1420 ± 106.8	2259 ± 144.2	0.014	oxphos	ubiquinol-cytochrome c reductase binding protein	M26700.1
HSPC051	218190_s_at	2274 ± 148.2	3325 ± 226.8	0.022	oxphos	ubiquinol-cytochrome c reductase complex (7.2 kD)	NM_013387.1
UQCRC1	201903_at	2754 ± 248.5	4553 ± 399.6	0.041	oxphos	ubiquinol-cytochrome c reductase core protein I	NM_003365.1
UQCRC2	200883_at	389 ± 32.8	999 ± 69.7	0.006	oxphos	ubiquinol-cytochrome c reductase core protein II	NM_003366.1
	212600_s_at	524 ± 46.8	1224 ± 64.8	0.006	oxphos	ubiquinol-cytochrome c reductase core protein II	AV727381
UQCRFS1	208909_at	1337 ± 95.5	2418 ± 123.3	0.006	oxphos	ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	BC000649.1
PDHA1	200980_s_at	881 ± 78.8	1721 ± 83.1	0.007	TCA cycle	pyruvate dehydrogenase (lipoamide) alpha 1	NM_000284.1
DLAT	212568_s_at	240 ± 19.6	636 ± 44.4	0.006	TCA cycle	dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex)	BF378872
	211150_s_at	315 ± 27.5	742 ± 57.6	0.008	TCA cycle	dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex)	J03866.1
	213149_at	86 ± 10.1	236 ± 15.8	0.009	TCA cycle	dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex)	AW299740
PDX1	203067_at	384 ± 23.3	626 ± 29.1	0.006	TCA cycle	Pyruvate dehydrogenase complex, lipoyl-containing component X; E3-binding protein	NM_003477.1
DLD	209095_at	1452 ± 95.3	2227 ± 145.4	0.008	TCA cycle	dihydrolipoamide dehydrogenase (E3 component of pyruvate dehydrogenase complex, 2-oxo-glutar	J03620.1
ACO2	200793_s_at	397 ± 44.7	1427 ± 70.7	0.004	TCA cycle	aconitase 2, mitochondrial	NM_001098.1
SUCLG1	217874_at	1377 ± 95.9	2529 ± 107.1	0.004	TCA cycle	succinate-CoA ligase, GDP-forming, alpha subunit	NM_003849.1
IDH3A	202069_s_at	215 ± 29.4	1010 ± 74.6	0.005	TCA cycle	isocitrate dehydrogenase 3 (NAD+) alpha	AI826060
	202070_s_at	255 ± 34.1	1142 ± 65.2	0.005	TCA cycle	isocitrate dehydrogenase 3 (NAD+) alpha	NM_005530.1
IDH3B	210418_s_at	443 ± 27.7	690 ± 43.0	0.010	TCA cycle	isocitrate dehydrogenase 3 (NAD+) beta	AF023265.1
	210014_x_at	505 ± 30.1	822 ± 58.4	0.012	TCA cycle	isocitrate dehydrogenase 3 (NAD+) beta	AF023266.1
OGDH	201282_at	235 ± 23.9	641 ± 53.2	0.007	TCA cycle	oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)	NM_002541.1

