The transcriptional coactivator PGC-1α as a modulator of ERRα and GR signaling: function in mitochondrial biogenesis

Inauguraldissertation

Zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

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aus Remseck, Deutschland

Basel, 2004

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

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Basel, den 06.04.2004

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Abbreviations

Aa	amino acids
AF	activation function
DBD	DNA binding domain
ER	estrogen receptor
ERE	estrogen response element
ERR	estrogen related receptor
ERRE	estrogen related receptor response element
GFP	green fluorescent protein
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HNF	hepatocyte factor
LBD	ligand binding domain
LRH	liver receptor homologue
LXR	liver x receptor
NLS	nuclear localization signal
MAPK	mitogen activated kinase
MCAD	medium-chain acyl-coenzyme A dehydrogenase
MEF	myocyte enhancing factor
MR	mineralocorticoid receptor
NID	nuclear receptor interacting domain
NRF	nuclear respiratory factor
OXPHOS	oxidative phosphorylation
PPAR	peroxisome proliferator-activated receptor
PERC	PGC-1 related estrogen receptor coactivator
PGC	peroxisome proliverator-activated receptor coactivator
PR	progesterone receptor
PRC	PGC-1 related coactivator
PEPCK	phosphoenol pyruvate carboxykinase
RAR	retinoic acid receptor
RS	arginine serine rich domain
RRM	RNA recognition motif
RXR	retinoid x receptor
TR	thyroid receptor
siRNA	small interfering RNA
SHP	small heterodimer partner
VDR	vitamin D receptor

Abstract

The nuclear receptor family represents a large class of transcription factors that regulate metabolism, differentiation and development. Most of the nuclear receptor family members are orphan receptors, so called because no ligands were known when they were identified. Although for some receptors ligands have been identified by now, many receptors, such as the estrogen related receptor α (ERRα), still remain orphan. The activity of all nuclear receptors requires the recruitment of coregulators, which are able to enhance or repress their activity. Our work has focused on PGC-1α, which responds to physiological signals, such as cold, fasting and exercise, and was characterized as an important factor in the regulation of energy homeostasis and metabolic pathways. In my thesis work, we demonstrate that PGC-1α regulates the expression and activity of the orphan nuclear receptor ERRα. Our findings suggest that PGC-1α may act as a protein ligand, substituting for the lack of small lipohilic ligands for this receptor. The expression of PGC-1α and ERRα is parallel in tissues with high energy demand, and induced in vivo when animals are exposed to cold. Furthermore, our studies demonstrate that ERRα is important for PGC-1α signaling, since diminished ERRα levels significantly reduce the induction of mitochondrial biogenesis by PGC-1α. Binding sites for ERRα are observed in many genes encoding for mitochondrial proteins, and in vitro studies suggest that ERRα activates the transcription of at least a subset of the genes by binding to their promoters. Furthermore, ERRα fused to the potent VP16 activation domain is sufficient to induce mitochondrial biogenesis. We suggest that PGC-1α and ERRα regulate the transcription of genes encoding mitochondrial proteins in response to metabolic requirements.

Previous studies from our lab identified PGC-1α as a potent regulator of glucocorticoid receptor (GR) function *in vitro*. In support of our studies, other groups have shown that PGC-1α coactivates GR on the PEPCK promoter, the key enzyme of gluconeogenesis. Further data has shown that glucocorticoids and glucagon regulate the expression of PGC-1α. This led us to investigate the role of PGC-1α in GR signalling in SAOS2 cells. Our preliminary data suggest that glucocorticoids strongly

influence PGC-1α signaling, enhancing some PGC-1α pathways and suppressing others. Finally, our data provide support to the hypothesis that PGC-1α is not a general enhancer of glucocorticoid responses, but rather provides specificity to GR signalling. PGC-1α expression leads to the activation of a distinct set of genes by GR. Future studies should provide more insight into this relationship.

Chapter I: Introduction

Overview of transcriptional regulation by nuclear receptors

All cellular processes involved in development, differentiation, cell growth and metabolism are constantly regulated by the transcriptional activation or repression of many different genes. The misregulation of even a single component often leads to disease, such as obesity, diabetes or cancer (Rosmond, 2002; Smith and Kantoff, 2002; Spiegelman and Flier, 2001; Tenbaum and Baniahmad, 1997). Therefore, the control of gene expression and the mechanisms of achieving specificity in transcriptional pathways have attracted increasing amount of attention in the last two decades (reviewed in Orphanides and Reinberg, 2002). The transcription of genes is regulated in a highly organized fashion to ensure protein expression in a spatially and temporally defined manner. Mechanisms must exist that allow cells to integrate intracellular and extracellular signals to their differentiation state, cell cycle stage or metabolic state, and ensure appropriate transcriptional responses.

One class of extracellular signals are steroid hormones. These are small lipophilic molecules that are produced by endocrine glands and transported through the blood, and that can diffuse through the plasma membrane to the cell interior. They exert their transcriptional effects by binding and activating nuclear receptors, which are among the most intensively studied and probably best-understood transcription factors to date (reviewed in Aranda and Pascual, 2001; Mangelsdorf et al., 1995). Several facts have established nuclear receptors as valuable tools for studying the mechanisms that provide specificity in transcriptional regulation. First, nuclear receptors are important modulators of all aspects of physiology. Second, the expression of many nuclear receptors, for example the glucocorticoid receptor (GR), is ubiquitous (Jenkins et al., 2001), yet the responses they elicit are cell type- or physiological state-dependent. Third, nuclear receptors are regulated through small lipophilic ligands, which are good experimental tools for turning on and of the activity of the receptors, as well as have therapeutic applications.

For several years, the regulation of transcription by nuclear receptors was imagined in a simple way. Hormones 'slip' into the cells and 'waken' up the inactive receptor, which then binds to DNA and activates transcription. However, things are not as simple as they seem. First, nuclear receptors activate or repress transcription mostly, but not always, in a ligand-dependent manner. The identification of the first steroid-related receptors, the estrogen related receptors (ERRs), for which no ligand was known, founded the subgroup of orphan nuclear receptors (Giguere et al., 1988). Today, many dietary lipids and endogenous metabolites have been identified as ligands for some of the orphan nuclear receptors. These receptors are thought of as important metabolic sensors and targets for drug development (reviewed in Blumberg and Evans, 1998; Giguere, 1999; Moller, 2001). Second, increasing evidence suggests that nuclear receptors are not sufficient by themselves to interact with RNA polymerase II and activate or repress transcription. In fact, the transcriptional activity of nuclear receptors is modulated through several different classes of coregulators. In the last few years, biochemical and yeast two hybrid approaches have identified many nuclear receptor interacting proteins that act as coregulators, leading either to the activation (coactivators) or repression (corepressors) of transcription (reviewed in Glass and Rosenfeld, 2000; McKenna et al., 1999; Naar et al., 2001).

By definition, coactivators or corepressors do not interact directly with DNA, but are recruited to regulatory regions of target genes via protein-protein interactions with DNA binding transcription factors. Once recruited, they exert several different activities that may modify chromatin, the basal transcription machinery factors and/or RNA polymerase II (reviewed in Collingwood et al., 1999; Glass and Rosenfeld, 2000; McKenna et al., 1999). The first nuclear receptor coactivators proposed in the 1980s were the binding proteins of the basic transcription factor TFIID, namely the TATA-binding protein (TBP) (Hahn et al., 1989; Horikoshi et al., 1989; Kao et al., 1990) and the TBP-associated factors (TAFs) that built a bridge between DNA-specific transcription factors and the basal transcriptional machinery (Dynlacht et al., 1991; Pugh and Tjian, 1990). A few years later, a new class of coregulators was defined with the identification of a 160-kDa estrogen receptor (ER)-associated protein (ERAP-160; (Halachmi et al., 1994)), which later on turned out to be a splicing variant of the steroid receptor coactivator-1 ((SRC-1); Kamei et al., 1996; Onate et al.,

1995). The group of M.G. Parker, at the same time, identified the coregulators RIP80, RIP140 and RIP160 (Cavailles et al., 1994), which interact with ER in the presence of an agonist ligand. Shortly after, the characterization of GRIP-170, a 170 kDa GR-interacting protein (Eggert et al., 1995), demonstrated that this class of cofactors is essential for the transcriptional activity of the nuclear receptors, and suggested that it may be a functionally limiting component. To date, more than 50 coactivators and several corepressors of nuclear receptors have been identified (reviewed Collingwood et al., 1999; Glass and Rosenfeld, 2000; McKenna et al., 1999). The different coregulators have been proposed to regulate transcription either alone, sequentially, combinatorially or as big multiprotein complexes.

Increasing efforts in the last few years aim in unraveling the mechanisms of transcriptional regulation through coregulators, and elucidating their biological roles (reviewed in Glass and Rosenfeld, 2000; McKenna et al., 1999; Naar et al., 2001). While a few coregulators are expressed in a tissue-specific manner, most are expressed ubiquitously, similar to what has been observed for several nuclear receptors. Therefore, the presence of many different coactivators, corepressors and nuclear receptors in the same tissue and at the same time seems to be a common theme. Given that each coregulator can interact with multiple nuclear receptors and vice versa, understanding the mechanisms that lead to specific transcription factor – coregulator complexes, and the biological roles of these specific partnerships is fundamental.