MDH2	213333_at	189 ± 14.4	375 ± 21.2	0.008	TCA cycle	malate dehydrogenase 2, NAD (mitochondrial)	AL520774
MUT	202960_s_at	208 ± 14.7	392 ± 23.9	0.009	TCA cycle	methylmalonyl Coenzyme A mutase	NM_000255.1
CS	208660_at	1580 ± 148.1	2867 ± 162.7	0.018	TCA cycle	citrate synthase	BC000105.1
GOT2	200708_at	1359 ± 123.2	2063 ± 115.3	0.023	TCA cycle	glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2)	NM_002080.1
MDH2	209036_s_at	1143 ± 93.4	1632 ± 89.7	0.033	TCA cycle	malate dehydrogenase 2, NAD (mitochondrial)	BC001917.1
SUCLA2	202930_s_at	236 ± 48.4	582 ± 40.9	0.049	TCA cycle	succinate-CoA ligase, ADP-forming, beta subunit	NM_003850.1
CPT1A	203633_at	196 ± 19.5	407 ± 22.5	0.009	Fatty acid oxidation (FAO)	carnitine palmitoyltransferase 1A (liver)	BF001714
FACL3	201661_s_at	241 ± 25.9	425 ± 34.7	0.027	Fatty acid oxidation (FAO)	fatty-acid-Coenzyme A ligase, long-chain 3	NM_004457.2
	201662_s_at	500 ± 37.3	967 ± 45.8	0.004	Fatty acid oxidation (FAO)	fatty-acid-Coenzyme A ligase, long-chain 3	D89053.1
ACADM	202502_at	705 ± 62.7	1305 ± 68.8	0.008	Fatty acid oxidation (FAO)	acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain	NM_000016.1
HADHA	208631_s_at	907 ± 57.8	1323 ± 96.7	0.030	Fatty acid oxidation (FAO)	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydra	U04627.1
HADHB	201007_at	1540 ± 88.7	2040 ± 107.8	0.015	Fatty acid oxidation (FAO)	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydra	NM_000183.1
ACADVL	200710_at	304 ± 28.1	463 ± 27.1	0.025	Fatty acid oxidation (FAO)	acyl-Coenzyme A dehydrogenase, very long chain	NM_000018.1
BAK1	203728_at	203 ± 16.6	444 ± 30.5	0.006	apoptosis	BCL2-antagonist/killer 1	NM_001188.1
PDCD8	205512_s_at	268 ± 19.1	400 ± 24.8	0.013	apoptosis	programmed cell death 8 (apoptosis-inducing factor)	NM_004208.1
BCL2L1	212312_at	149 ± 10.8	251 ± 24.3	0.034	apoptosis	BCL2-like 1	AL117381
MFN2	201155_s_at	1034 ± 78.2	2160 ± 87.3	0.005	morphology	mitofusin 2	NM_014874.1
	216205_s_at	316 ± 34.0	760 ± 67.6	0.015	morphology	mitofusin 2	AK021947.1
OPA1	212213_x_at	270 ± 25.8	458 ± 23.8	0.015	morphology	optic atrophy 1 (autosomal dominant)	AB011139.1
IMMT	200955_at	987 ± 67.3	1559 ± 106.9	0.018	morphology	inner membrane protein, mitochondrial (mitofilin)	NM_006839.1
DNM1L	203105_s_at	373 ± 23.4	535 ± 35.7	0.023	morphology	dynamin 1-like	NM_012062.1
MIRO-2	221789_x_at	351 ± 20.2	472 ± 23.6	0.008	morphology	mitochondrial Rho 2	AK024450.1
	65770_at	501 ± 30.0	733 ± 37.9	0.008	morphology	mitochondrial Rho 2	AI024450.1
	222131_x_at	338 ± 26.0	500 ± 35.2	0.023	morphology	mitochondrial Rho 2	BC004327.1
TPT	220865_s_at	127 ± 9.0	287 ± 16.6	0.005	coenzyme Q biosynthesis	trans-prenyltransferase	NM_014317.2
GK	207387_s_at	41 ± 3.1	60 ± 3.4	0.005	carbohydrate metabolism	glycerol kinase	NM_000167.1
FDXR	207813_s_at	95 ± 6.0	213 ± 22.1	0.012	steroid biogenesis	ferredoxin reductase	NM_004110.2