Nuclear receptors

Nuclear receptor structure

The classical structural features of this family of transcription factors are, first, the two well conserved zinc finger domains that bind to DNA (DBD), and second, a C-terminal domain that binds ligand (ligand binding domain, LBD) and carries a transcriptional activation function (reviewed in Aranda and Pascual, 2001). In addition, all nuclear receptors harbor nuclear localization signal (NLS). Nuclear receptors also have a variable N-terminal domain, which in some cases carries a second transcriptional activation function. Accordingly, nuclear receptors can be divided into five regions based on structural and functional similarities (see figure 1): the variable N-terminal region (A/B) that may contain a transcriptional activation function (AF-1); the conserved DBD, which also includes a dimerization domain (C); variable hinge region (D); a conserved LBD with a second dimerization domain and the conserved transcriptional activation function AF-2 (E); and a variable C-terminal region (F). Whereas the AF-1 activity can function independently of ligand binding, the AF-2 activity is strictly ligand-dependent. (reviewed in Warnmark et al., 2003). The AF-2 domain is essential to determine the binding of the appropriate coactivator or corepressor molecule to the receptor.

Nuclear receptors are all thought to be evolutionarily related and have possibly derived from a common ancestral gene via gene duplication and/or exon shuffling (Laudet, 1997; Sluder et al., 1999). The regions C (DBD) and E (LBD) represent the most conserved elements (Evans, 1988; Green and Chambon, 1988).

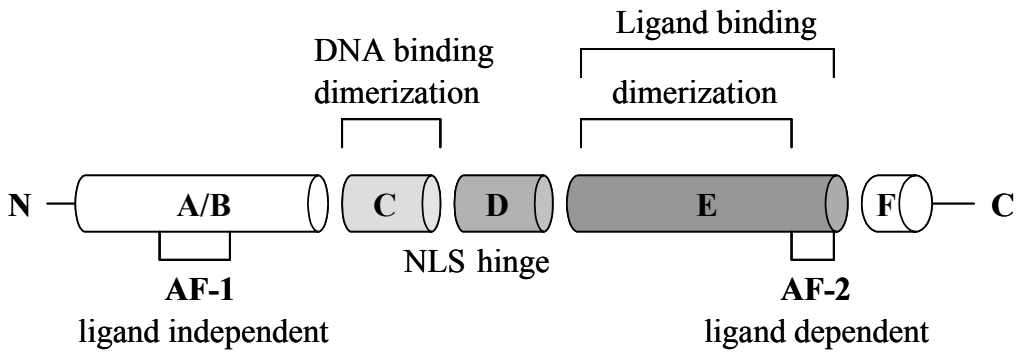

Figure 1. Nuclear receptor domains
Structure of a classical nuclear receptor. Typical domains and functions are indicated. AF =activation domain, NLS = nuclear localization domain. (Adapted from Mangelsdorf et al., 1995).

Classification of the nuclear receptor family

The family of nuclear receptors represents the biggest group of transcription factors known. Although structurally related, they carry very different functions in the regulation of homeostasis, metabolism, cell cycle and development, and bind structurally diverse ligands. Moreover, for several of the orphan receptors no physiological ligand has been identified up to now (Blumberg and Evans, 1998; Giguere, 1999). Although the majority of nuclear receptors consist of the classical features described in the previous section, some members contain only a subset of them. For example, SHP (small heteromeric partner) and DAX-1, two closely related members, are atypical receptors that have a LBD but lack a DBD. They seem to heterodimerize with other nuclear receptors, via the LBD, and serve as repressors (Howell et al., 1998; Seol et al., 1996; Wan et al., 2000). In addition to the classification on the basis of sequence similarity, nuclear receptors have been classified into three groups, based on the nature of their ligands (see figure 2; reviewed in (Chawla et al., 2001).

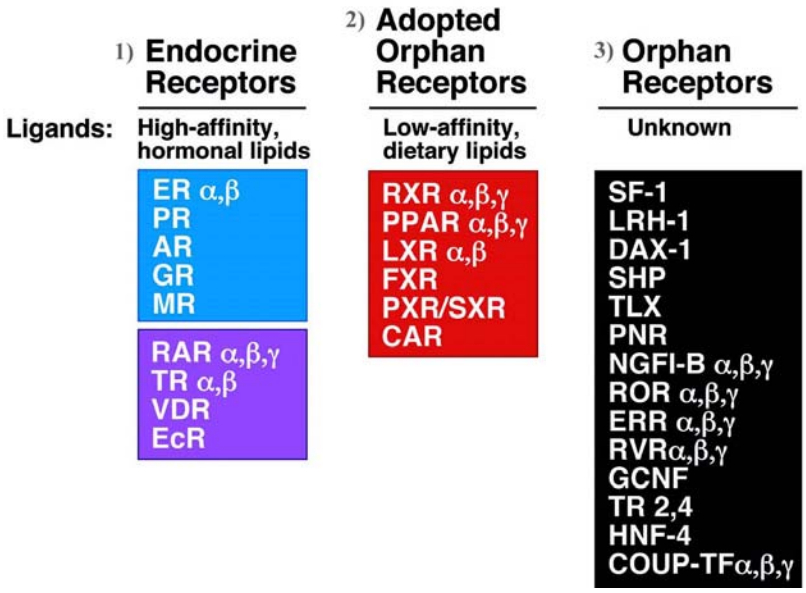

Figure 2. Nuclear receptors can be classified in three groups according to their ligands. (Adapted from Chawla et al., 2001).

1) Classical endocine receptors with high affinity hormonal ligands

They include the **steroid hormone receptor** subfamily (glucocorticoid (GR), mineralocorticoid (MR), estrogen (ER), progesterone (PR) and androgen receptors (AR)) and represent the initial group of nuclear receptors cloned in the mid 1980s. They harbor the classical structural features that were described before and that have defined the family, and seem to act dependent on ligand binding (reviewed in Aranda and Pascual, 2001; Chawla et al., 2001). This family also includes receptors with high affinity for some dietary components, such as vitamin A and D, as well as the receptors for thyroid hormone (retinoic acid receptor (RAR), vitamin D receptor (VDR), thyroid hormone receptor (TR)).

2) "Metabolic sensors" or "adopted" orphan receptors with low affinity for dietary lipids or intermediary metabolites.

Nuclear receptors of this class are activated by a diverse group of nutrient components or intermediary metabolites (e.g. fatty acids, bile acids and others), act as **'metabolic sensors',** and contain the 'adopted' members of the orphan receptor family. Interestingly, their major role is in metabolism, and in particular the regulation of lipid and xenobiotic metabolism. For example, PPARα is expressed in tissues with high capacity for fatty acid oxidation, like heart, liver, kidney and brown fat, and is activated by fatty acids (Gottlicher et al., 1992). LXRs and FXR are activated by oxysterols and bile acids, respectively, and potently regulate cholesterol and bile acid metabolism (Repa and Mangelsdorf, 2000). Most receptors here bind DNA as heterodimers with RXR (Yu et al., 1991), although formation of heterodimers with RXR is not an exclusive characteristic of this class (reviewed in Giguere, 1999).

3) Orphan receptors with unknown ligands

The third class of nuclear receptors contains the still **orphan receptors**, which seem to be active in the absence of any known ligand. Besides SHP and DAX-1, referred to previously, COUP-TFs acts often as repressors of transcription. This repressor function may be exerted by competing with other receptors for binding to DNA or for heterodimerization with RXR, as well as by having active repression domains that recruit corepressor complexes (reviewed in Pereira et al., 2000; Shibata et al., 1997). Some of the receptors in this group may "bona fide" orphans. The recent elucidation of the crystal structure of the NURR-1 LBD showed the ligand-binding pocket filled with hydrophobic residues and thereby, unlikely to be available for binding small ligands (Wang et al., 2003). Furthermore, NURR-1 lacks the classical coactivator interaction domain, and is very likely regulated through signaling molecules like receptor tyrosine kinases kinases (Wang et al., 2003). Similar observations have been made for the steroidogenic factor SF-1 (Desclozeaux et al., 2002) and the Rev-ErbA subfamily members (Renaud et al., 2000). Other members of this class include LRH-1, which regulates lipid metabolism in collaboration with the described LXRs and FXR, HNF4, which plays a role in gluconeogenesis and the ERR family members, the first orphan receptors described (reviewed in Giguere, 1999; Repa and Mangelsdorf, 2000).

The glucocorticoid receptor

Glucocorticoid hormones were already used in the middle of the 20th century, before the molecular identification of the glucocorticoid receptor (GR), as anti-inflammatory agents. The isolation of the active component and the use as efficient drug against rheumatoid arthritis led to the Nobel prize for Tadeus Reichstein, Edward Kendall and Philip Hench in 1950 (reviewed in Bonnelye and Aubin, 2002; Neeck, 2002). The glucocorticoid receptor (GR), was one of the first steroid receptors to be cloned, in 1985 (Hollenberg et al., 1985; Weinberger et al., 1985).

Activation pathway of glucocorticoid receptor signaling

In the absence of hormone, GR is part of a multiprotein complex in the cytoplasm (Nathan and Lindquist, 1995; Picard et al., 1990; Sanchez et al., 1985). This large complex consists of the two essential heat shock proteins hsp90 and hsp70, and several other chaperones and immunophilins. Formation of the complex depends on the LBD of the receptor, which interacts with Hsp90 (Pratt and Toft, 2003). The multiprotein complex keeps the receptor in a transcriptionally inactive state, while allowing ligand binding and even facilitating the folding of the LBD into a high affinity binding-pocket for the ligand ligand (Picard et al., 1990). Apparently, this is not a rigid situation, and the receptor is dynamically passing in and out of the nuclei of hormone-free cells, even though accumulating in the cytoplasm. Upon binding of hormone to the receptor, the ligand-bound receptor dissociates from the chaperone complex, translocates to the nucleus, and binds to specific DNA sequences, termed glucocorticoid response elements (GREs).

Three types of binding sites have been described for GR LBD (reviewed in Almawi and Melemedjian, 2002). At these sites, GR can activate or repress transcription, depending on the sequence of the GRE, the available coactivators and other non-receptor DNA-binding transcription factors. **1)** A simple GRE consists of an imperfect palindrome with two hexamer half-sites separated by 3 base pairs. The recruitment of specific coactivators and/or the displacement of other activating transcription factors seems to then lead to the activation or repression of transcription (Meyer et al., 1997; Rogatsky et al., 2002; Stromstedt et al., 1991).

2) A composite GRE was described first for the promoter of the proliferin gene, where GR represses transcription. GR binds directly to this composite GRE, but the availability of AP-1 factors that bind in the vicinity, or possibly also coactivators, determines whether GR represses or activates transcription (Diamond et al., 1990; Rogatsky et al., 2002). **3)** On tethering response elements, GR does not bind DNA directly, but affects transcription through an interaction with other transcription factors like NfκB or AP-1. Via this mechanism, GR is proposed to exert its immunosuppressive effects and its ability to inhibit NfκB-dependent (Caldenhoven et al., 1995; Heck et al., 1997; Ray and Prefontaine, 1994; Scheinman et al., 1995).

The role of GR in stress responses

Glucocorticoids are known for their role in regulating important components of the transcriptional response to stress. In the following, some of the better studied effects of glucocorticoids in metabolism will be described. At this point, it should be noted, that most glucocorticoid responses are exerted by GR. However, the mineralocorticoid receptor also posseses a high affinity for glucocorticoids, and some of the responses are likely to be mediated by this receptor. Glucocorticoid release is controlled by the hypothalamic-pituitary axis, in a diurnal rhythm-dependent manner and in response to stress (Jacobson and Sapolsky, 1991). After the first wave of stress hormones (catecholamines, glucagons and growth hormone), glucocorticoids conduct, as second 'wave', part of the metabolic response to stress, exerting effects on glucose, lipid, protein and nucleotide metabolism. In addition, they influence the electrolyte and calcium homeostasis and the immune system. From an evolutionary perspective, stress is usually caused by predators, and the trancriptional response facilitates the 'fight or flight' behavior. The main role of glucocorticoids in metabolism is to raise blood glucose levels, by mobilizing existing energy stores, and provide energy to the brain. The metabolic actions of glucocorticoids in stress responses are complex and have been defined into the following three types responses (Sapolsky et al., 2000).

1) Permissive actions of glucocorticoids, where the presence of the hormone prior to the stressor influences strongly the response to the first wave of "stress hormones" (catecholamines, glucagons and growth hormone). An example of this permissive action is the increase of glycogenolysis, lipolysis and hepatic gluconeogenesis.

2) The **stimulating** actions of glucocorticoids enhance the response to the first wave of "stress hormones". In this action, GR induces proteolysis in various muscle types, inhibits protein synthesis, and keeps lipolysis active in fat cells, thereby providing substrates for gluconeogenesis in liver. GR also directly stimulates gluconeogenesis in liver. To assure the supply of glucose for the brain, glucocorticoids also inhibit glucose uptake in the peripheral tissues.

3) The **preparativ**e function of glucocorticoids can often be **suppressive** and induce opposite effects. They are important to prepare for the next stressor. One example of a preparative function, which is contradictary to the glycogenolysis at the beginning of the stress response, is that glucocorticoids can also induce glycogen storage in the liver.

Glucocorticoids as therapeutic drugs

The immunosuppressive function of GR, which is thought of as important for protecting the body from the actual stress response, has led to the use of synthetic glucocorticoids for the treatment of many different immune diseases, like rheumatic arthritis, asthma, collagen vascular diseases and more. GR is expressed and active in almost all tissues, affecting many different aspects of metabolism and cell growth, by regulating the expression of target genes in a cell-type and physiological state-dependent manner. Thus, it is not surprising that therapeutic treatment with glucocorticoids, particularly when long-term stress, leads to undesired effects, such as increases in blood glucose levels (a condition predisposing to diabetes) and osteoporosis. Pharmaceutical companies have spent a lot of effort to find synthetic ligands that maximize the desired effects (e.g. immunosuppression) while minimizing effects on blood glucose levels and the bone. These efforts have been partially successful and have generated ligands with preferential effects on GR-mediated responses. Interestingly, the underlying mechanism seems to be that these ligands encourage the interaction of GR with a specific subset, rather than all coactivators, suggesting that distinct coactivators may be utilised at different promoters, pathways, or cell types (Miner, 2002).

The ERR family

Estrogen-related receptor α (ERRα) and ERRβ represent the first orphan receptors identified, based on their sequence similarity with ER (Giguere et al., 1988). The third member of this family, ERRγ, exists as multiple tissue-specific, alternatively spliced isoforms (Eudy et al., 1998). ERRs are highly similar at the amino acid level, with the highest identity being between ERRβ and ERRγ (77%). All three family members bind as homodimers to the extended half-site TNAAGGTCA, which is also the binding site for the orphan receptor SF-1 (Vanacker et al., 1999a). Heterodimerization of ERRs with ERα, and of ERRα with ERRγ has also been proposed (Huppunen and Aarnisalo, 2004; Johnston et al., 1997; Yang et al., 1996a). Furthermore, several studies have demonstrated that the ERR family members can bind to classical estrogen response elements (EREs) and compete with ERα for binding to these sites (Johnston et al., 1997; Kraus et al., 2002; Vanacker et al., 1999b; Zhang and Teng, 2001). The DBDs of the three ERRs are highly identical (around 90 %) and the receptors are likely to be co-expressed in some tissues (Bonnelye et al., 1997a; Giguere et al., 1988; Heard et al., 2000; Hong et al., 1999; Pettersson et al., 1996). At present, it remains unclear whether the three ERRs have overlapping functions, or carry distinct biological roles.

ERRα

ERRα is expressed widely, but at different levels in different tissues. During mouse embryonic development, high levels of ERRα are detected at sites of ossification (Bonnelye et al., 1997a). Furthermore, ERRα transcripts are found in heart, muscle, kidney, specific areas of the brain and in the digestive tract, with increasing levels during later developmental stages. In adults, ERRα is mainly expressed in tissues with high β-fatty acid oxidation activity like heart, kidney and brown fat, but also in brain and muscle (Bonnelye et al., 1997b; Sladek et al., 1997).

The physiological role of ERRα is still debated, although several functions have been suggested. In vitro studies have proposed that ERRα modulates estrogen signaling, in more than one way: (1) by activating classical estrogen target genes, in the absence of

estrogens; (2) by competing with ERα for EREs, and thereby antagonizing ERα function, (3) via direct physical interaction with ERα and (4) by regulating the expression of the aromatase gene, and hence production of estrogens (Johnston et al., 1997; Kraus et al., 2002; Vanacker et al., 1999b; Yang et al., 1996a; Zhang and Teng, 2001).

The high levels of expression of ERRα at ossification sites in developing mouse embryos have led to a proposed function in the regulation of bone formation. In support of such a role, ERRα induces the expression of osteopontin by binding directly to the promoter of this gene (Vanacker et al., 1998), and has been shown to induce bone nodule formation *in vitro* (Bonnelye et al., 2001; Bonnelye et al., 1997a).

The expression pattern of ERRα in adult mice (highest in heart, kidney and brown fat) have led to a proposed role in b-fatty acid oxidation. Importantly, ERRα binds the promoter of the gene encoding the medium-chain acyl coenzyme A dehydrogenase (MCAD), a key enzyme in β-fatty acid oxidation, and regulates its expression (Sladek et al., 1997; Vega and Kelly, 1997).