ALAS1	205633_s_at	539 ± 38.8	956 ± 48.0	0.009	heme biosynthesis	aminolevulinate, delta-, synthase 1	NM_000688.1
LIAS	214045_at	101 ± 9.9	160 ± 7.1	0.027	lipoic acid synthesis	lipoic acid synthetase	BF056778
MUT	202959_at	111 ± 10.2	193 ± 8.3	0.014	AS catabolism	methylmalonyl Coenzyme A mutase	AI433712
AUH	205052_at	89 ± 6.4	205 ± 18.7	0.010	mRNA degradation	AU RNA binding protein/enoyl-Coenzyme A hydratase	NM_001698.1
LRPPRC	211971_s_at	501 ± 35.3	901 ± 48.3	0.006	RNA processing	leucine-rich PPR-motif containing	AF052133.1
	211615_s_at	521 ± 50.2	856 ± 46.5	0.027	RNA processing	leucine-rich PPR-motif containing	M92439.1
AFG3L2	202486_at	305 ± 30.9	610 ± 37.1	0.012	proteolysis	AFG3 ATPase family gene 3-like 2 (yeast)	NM_006796.1
HSPA9B	200692_s_at	1337 ± 73.0	1846 ± 120.6	0.022	proliferation	heat shock 70kDa protein 9B (mortalin-2)	NM_004134.1
	200691_s_at	1464 ± 94.2	1984 ± 114.8	0.027	proliferation	heat shock 70kDa protein 9B (mortalin-2)	BC000478.1
	200690_at	228 ± 17.4	314 ± 19.0	0.045	proliferation	heat shock 70kDa protein 9B (mortalin-2)	AA927701
MTCP1	205106_at	35 ± 2.5	58 ± 3.2	0.010	proliferation	mature T-cell proliferation 1	NM_014221.1
VDAC2	211662_s_at	2102 ± 155.9	3378 ± 150.1	0.006	ion channel	voltage-dependent anion channel 2	L08666.1
CYP24A1	206504_at	15 ± 0.8	35 ± 2.0	0.006	calcium homeostasis	cytochrome P450, family 24, subfamily A, polypeptide 1	NM_000782.1
GBAS	201816_s_at	120 ± 9.5	204 ± 15.0	0.011	protein trafficking	glioblastoma amplified sequence	NM_001483.1
AKAP1	201674_s_at	552 ± 31.5	806 ± 54.6	0.014	signaling	A kinase (PRKA) anchor protein 1	BC000729.1
	210625_s_at	334 ± 19.0	468 ± 31.0	0.018	signaling	A kinase (PRKA) anchor protein 1	U34074.1
CGI-51	201569_s_at	157 ± 15.9	518 ± 37.8	0.005	membrane protein sorting	CGI-51 protein	NM_015380.1
	201570_at	356 ± 38.2	958 ± 56.6	0.005	membrane assembly	CGI-51 protein	NM_015380.2
C14orf2	202279_at	354 ± 27.7	718 ± 37.7	0.005	unknown, predicted	chromosome 14 open reading frame 2	NM_004894.1
	210532_s_at	1961 ± 146.1	2786 ± 161.3	0.015	unknown, predicted	chromosome 14 open reading frame 2	AF116639.1
C21orf33	202217_at	851 ± 75.9	1527 ± 116.2	0.024	unknown, predicted	chromosome 21 open reading frame 33	NM_004649.1
	210667_s_at	316 ± 35.0	587 ± 65.1	0.150	unknown, predicted	chromosome 21 open reading frame 33	D86062.1
DKFZP564B16	202427_s_at	837 ± 58.7	1441 ± 66.4	0.003	unknown, predicted	DKFZP564B167 protein	NM_015415.1
KIAA0446	32091_at	179 ± 13.3	311 ± 27.5	0.032	unknown, predicted	KIAA0446 gene product	AB007915
	212683_at	230 ± 12.9	373 ± 24.2	0.013	unknown, predicted	KIAA0446 gene product	AL526243
LOC51064	217751_at	383 ± 24.0	602 ± 40.3	0.012	unknown, predicted	glutathione S-transferase subunit 13 homolog	NM_015917.1
NOC4	218057_x_at	452 ± 28.4	735 ± 51.0	0.018	unknown, predicted	neighbor of COX4	NM_006067.1