The recent generation of ERRα knockout mice by V. Giguere (Luo et al., 2003), supports a role for ERRα in lipid metabolism. The disruption of the ERRα gene leads to viable mice that have reduced adipose tissue. Although no differences in the energy expenditure, the fasting glucose levels or the serum free fatty acid or triglyceride levels have been observed, these mice are resistant to high-fat diet-induced obesity. Gene expression profiling of isolated adipocytes have disclosed an altered regulation of enzymes involved in fat metabolism. Strikingly, MCAD is upregulated, suggesting a repressor function for ERRα at this gene (Luo et al., 2003). The reasons for the decreased adipocity and resistance to obesity are, however, currently unclear.

ERRβ

ERRβ expression is highly specific in extra-embryonic tissues during the early embryonic development. ERRβ mRNA levels could be detected in a subset of cells in the extra-embryonic ectoderm at day 5.5 post-coitum and more prominently after day 6.5 p.c. in ectodermally derived cells that later on form the chorion. In adults, ERRβ expression could be detected only in low levels in the liver, stomach, skeletal muscle, heart and kidney (Chen et al., 1999b; Giguere et al., 1988). Disruption of ERRβ in mice demonstrated clearly that ERRβ is essential for normal placental formation. ERRβ-/- mice show abnormal chorion formation, placental failure and impaired trophoblast stem cell differentiation (Luo et al., 1997).

ERRγ

ERRγ transcripts are detected at high levels in both the embryo and adults. During development, major sites of expression are the fetal brain, with lower levels in the kidney, lung and liver. In human adults, ERRγ mRNA is expressed at high levels in the lung, bone marrow, brain and adrenal gland, lower in the thyroid gland, spinal cord and trachea (Eudy et al., 1998). The expression pattern in adult mice looks different, with high levels in specific areas of the brain brain (Hermans-Borgmeyer et al., 2000; Lorke et al., 2000), kidney, testis, spleen and lower levels in lung ((Eudy et al., 1998)). In other studies, high levels of ERRγ expression are detected in the adult mouse heart, and modest expression is seen in muscle (Hong et al., 1999; Susens et al., 2000). The function of the third member of the ERR family of orphan receptors remains unclear, even though some target genes, such as the SHP orphan receptor gene, have been proposed (Sanyal et al., 2002).

Ligands for the orphan receptor family of ERRs

During the course of identifying new ligands for the family of orphan nuclear receptors, a new concept emerged: the "reverse endocrinology". Instead of identifying a receptor for a physiologically characterized hormone, as was the case with steroid hormones and receptors, orphan receptors were used to search for new hormones (Blumberg and Evans, 1998; Giguere, 1999). In the case of ERRα, initial studies

reported that it could be activated by a component present in fetal calf serum (Vanacker et al., 1999a), while subsequent studies suggest that the three ERRs are constitutively active, in the absence of any ligand (Chen et al., 2001; Xie et al., 1999). Searches for ligands for the ERRs have been successful in identifying only synthetic antagonists. Because of the similarity of ERRs with ERs, ligands with estrogen-like activity have been considered. The results are contradictory and differ somewhat from group to group, but suggest that toxaphene, chlordane, diethylstilbestrol (DES) and 4-hydroxytamoxifen (OHT) can act as antagonists of ERRβ and ERRγ, but not of ERRα (Coward et al., 2001; Tremblay et al., 2001; Yang and Chen, 1999). Finally, elucidation of the crystal structure of the ERRγ ligand-binding domain bound to a peptide derived from the SRC-1 coactivator, shows that the ERRγ LBD can adopt an active conformation already in the absence of any ligand (Greschik et al., 2002). The question is still open, if an agonist ligand can exists for the ERRs. A recent publication has proposed that flavones and isoflavone phytoestrogens may enhance the activity of ERRs (Suetsugi et al., 2003).

Coregulators of transcription

Nuclear receptors are not able to interact directly with RNA polymerase II. Their function therefore depends on coregulators, which serve as intermediate factors between the nuclear receptors and the basal transcriptional machinery, and which determine the activator or repressor function of the receptor. Coregulators can be divided into two main classes: coactivators, which activate transcription, and corepressors, which lead to transcriptional repression. In the context of this thesis, I will focus on the class of coactivators.

Coactivators. Coactivators bind to nuclear receptors mostly dependent, but also independent of ligand availability, and are able to switch the nuclear receptors from an inactive to an active state. The interaction surface of all coactivators is very similar. They bind to nuclear receptors via multiple nuclear-receptor interaction domains (also called NR boxes) that contain the sequence LXXLL (L=leucine, x=any amino acid) located in an amphiphatic a-helix. These motifs have been shown to be necessary and sufficient to mediate binding with nuclear receptors (Heery et al., 1997; Hu and Lazar, 1999; Nagy et al., 1999; Perissi et al., 1999; Radhakrishnan et al., 1997; Yamamoto et al., 1998). Whereas most coactivators interact with the AF-2 domain of the nuclear receptors, some coactivators, such as SRCs and DRIP150 can also interact with the AF-1 domain or synergistically with both the AF-1 and the AF-2 (Benecke et al., 2000; Hittelman et al., 1999). The structural basis for these interactions is however not well understood yet.

Corepressors. The two main corepressors are N-CoR and SMRT, which bind to nuclear receptors in the absence of ligand or the presence of antagonist ligand (Chen and Evans, 1995; Horlein et al., 1995; Lavinsky et al., 1998; Zhang et al., 1998). Corepressors harbor conserved NR interacting domains, referred to as CoRNR box, with the motif LXXI/HIXXXI/L. This motif seems to represent a prolonged form of the coactivator motif LXXLL, with an extended α-helix (Hu and Lazar, 1999; Nagy et al., 1999; Perissi et al., 1999; Xu et al., 2002). Disruption of the N-CoR gene blocks the development of the CNS, erythrocytes and thymocytes, suggesting that N-CoR

repressor activity is essential for organ development (Hermanson et al., 2002). Although the available data are contradictory, N-CoR and SMRT seem to execute their repression function as complexes with histone deacetylases Sin3, HDAC1, HDAC2 and other components (Guenther et al., 2000; Li et al., 2000; Underhill et al., 2000; Wen et al., 2000). The isolation of additional complexes with varying components suggest that N-CoR and SMRT act in a tissue- and promoter-specific manner. Furthermore, N-CoR and SMRT conduct short- and long-term repression functions not only for nuclear receptors but also many other transcription factors, like Mad, and play a role as general repressors of transcription.

Structural data have revealed that most of the coactivators and corepressors bind to an overlapping binding surface on the nuclear receptors (Hu and Lazar, 1999; Nagy et al., 1999; Xu et al., 2002). This region, also known as AF-2 domain, is located in the well-conserved LBD (see figure 3). Helix 12, which is located in the AF-2, seems to be the major determinant for the binding of coactivators and to act as a switch. In the absence of ligand, the pocket is ‘open’ and corepressors are able to bind (figure 3). When an agonist ligand binds, helix 12 moves over the ligand binding pocket; this conformational change creates a new pocket that enables coactivator binding (Bourguet et al., 1995; Nolte et al., 1998). The actual mechanism may be more complex, since several intermediates seem to be possible. Moreover, some corepressors, like RIP140, are able to interact with agonist-bound receptor and repress transcription (Lee et al., 1998).

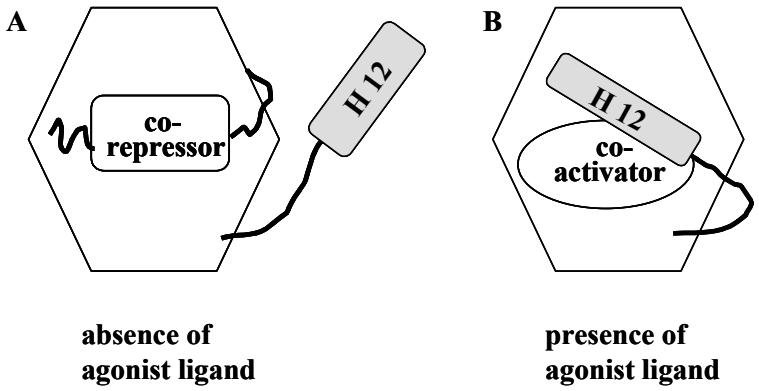

Figure 3. Model for corepressor and coactivator binding to the nuclear receptor LBD in the absence and presence of agonist ligand. (Adapted from Nagy et al., 1999).

Types of coactivators

More than 50 nuclear receptor coactivators have been described. With the exception of the nuclear receptor interacting motif LXXLL that they have in common, the different coactivators are structurally quite diverse proteins. The large number of coactivators and coactivator complexes suggests that they carry diverse roles, such as integrating distinct signals, conferring tissue and promoter-specific regulation, and/or acting via distinct mechanisms. In the following section, the different types of coactivators, grouped according to their mechanism of action, will be described:
1) ATP-dependent chromatin remodeling complexes, **2)** histone modifying complexes, **3)** bridging factors, **4)** other coactivators of transcription. (reviewed in Glass and Rosenfeld, 2000; McKenna et al., 1999; Naar et al., 2001).

1) ATP-dependent chromatin remodeling complexes

DNA is condensed in chromatin, where the small units, the nucleosomes, consist of DNA coiled around an octamer of histone proteins. In the last two decades, it has been shown that nucleosomes can repress transcription and be directly linked to transcriptional activation (Akey and Luger, 2003; Khorasanizadeh, 2004). A layer at

which regulation of transcription can be exerted has been appreciated by the identification of a number of coactivators that seem to affect chromatin structure, making the DNA accessible to the transcription machinery. Two related remodeling complexes have been described in yeast: The SWI/SNF complex, also conserved in mammalian cells, and the RSC (remodeling the structure of chromatin) complex (Cairns et al., 1994; Peterson et al., 1994; Wang et al., 1996). One of the most conserved elements of this complex is the SWI2/SNF2 protein (termed brg-1, or brahma-related gene-1 in humans), which contains the ATPase activity that remodels the nucleosomes (Khavari et al., 1993; Laurent et al., 1993). The SWI2/SNF2-family of DEAD/H ATPases and DNA helicases contains furthermore many members that play roles in DNA repair or recombination, chromosome segregation and cell cycle progression (Pollard and Peterson, 1998).

2) Histone modifying coactivators

A different group of chromatin modifying coactivators includes proteins that enzymatically modify histones. Since the early discovery of histone acetylation (Allfrey et al., 1964), the hypothesis has been put forward that the rate of transcriptional activity is directly linked to the grade of histone acetylation (Pazin and Kadonaga, 1997). Yeast GCN5, the first coactivator identified to exhibit histone acetylase (HAT) activity (Brownell et al., 1996), leads to hyperacetylation of lysine residues in the short amino-terminal domain of free histones and inhibits the higher order folding of nucleosomes. As a consequence, specific promoter areas of genes are accessible for the transcription machinery. These findings have received further support by the identification of the mammalian ortholog p/CAF (Yang et al., 1996b) and other coactivators with HAT activity like CBP, the adenovirus E1A binding protein p300 (Bannister and Kouzarides, 1996; Ogryzko et al., 1996) and $TAF_{II}250$ (Mizzen et al., 1996). The yeast p/CAF and GCN5 have been shown to exist as big multisubunit coactivator complexes named ADA or SAGA complexes (Grant et al., 1997; Grant et al., 1998). They contain several different components like ADA proteins but also TBPs and TAFs, and they connect nuclear receptors to the basal transcription machinery. Whereas the mammalian counterpart also contains ADA and

TAF proteins (Ogryzko et al., 1998), the interaction to the core machinery has not yet been shown.
The ubiquitously expressed CBP and its functional homolog p300 serve as ligand-dependent coactivators for several nuclear receptors (Almlof et al., 1998; Smith et al., 1996) and other transcription factors like CREB (Nakajima et al., 1996). Studies have shown that p300/CBP interact with other nuclear receptor coactivators like SRC-1 (Kamei et al., 1996; Yao et al., 1996) or coactivator complexes like the P/CAF (p300/CBP associated factor) mutliprotein complex (Yang et al., 1996b). Interestingly, p300/CBP and P/CAF do not only acetylate histones, but also the general transcription factors and others (Imhof et al., 1997).

The p160 family of coactivators contains proteins with the molecular mass of about 160 kDa that interact with nuclear receptors in a ligand-dependent manner (Cavailles et al., 1994; Halachmi et al., 1994; Kurokawa et al., 1995). Biochemical and yeast two-hybrid assays have led to the identification of three members: SRC-1 (steroid receptor coactivator 1), SRC-2 and SRC-3 (Anzick et al., 1997; Chen et al., 1997; Hong et al., 1997; Kamei et al., 1996; Onate et al., 1995; Takeshita et al., 1997; Torchia et al., 1997). In addition to the highly conserved basic helix-loop-helix (bHLH) PAS domain, that can be found in all family members, SRC-1 and SRC-3 possess a weak intrinsic HAT activity. The importance of this HAT activity is not clear, given that SRC-1 also interacts with and recruits other coactivators that posses enzymatic activities, like the histone acetylase CBP and the methyltransferase CARM1 (Chen et al., 1999a; Kamei et al., 1996; Yao et al., 1996). The p160 family members interact not only with the AF-2, but also the AF-1 domain of nuclear receptors, as shown for SRC-1 and SRC-2, suggesting that they built a bridge between both activation domains (Alen et al., 1999; Bevan et al., 1999; Ma et al., 1999; Webb et al., 1998).

3) Bridging coactivators- the mediator complex

One of the best characterized coactivator complexes is the mediator complex, identified initially in yeast (Kim et al., 1994). In mammalian cell systems, several similar mediator-type complexes have been isolated, also known as TRAP, DRIP, ARC complex, two smaller complexes CRSP and PC2 (Malik et al., 2000; Ryu et al., 1999), and two complexes that may mediate a form of repression, SMCC and NAT (Boyer et al., 1999; Fondell et al., 1996; Gu et al., 1999; Naar et al., 1999; Rachez et al., 1998). It seems possible, that all of these complexes represent only subcomplexes of the same one mediator complex (reviewed in (Glass and Rosenfeld, 2000; Naar et al., 2001)).

The members of the mediator complex do not exert a HAT or any other enzymatic activity, but enhance the transcriptional activity of several nuclear receptors like TR (Fondell et al., 1999) and VDR (Rachez et al., 1998), as well as of other factors such as SREBP-1a and Sp1 (Naar et al., 1999). The ability to interact with the RNA polymerase II implements a role as bridging complex to the basal transcription machinery (Rachez et al., 1999). Disruption of the common component TRAP220 leads to embryonic lethality in mice, which suggested that the ligand-dependent interaction of the mediator complex with nuclear receptors is dependent on TRAP220 (Treuter et al., 1999; Yuan et al., 1998).

The reason for the identification of so many different complexes (i.e. ATP remodeling complexes, HAT complexes, mediator, and others) is not yet fully understood, but Glass and Rosenfeld (Glass and Rosenfeld, 2000) have proposed that the different coactivator complexes may act sequentially, combinatorially or in parallel at different promoters (see figure 4).

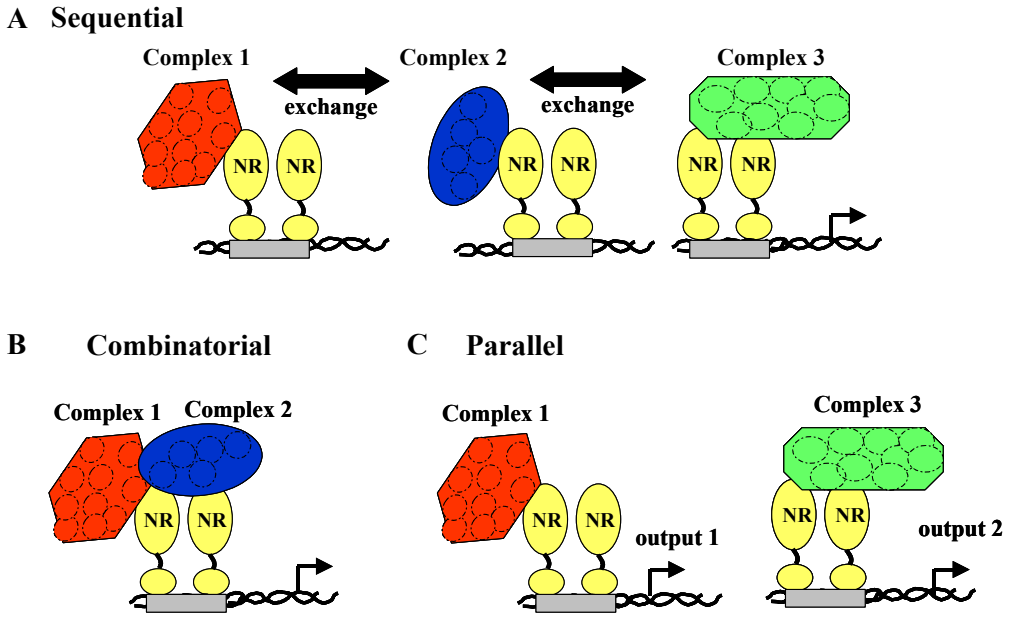

Figure 4. Utilization of multiple coactivator complexes.

(A) Sequential recruitment to the same promotor sequence. In this model, one coactivator complex could be necessary to prepare for the next**. (B)** Combinatorial recruitment of several coactivator complexes could be required for physiologic levels of expression on specific promotors. **(C)** Parallel utilization of coactivator complexes describes the recruitment of different coactivator complexes in response to distinct signaling pathways or in different tissues. (Adapted from Glass and Rosenfeld, 2000).

4) Other coactivators of transcription

Some coactivators cannot be classified in any of the described groups yet. The essential yeast gene Rsp5 and its human homolog NEDD4 have been demonstrated to enhance GR and PR transcription in yeast and mammalian cells *in vitro* (Imhof and McDonnell, 1996). Subsequent studies have revealed that NEDD4 ubiquitinates the largest subunit of RNA polymerase II *in vitro* and possibly mediates its UV-induced proteolytic degradation by the proteosome (Beaudenon et al., 1999). Another coactivator with ubiquitination capacity is the E6-associated protein E6-AP (Huibregtse et al., 1991), which seems to interact with NEDD4 to regulate transcriptional activation of steroid receptors by ubiquitin-ligase complexes (McKenna et al., 1998).