B. List of genes that encode mitochondrial proteins and that were upregulated by PGC-1α in all three experiments, but did not pass the one-way ANOVA test (P>0.05)

Gene	Probe set	Expression , control	Expression , +PGC-1α	Significance	Classification	Description	RefSeq
TUFM	201113_at	1396 ± 93.2	1882 ± 129.7	> 0.05	protein synthesis	Tu translation elongation factor, mitochondrial	NM_003321.1
MRPL4	218105_s_at	705 ± 65.7	1090 ± 89.8	> 0.05	protein synthesis	mitochondrial ribosomal protein L4	NM_015956.1
MRPL16	217980_s_at	264 ± 26.3	402 ± 27.7	> 0.05	protein synthesis	mitochondrial ribosomal protein L16	NM_017840.1
TIMM44	203092_at	179 ± 11.3	261 ± 29.0	> 0.05	protein transport	translocase of inner mitochondrial membrane 44 homolog (yeast)	AF026030.1

	203093_s_at	182 ± 12.7	264 ± 28.1	> 0.05	protein transport	translocase of inner mitochondrial membrane 44 homolog (yeast)	NM_006351.1
TOMM22	217960_s_at	502 ± 49.8	804 ± 89.0	> 0.05	protein transport	translocase of outer mitochondrial membrane 22 homolog (yeast)	NM_020243.1
NDUFS8	203190_at	558 ± 35.7	711 ± 55.7	> 0.05	oxphos	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa (NADH-coenzyme Q reductase)	NM_002496.1
NQO2	203814_s_at	389 ± 37.5	634 ± 85.8	> 0.05	oxphos	NAD(P)H dehydrogenase, quinone 2	NM_000904.1
OXA1L	208717_at	689 ± 72.4	1011 ± 76.9	> 0.05	oxphos	oxidase (cytochrome c) assembly 1-like	BC001669.1
ATP5D	213041_s_at	567 ± 55.0	849 ± 94.8	> 0.05	oxphos	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	BE798517
ATPAF2	213057_at	98 ± 8.6	132 ± 12.5	> 0.05	oxphos	ATP synthase mitochondrial F1 complex assembly factor 2	AW118608
NDUFA3	218563_at	992 ± 68.5	1482 ± 135.5	> 0.05	oxphos	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 3, 9kDa	NM_004542.1
FH	214170_x_at	400 ± 34.5	534 ± 47.6	> 0.05	TCA cycle	fumarate hydratase	AA669797
PDHB	208911_s_at	266 ± 17.4	364 ± 28.0	> 0.05	TCA cycle	pyruvate dehydrogenase (lipoamide) beta	M34055.1
CRAT	209522_s_at	178 ± 13.7	238 ± 15.5	> 0.05	Fatty acid oxidation (FAO)	carnitine acetyltransferase	BC000723.1
MTRR	203200_s_at	187 ± 21.6	267 ± 21.5	> 0.05	AS metabolism	5-methyltetrahydrofolate-homocysteine methyltransferase reductase	NM_024010.1
SOD2	215223_s_at	700 ± 83.7	1336 ± 231.6	> 0.05	oxidadive stress	superoxide dismutase 2, mitochondrial	W46388
	216841_s_at	110 ± 16.9	223 ± 52.3	> 0.05	oxidadive stress	superoxide dismutase 2, mitochondrial	X15132.1
SYNJ2BP	219156_at	22 ± 1.7	37 ± 6.6	> 0.05	unknown, predicted	synaptojanin 2 binding protein	NM_018373.1

C. TFAM (mtTFA) was assigned a PGC-1α up-regulated status in only two of the three experiments, but was confirmed as up-regulated by quantitative RT-PCR

Expression shows the mean ± SEM of values from all three experiments
The significance of the increase in PGC-1α expressing cells compared to control (β-gal-expressing cells) was calculated using values for all three experiments and the one-way ANOVA test.

Gene	Probe set	Expression , control	Expression , +PGC-1α	Significance	Classification	Description	RefSeq
TFAM	203177_x_at	183 ± 14.6	262 ± 15.7	0.040	transcription/replication	transcription factor A, mitochondrial (mtTFA)	NM_003201.1
	203176_s_at	100 ± 8.3	130 ± 12.2	> 0.05	transcription/replication	transcription factor A, mitochondrial (mtTFA)	BE552470
	208541_x_at	187 ± 11.2	197 ± 14.2	> 0.05	transcription/replication	transcription factor A, mitochondrial (mtTFA)	NM_012251.1

D. List of genes that encode non-mitochondrial proteins classified as "co-regulated with mitochondrial genes", [Mootha, V.K. *et al.* (2003) *Cell* 115, 629-640], and that were up-regulated significantly by PGC-1α in all three experiments

The significance of the increase in PGC-1α expressing cells compared to control (β-gal-expressing cells) was calculated using the one-way ANOVA test.