SRA (steroid receptor RNA activator) represents a nuclear receptor coactivator different from all known coregulators (Lanz et al., 1999). It functions as a RNA transcript, and interacts with the AF-1 domain of nuclear receptors in the absence of ligands. Giguere and coworkers have also suggested a ligand dependent interaction with the AF-2 of nuclear receptors, like classical coactivators, and the regulation by MAPK pathways (Deblois and Giguere, 2003). Interestingly, SRA seems to be recruited by SRC-1 and to furthermore interact with another uncommon family of coactivators, the DEAD-box conatining RNA helicases p72 and p68 (Lanz et al., 1999; Watanabe et al., 2001), to acts as a bridge for the AF-1 and AF-2 domain activity of nuclear receptors.

The PGC-1 family: inducible, tissue-specific coactivators

Structural features of PGC-1α

PGC-1 or PGC-1α, as it has been renamed after the identification of its homologues, was the founder of a new group of coactivators. It does not posses any enzymatic activity, but shows several interesting features (see figure 5). At the N-terminus of PGC-1α lies a potent acidic transactivation domain. PGC-1α contains three leucine-rich LXXLL motifs, L1, L2 and L3 that reside in predicted α-helices. L1 is located in the acidic N-terminal transcriptional activation domain and is conserved between the PGC-1α family members (Kressler et al., 2002). Interestingly, Mutation of the L1 motif strongly influenced the transactivation function, which suggested that it is part of a region that is important for the interaction with other factors (Kressler et al., 2002; Puigserver et al., 1999). Leucine motifs L2 and L3 reside in two nuclear receptor interaction domains (NIDs). Motif L2 in particular serves as the major interaction surface for GR ER, PPARs, RXR, LXR and probably others (Delerive et al., 2002; Knutti et al., 2001; Oberkofler et al., 2003; Tcherepanova et al., 2000; Vega et al., 2000). Furthermore, mutations in motifs L2 and L3 have been shown to increase PGC-1α transcriptional activity possibly by disruption of the interaction with a repressor (Knutti, 2001, see later section). Besides the described L2 and L3 motifs, PGC-1α harbours other interaction domains, proposed to bind PPARγ, NRF-1 and the

muscle-specific transcription factor MEF2C, which are not yet well defined ((Michael et al., 2001; Puigserver et al., 1998; Wu et al., 1999), see figure 5).

At the C terminus, PGC-1α harbours two motifs that are atypical for coactivators: a serine/arginine-rich (RS) domain and an RNA recognition motif ((RRM); (Knutti et al., 2000)). Strikingly, both RRM and RS domains are characteristic features of SR splicing factors, components and/or regulators of the spliceosome (reviewed in Graveley, 2000; Hastings and Krainer, 2001; Reed and Magni, 2001). RRM motifs determine substrate specificity and can interact with RNA as well as other proteins, while the RS domains are shown to be important for protein-protein interactions. Although the mechanism is not yet understood, PGC-1α has been suggested to be involved in RNA processing (Monsalve et al., 2000). Three different observations, made by Monsalve and coworkers, support this PGC-1α function: First, PGC-1α, through its C-terminal domain, associates with several splicing factors, SRp75, SRp55 and SRp40 and the elongating form of RNA polymerase II. Second, immunofluorescence studies suggest that PGC-1α colocalizes with splicing factors in nuclear specles. Third, PGC-1α seems to modulate the processing of a fibronectin minigene. The current model for the mechanism by which PGC-1α regulates gene expression proposes therefore that PGC-1α couples transcription and pre-mRNA splicing: after the recruitment of PGC-1α on a specific target gene by a nuclear receptor, PGC-1α seems to bind to SRC-1, CBP, and possibly the mediator complex (Puigserver et al., 1999; Surapureddi et al., 2002; Wallberg et al., 2003). Next, it is suggested that CBP induces chromatin remodelling, whereas the mediator complex bridges to the basic transcription machinery. The N-terminus of PGC-1α possibly also binds to polymerase II (CTD; (Monsalve et al., 2000). Finally, PGC-1α is proposed to interact with the elongation form of polymerase II, and to regulate mRNA splicing (Monsalve et al., 2000). However, it has to be emphasized here, that although the interactions between these molecules has been demonstrated, additional experiments are still needed to confirm this model.

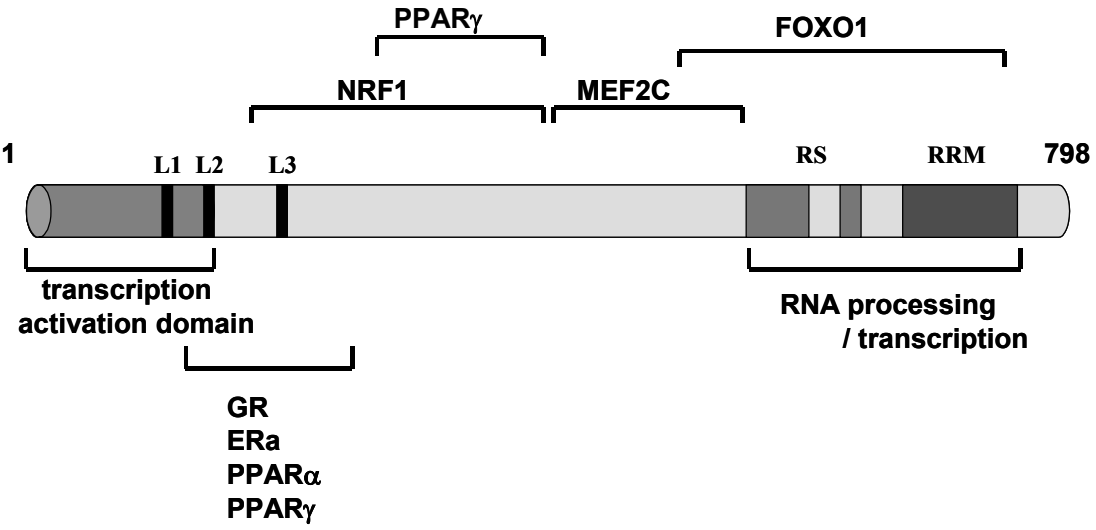

Figure 5. Structure of PGC-1α

Parenthesis denote the interaction domain with different transcripton factors, the N-terminal transactivation domain and the RNA processing domain with the RS= serin-arginin rich streches, RRM= RNA recognition motif. L1, L2 and L3 represent the three leucine LXXLL motifs.

Physiological role of PGC-1α

In contrast to many coactivators that are ubiquitously expressed, PGC-1α is expressed in a tissue-specific manner and induced by specific metabolic signals. PGC-1α expression is highest in tissues with a high density of active mitochondria, like heart, skeletal muscle, brown adipose tissue (BAT), kidney, liver and brain (Esterbauer et al., 1999; Knutti et al., 2000; Puigserver et al., 1998). In addition, PGC-1α is induced in physiologic states that display specific energy demands, such as exposure to cold, fasting and physical exercise. (Goto et al., 2000; Herzig et al., 2001; Lehman et al., 2000; Puigserver et al., 1998)

PGC-1α role in adaptive thermogenesis

Adaptive thermogenesis is a process tightly associated to the function of mitochondria and energy expenditure. This programme is switched on in response to exposure to cold and overfeeding, and leads to the production of heat instead of energy through

the uncoupling of the respiratory chain (reviewed in Puigserver and Spiegelman, 2003). Interestingly, PGC-1α is strongly induced in the brown fat and muscle (i.e. thermogenic tissues) of mice upon exposure of the animals to cold. Overexpression studies reveal that PGC-1α is capable of upregulating molecular components of the adaptive thermogenesis, e.g. the UCPs (uncoupling proteins), a process likely to depend on the interaction of PGC-1α with PPARα , PPARγ, RAR and probably TR (Puigserver et al., 1998).

PGC-1α regulates mitochondrial biogenesis in response to specific signals

Mitochondria provide cellular energy in the form of ATP. The mitochondrial content and respiration efficiency vary greatly from cell type to cell type and reflect the energy demand defined by the physiological status of the cell (reviewed in Moyes and Hood, 2003). The modulation of mitochondrial functions is a complex process, which requires the coordinate expression of mitochondrial and nuclear encoded proteins. Studies have shown that PGC-1α levels are increased after exercise in muscle, a situation with high energy requirements (Goto et al., 2000). Furthermore, PGC-1α upregulation is detected in the heart of mice directly after birth, shortly before a strong increase of mitochondrial biogenesis and oxidative metabolism (Lehman et al., 2000). Ectopic expression of PGC-1α in adipocytes, myocytes and cardiomyocytes induces the biosynthesis of mitochondria and increases cellular respiration (Goto et al., 2000; Lehman et al., 2000; Puigserver et al., 1998; Wu et al., 1999). PGC-1α seems to regulate mitochondrial biogenesis in adipocytes and myocytes through the induction and coactivation of NRF-1, and possibly NRF-2, which enhance the expression of key factors in mitochondrial transcription and replication, such as mtTFA (Wu et al., 1999).

PGC-1α function in glucose metabolism

An additional situation, where PGC-1α upregulation has been observed, is in the liver and heart of fasted animals (Lehman et al., 2000; Yoon et al., 2001). In the fasting state, gluconeogenesis is increased in the liver so as to ensure glucose availability to tissues like the brain. Overexpression of PGC-1α induces the expression of PEPCK and glucose-6-phosphatase, two key enzymes of gluconeogenesis, through the coactivation of HNF4, GR and FOXO1 (Herzig et al., 2001; Puigserver and

Spiegelman, 2003; Yoon et al., 2001). Additional studies reveal that PGC-1α may also control the glucose uptake in peripheral tissues, through the induction of the insulin-sensitive glucose transporter GLUT4 in muscle (Michael et al., 2001). This induction may be mediated through the interaction with MEF2C, a muscle and heart specific transcription factor. The effect of PGC-1a on GLUT4 is however not yet clear, as GLUT4 is reported to be down-regulated in the muscle of transgenic mice overexpression PGC-1α (Miura et al., 2003).

PGC-1α is involved in diabetes

Consistent with PGC-1α increasing glucose production, PGC-1α levels have been reported to be raised in the livers of modeldiabetic mice (Yoon et al., 2001). Genetic studies indicate further that mutations in the PGC-1α gene locus may increase the susceptibility of patients to diabetes type II (Ek et al., 2001; Hara et al., 2002). The contribution of PGC-1α to diabetes seem however to be complex. While increased PGC-1α levels and activity in liver may contribute to increased glucose, decreased PGC-1α levels and activity in muscle may also contribute to the diabetic phenotype. Mootha and coworkers (Mootha et al., 2003) adopted recently a very elegant approach with the help of expression profiling and new in silico-techniques, to identify the oxidative phosphorylation genes (OXPHOS) as a coordinately downregulated gene set in muscle biopsies of diabetes patients. Interestingly, PGC-1α expression seemed to be also repressed in these patients, implying a role for PGC-1α in the downregulation of mitochondrial biogenesis and the decreased energy expenditure during diabetes. (Mootha et al., 2003; Patti et al., 2003).

Regulation of PGC-1α activity

PGC-1α is upregulated in a temporally and spatially defined manner. Regulation has to be tight, since both increased and decreased levels could be contributing to diseases like diabetes. This leads to the question, which signalling pathways are involved in the regulation of PGC-1α.

Regulation during adaptive thermogenesis

Initial studies showed PGC-1α to be regulated by the β-adrenergic receptor in response to cold (Boss et al., 1999; Puigserver et al., 1998). PGC-1α levels are also regulated by the adipocyte-derived hormone leptin, which regulates food uptake and energy expenditure and counts as an important factor controlling adaptive thermogenesis (Ahima and Flier, 2000). Interestingly, PGC-1α levels are decreased in mice that are leptin-deficient or not responsive to leptin, and upregulated in hyperleptinemic rats (Kakuma et al., 2000).

Mechanisms regulating PGC-1α in mitochondrial biogensis

Studies in transgenic mice have revealed a complex interplay between MEF2 and the histone-deacetylase HDAC5 that lead to the regulation of PGC-1α expression and mitochondrial biogenesis in myocytes, possibly in response to CaMK IV (Czubryt et al., 2003). In response to exercise, Ca2+ levels rise in muscle cells, inducing calcineurin and CaMK IV. Interestingly, PGC-1α levels are induced in transgenic mouse lines expressing constitutively active CaMK IV (Wu et al., 2002). Further studies have led to the proposal of an autoregulatory loop, in which CREB phosphorylated by CaMK IV and MEF2 activated by calcineurin A bind to the PGC-1α promoter, and in co-operation with PGC-1α itself, lead to an increase in PGC-1α expression (Handschin et al., 2003).

The regulation of PGC-1α in glucose metabolism

The main regulators of the fasting state are glucagon, which is acting through the cAMP pathway, and glucocorticoids. After cAMP levels rise, protein kinase A (PKA) exerts increased activity and activates the cAMP-response element binding protein (CREB). The treatment of hepatic cells with cAMP lead to an upregulation of PGC-1α, which is further potentiated by glucocorticoids (Yoon et al., 2001). The PGC-1α promoter harbors binding sites for CREB and seems to be regulated by this factor (Herzig et al., 2001).

Posttranslational mechanisms regulating PGC-1α

Studies from our lab and from others have demonstrated that PGC-1α is also regulated by posttranslational mechanisms (Knutti et al., 2001; Puigserver et al.,

2001). Interestingly, the stress-responsive kinase p38 seems to phosphorylate PGC-1α on three residues close to the nuclear receptor interaction domains harboring the L2 and L3 motif. Several findings suggest that this phosphorylation induces the release of a molecular repressor of PGC-1α (Knutti et al., 2001): **1)** mutation of the L2 and the L3 motifs lead to icreased PGC-1α activity; **2)** coexpression of a shorter version of PGC-1α that has motifs L2 and L3 competes for repressor binding and increases PGC-1α activity; **3)** a constitutively active upstream kinase of p38 increases the activity of wild-type, but not of an L2/3A mutant PGC-1α (Knutti et al., 2001). Recent studies by the Spiegelman group have identified the p38-sensitive, L2/3 interacting repressor as the coregulator p160 myb (Fan et al., 2004). Moroever, studies by Ichida et al have proposed the orphan receptor ERRα, which also interacts with L2 and L3, to act as a repressor of PGC-1α (Ichida et al., 2002).

PGC-1 family members

PGC-1-related coactivator (**PRC**), the first homologue of PGC-1α, was identified due to its homology in the N-terminus (Andersson and Scarpulla, 2001). Even though the overall sequence similarity with PGC-1α is quite low, the domain pattern is highly similar. Both coactivators contain the N-terminal acidic transactivation domain, the nuclear receptor interaction (LXXLL) motif, the proline rich region, the RS domain and the RNA binding domain. Elucidation of the expression levels showed that PRC is ubiquitously expressed with higher levels in skeletal muscle and heart (Andersson and Scarpulla, 2001). One common function of PGC-1α and PRC is the interaction with NRF1 and the induction of mitochondrial biogenesis. A distinctive characteristic is that PRC expression is not regulated by exposure to cold, but rather during the cell cycle, suggesting a role for PRC distinct from that of PGC-1α.

Recently, a second homologue named **PGC-1β** (mouse) or PERC (human) was cloned by us and others (Kressler et al., 2002; Lin et al., 2002). Sequences of PGC-1α, β and PRC display around 45 to 46 % (over 450 aa) similarity in the C-terminus; whereas all three proteins contain a RNA recognition motif, PGC-1β does not contain an RS domain. The N-terminus of all three proteins shows a conserved L1 motif and a

conserved nuclear receptor interaction domain (L2). In addition, PGC-1β harbors an additional, unique to PGC-1β NID. RNA expression analysis shows PGC-1β to be similarly expressed as PGC-1α, with high levels in heart, skeletal muscle, BAT and medium levels in liver, brain, WAT, adrenal gland and kidney (Kressler et al., 2002; Lin et al., 2002). Strikingly, PGC-1β does not show a specific upregulation in response to cold or fasting.

We have observed that PGC-1β, in contrary to PGC-1α, shows much higher specificity in the interaction with nuclear receptors (Kressler et al., 2002). PGC-1β, or PERC (PGC-1 related estrogen receptor coactivator) shows a high preference in enhancing the activity of ERα, over that of many other nuclear receptors. Moroever, PGC-1β converted tamoxifen from an antagonist to an agonist of ERα in osteoblast cells in a cell-type and promoter-specific manner. Based on these findings, we have proposed a role for PGC-1β in the regulation of estrogen signaling. In disagreement with these findings, other labs report that PGC-1β can coactivate PPARγ, TRβ, GR and HNF4 (Lin et al., 2002; Meirhaeghe et al., 2003), and more strikingly, NRF-1 (Lin et al., 2002). Finally, we and others have seen that PGC-1β is an effective coactivator of the orphan receptor ERRα ((Kamei et al., 2003), Kressler unpublished). Interestingly, ectopically expressed PGC-1β like PGC-1α can also induce mitochondrial biogenesis in myoblasts (Meirhaeghe et al., 2003) and hepatocytes (Lin et al., 2002). PGC-1α- and PGC-1β-induced mitochondria do however show some functional differences, suggesting that the two coactivators have overlapping but not identical functions (St-Pierre et al., 2003). Studies in muscle of prediabetic and diabetic patients also showed increased levels of PGC-1β, besides PGC-1α and the OXPHOS genes (Patti et al., 2003).

Consistent with a role of PGC-1β in controlling OXPHOS genes, transgenic mice overexpressing PGC-1β have a higher oxygen consumption and are resistant to highfat diet-induced, as well as genetically determined obesity.