Gene	Probe set	Expression , control	Expression , +PGC-1α	Significance	Classification	Description	RefSeq
ESRRA	1487_at	214 ± 20.6	658 ± 36.8	0.002	transcription factor	estrogen-related receptor alpha	L38487
REA	201600_at	3432 ± 216.2	4873 ± 241.1	0.005	transcriptional corepressor	repressor of estrogen receptor activity	NM_007273.1
MB	204179_at	88 ± 7.6	212 ± 15.2	0.003	oxygen transport	myoglobin	NM_005368.1
SLC31A1	203971_at	216 ± 14.6	365 ± 16.7	0.002	copper homeostasis	solute carrier family 31 (copper transporters), member 1	NM_001859.1
LCE	204256_at	76 ± 8.3	148 ± 16.2	0.043	lipid metabolism	long-chain fatty-acyl elongase	NM_024090.1
PHB	200659_s_at	430 ± 28.3	599 ± 41.3	0.027	tumor suppressor	prohibitin	NM_002634.2
LPIN1	212276_at	228 ± 19.3	403 ± 38.7	0.023	differentiation, adipocytes/muscl	lipin 1	D80010.1
	212274_at	260 ± 23.6	430 ± 25.7	0.014	differentiation, adipocytes/muscl	lipin 1	D80010.1
TOB1	202704_at	84 ± 5.5	157 ± 11.8	0.011	signaling	transducer of ERBB2, 1	AA675892
PPP1R3C	204284_at	259 ± 18.3	661 ± 58.6	0.007	signaling	protein phosphatase 1, regulatory (inhibitor) subunit 3C	N26005
STARD7	200028_s_at	1915 ± 140.6	3050 ± 139.4	0.006	signaling	START domain containing 7	NM_020151.1
SMPD1	209420_s_at	283 ± 24.6	400 ± 20.1	0.038	sphingomyelin metabolism	sphingomyelin phosphodiesterase 1, acid lysosomal (acid sphingomyelinase)	M59916.1
AKIP	218580_x_at	1515 ± 113.2	2204 ± 153.9	0.035	proteasome degradation	aurora-A kinase interacting protein	NM_017900.1
CLCNKA	207047_s_at	47 ± 3.9	124 ± 8.2	0.003	excretion	chloride channel Ka	NM_004070.1
---	213758_at	90 ± 7	175 ± 8.8	0.003	unknown	Homo sapiens cDNA: FLJ23483 fis, clone KAIA04052.	AW337510
C20orf45	217851_s_at	30 ± 2.2	57 ± 3.8	0.006	unknown	chromosome 20 open reading frame 45	NM_016045.1
DKFZP434K04	212228_s_at	428 ± 38.5	1039 ± 41.5	0.002	unknown	hypothetical protein DKFZp434K046	AC004382
FLJ20420	217972_at	778 ± 52	1357 ± 61.5	0.003	unknown	hypothetical protein FLJ20420	NM_017812.1
GHITM	209248_at	1254 ± 99.1	2120 ± 111.4	0.007	unknown	growth hormone inducible transmembrane protein	AL136713.1
	209249_s_at	1989 ± 129.1	2802 ± 129.3	0.003	unknown	growth hormone inducible transmembrane protein	AF131820.1
HIG1	217845_x_at	2267 ± 127.5	2925 ± 144	0.003	unknown	likely ortholog of mouse hypoxia induced gene 1	NM_014056.1
HSPC125	219006_at	362 ± 30.6	930 ± 38.3	0.002	unknown	HSPC125 protein	NM_014165.1
MDS029	218597_s_at	527 ± 37.4	933 ± 80	0.018	unknown	uncharacterized hematopoietic stem/progenitor cells protein MDS029	NM_018464.1
MGC2198	209329_x_at	941 ± 61.1	1427 ± 75.6	0.003	unknown	hypothetical protein MGC2198	BC000587.1
MGC4276	209273_s_at	365 ± 28	552 ± 38.8	0.019	unknown	hypothetical protein MGC4276 similar to CG8198	BG387555
	209274_s_at	444 ± 32	708 ± 43.5	0.005	unknown	hypothetical protein MGC4276 similar to CG8198	BC002675.1

E. Expression values for probe sets representing NRF-1 and NRF-2 isoforms (GABPB1, GABPB2, GABPA).

Expression shows the mean ± SEM of values from all three experiments
nd, not determined

Gene	Probe set	Expression , control	Expression , +PGC-1α	Significance	Classification	Description	RefSeq
NRF1	204652_s_at	124 ± 5.5	94 ± 5.5	nd	transcription	nuclear respiratory factor 1	NM_005011.1
	211279_at	109 ± 5.4	104 ± 6.3	nd	transcription	nuclear respiratory factor 1	L22454.1
	211280_s_at	56 ± 3.1	57 ± 3.0	nd	transcription	nuclear respiratory factor 1	L22454.1
GABPB1	204618_s_at	204 ± 16.0	185 ± 12.1	nd	transcription	GA binding protein transcription factor, beta subunit 1, 53kDa	NM_005254.2
GABPB2	206173_x_at	117 ± 5.6	99 ± 7.0	nd	transcription	GA binding protein transcription factor, beta subunit 2, 47kDa	NM_002041.2
GABPA	210188_at	70 ± 6.9	107 ± 18.3	nd	transcription	GA binding protein transcription factor, alpha subunit 60kDa	D13318.1

F. Genes from Lists A-D that have been reported as regulated by estrogen receptors in response to estradiol, tamoxifen or raloxifene in U2OS cells [Kian Tee, M. *et al.* (2004) *Mol. Biol. Cell.* **5**, 1262-1272]

Of 175 genes in lists A-D, 3 have been reported as regulated by estrogen receptor alpha or estrogen receptor beta in U2OS cells [Kian Tee, M. *et al.* (2004) *Mol. Biol. Cell.* **5**, 1262-1272]
We have not yet tested whether these 3 genes are regulated by PGC-1α in an ERRα-dependent manner.
Expression shows the mean ± SEM of values from all three experiments
The significance of the increase in PGC-1α expressing cells compared to control (β-gal-expressing cells) was calculated using the one-way ANOVA test.

Gene	Probe set	Expression , control	Expression , +PGC-1α	Significance	Classification	Description	RefSeq
NDUFA2	209224_s_at	703 ± 40.0	973 ± 76.6	0.037	oxphos	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2, 8kDa	BC003674.1
AKAP1	201674_s_at	552 ± 31.5	806 ± 54.6	0.014	signaling	A kinase (PRKA) anchor protein 1	BC000729.1
	210625_s_at	334 ± 19.0	468 ± 31.0	0.018	signaling	A kinase (PRKA) anchor protein 1	U34074.1
TOB1	202704_at	84 ± 5.5	157 ± 11.8	0.011	signaling	transducer of ERBB2, 1	AA675892

APPENDIX C: CURRICULUM VITAE

Roger Emter

Personal

Birth date	7th April 1974
Citizenship	Swiss Citizen
Languages	German (native), English, French (basic)
Marital status	single
Home address	In der Einhägi 9, 4142 Münchenstein, BL

Education

1990-1993	Gymnasium (High school), Typus B (Latin), Matura
1995-1999	University of Basel, Switzerland, Curriculum of Biology II (Biochemistry, Biophysical Chemistry, Cell Biology, and Molecular Genetics)
1999-2005	Ph.D. program, Biozentrum der Universität Basel, under supervision of Prof. Dr. Michael Hall, Dr. Anastasia Kralli, and Dr. Roger Clerc

Research experience

1998-1999	Diploma Thesis " Defect in ergosterol synthesis potentiates glucocorticoid signaling in yeast" with Dr. A. Kralli, Biozentrum der Universität Basel
1999-2005	Ph.D. Thesis with Dr. A. Kralli, Biozentrum der Universität Basel and The Scripps Research Institute, La Jolla, CA (2003-2004)

Teaching experience

1998-2001	Laboratory instructor, Biochemistry, Biozentrum der Universität Basel
2001-2002	Undergraduate seminar leader, Biozentrum der Universität Basel, seminar series "Übungen zu Grundlagen der allgemeinen Biologie" WS 2001-2002

Fellowships

2000-2002	Roche Research Foundation

Publications

Sitcheran R., **Emter R.**, Kralli A. and Yamamoto K. R. (2000). A genetic analysis of glucocorticoid receptor signaling: identification and characterization of Ligand-Effect Modulators in *S. cerevisiae*. Genetics 156(3), 963-972

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Catherine Teyssier, Han Ma, **Roger Emter**, Anastasia Kralli, Michael R. Stallcup. Activation of Nuclear Receptor Coactivator PGC-1α by Arginine Methylation. Submitted

APPENDIX D: ACKNOWLEDGEMENTS

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Thanks go also to all members of the Kralli lab in Basel and San Diego and all other people I met in the Biozentrum and The Scripps Research Institute for their support, for the scientific discussions and the great time we had.

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