Aim of my thesis

Regulation of transcription is a highly complex process that requires the orchestrated collaboration of numerous factors operating at several different levels. Extracellular factors like steroid hormones activate nuclear hormone receptors, which are released by the chaperone complex, translocate to the nucleus, bind to regulatory DNA sequences, interact with chromatin and the basic transcription machinery via coactivators, and regulate transcription (reviewed in Aranda and Pascual, 2001). Any of the steps in this pathway are potential targets for regulation, by different phosphorylation cascades or other mechanisms (reviewed in Weigel, 1996). Nuclear receptors like GR are ubiquitously expressed and posses the ability to interact with several different coactivators, which themselves are also able to coactivate numerous different transcription factors (reviewed in Glass and Rosenfeld, 2000; McKenna et al., 1999; Naar et al., 2001). Increasing evidence over the last few years suggests that coactivators may provide specificity to the physiological responses mediated by nuclear receptors (reviewed in Knutti and Kralli, 2001; Puigserver and Spiegelman, 2003).

The basic hypothesis of my PhD thesis is that some of the target gene specificity in GR signaling, but also in the signaling of other receptors, is determined by the interaction with the specific coactivator PGC-1α. To test this hypothesis, we established a cell culture model system, where we could activate GR in the absence and presence of PGC-1α, and ask if PGC-1α enhances the expression of all GR target genes, or if the presence of PGC-1α would reroute GR to the regulation of specific cellular programs. Briefly, PGC-1α was introduced into osteoblast progenitor SAOS2 cells that express no detectable levels of endogenous PGC-1α but have functional GR, and responses to glucocorticoids ± PGC-1α were analyzed by gene expression profiling on the Affymetrix U133A chips. The results could give information not only on how PGC-1α affects GR signaling, but also on potentially new programs regulated by PGC-1α in collaboration with other nuclear receptors expressed in these cells.

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Chapter II:

The transcriptional coactivator PGC-1 regulates the expression and activity of the orphan nuclear receptor ERRα

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The Journal of Biological Chemistry (2003), 278 (11). 9013-9018

Summary

The estrogen-related receptor alpha (ERRα) is one of the first orphan nuclear receptors identified. Still, we know little about the mechanisms that regulate its expression and its activity. In this study, we show that the transcriptional coactivator PGC-1, which is implicated in the control of energy metabolism, regulates ERRα at two levels. Firstly, PGC-1 induces the expression of ERRα. Consistent with this induction, levels of ERRα mRNA in vivo are highest in PGC-1 expressing tissues, such as heart, kidney, and muscle, and up-regulated in response to signals that induce PGC-1, such as exposure to cold. Secondly, PGC-1 interacts physically with ERRα and enables it to activate transcription. Strikingly, we find that PGC-1 converts ERRα from a factor with little or no transcriptional activity to a potent regulator of gene expression, suggesting that ERRα is not a constitutively active nuclear receptor but rather one that is regulated by protein ligands, such as PGC-1. Our findings suggest that the two proteins act in a common pathway to regulate processes relating to energy metabolism. In support of this hypothesis, adenovirus-mediated delivery of small interfering RNA for ERRα, or of PGC-1 mutants that interact selectively with different types of nuclear receptors, shows that PGC-1 can induce the fatty acid oxidation enzyme MCAD in an ERRα-dependent manner.

Introduction

The nuclear receptor ERRα was identified in 1988 as a protein that shares significant sequence similarity to known steroid receptors, such as the estrogen receptor (Giguere et al., 1988). ERRα and its relatives ERRβ and ERRγ form a small family of orphan nuclear receptors that are evolutionarily related to the estrogen receptors ERα and ERβ, and whose *in vivo* function is still unclear [(Giguere et al., 1988; Hong et al., 1999), reviewed in (Giguere, 2002)]. The three ERRs recognize and bind similar DNA sequences, which include estrogen response elements (EREs) recognized by ERα, as well as extended ERE half-sites that have been termed ERR response elements (Johnston et al., 1997; Sladek et al., 1997; Vanacker et al., 1999a; Vanacker et al., 1999b). Despite their high similarity to ligand-dependent receptors, ERRs seem

to regulate transcription in the absence of known natural lipophilic agonist ligands. Searches for ligands have so far identified only synthetic antagonists. 4-Hydroxytamoxifen, which binds ERRβ and ERRγ but not ERRα, and diethylstilbestrol, which binds all three ERRs, inhibit the ability of ERRs to activate transcription (Coward et al., 2001; Tremblay et al., 2001). In support of the pharmacological data, elucidation of the crystal structure of the ERRγ LBD suggests that the ERRs assume the conformation of ligand-activated nuclear receptors in the absence of ligand (Greschik et al., 2002), and that agonist ligands may not be required. These findings raise the question of how the activity of these nuclear receptors is regulated.

One way to control orphan receptor activity is to express the receptors in a temporally- and spatially-restricted manner. ERRα is expressed widely, however, particularly high ERRα mRNA levels have been noted at sites of ossification during development, and in heart, kidney, brown fat, and muscle in adults [(Bonnelye et al., 1997b; Bonnelye et al., 1997c; Shi et al., 1997; Shigeta et al., 1997; Sladek et al., 1997; Vanacker et al., 1998; Vega and Kelly, 1997), reviewed in (Giguere, 1999)]. Thus, differential expression of ERRα may contribute to the regulation of ERRα-mediated transcription. The mechanisms and signals that regulate ERRα expression are not clear.

The activity of orphan nuclear receptors may also be regulated at the protein level, via interactions with specific cofactors. ERRα has been reported variably as an activator, a repressor, or a DNA-binding factor with little activity, suggesting that cellular factors determine the ability of the ERRα protein to activate transcription (Bonnelye et al., 1997b; Johnston et al., 1997; Kraus et al., 2002; Lu et al., 2001; Sladek et al., 1997; Vanacker et al., 1998; Vanacker et al., 1999b; Xie et al., 1999; Zhang and Teng, 2000). Possible candidates for exerting such control are coactivators that interact with ERRα such as members of the p160 family of coactivators. Overexpression of p160 coactivators can indeed enhance ERRα mediated transcription at model reporters (Lu et al., 2001; Xie et al., 1999; Zhang and Teng, 2000). However, ERRα shows weak transcriptional activity in cells that express endogenous p160 coactivators (Lu et al., 2001; Sladek et al., 1997), suggesting that additional cofactors must be important.

PGC-1 is a transcriptional coactivator of many nuclear receptors, as well as specific other transcription factors like the nuclear respiratory factor 1 (NRF-1) and members of the MEF2 (myocyte enhancer factor 2) family (Knutti et al., 2000; Michael et al., 2001; Puigserver et al., 1998; Tcherepanova et al., 2000; Vega et al., 2000; Wu et al., 1999; Yoon et al., 2001). PGC-1 is expressed in a tissue-selective manner, with the highest mRNA levels found in heart, kidney, brown fat and muscle (Esterbauer et al., 1999; Knutti et al., 2000; Larrouy et al., 1999; Puigserver et al., 1998). Moreover, PGC-1 expression is induced in a tissue-specific manner by signals that relay metabolic needs. Exposure to cold leads to the induction of PGC-1 in brown fat and muscle, starvation induces PGC-1 expression in heart and liver, and physical exercise increases its expression in muscle (Goto et al., 2000; Herzig et al., 2001; Lehman et al., 2000; Puigserver et al., 1998; Yoon et al., 2001). PGC-1 function has been implicated in the control of energy metabolism, as PGC-1 expression stimulates mitochondrial biogenesis and modulates mitochondrial functions and utilization of energy [(Lehman et al., 2000; Vega et al., 2000; Wu et al., 1999), reviewed in (Knutti and Kralli, 2001)]. The nuclear receptors PPARγ, TRα, PPARα, HNF4 and GR, and the transcription factors NRF-1, MEF2C, and MEF2D interact with, and may recruit PGC-1 to the promoters of target genes that execute the metabolic effects of PGC-1. Additional transcription factors are likely to contribute to PGC-1 function.

In the study presented here, we show that PGC-1 regulates, first, the expression of ERRα mRNA and, second, the transcriptional activity of the ERRα protein. Our findings indicate that ERRα by itself is a poor activator of transcription, and that PGC-1 fulfils a specific role as a cofactor required for ERRα function. The interactions of PGC-1 and ERRα suggest that the two proteins act in a common pathway.

Experimental procedures

Plasmids and Adenoviral vectors. Expression plasmids for wild-type and mutant human PGC-1, and luciferase reporters pGK1, pΔLUC and pΔ(cERE)x2-Luc (referred in this study as pERE-Luc) have been described (Knutti et al., 2001; Kressler et al., 2002). pSG5-mERRα for the expression of full-length mouse ERRα was a gift of J.-M. Vanacker (Bonnelye et al., 1997b). The human ERRα ligand binding domain (LBD) was amplified by PCR, using HeLa cDNA and primers CGAATTCATATGGGGCCCCTGGCAGTCGCT
and GCTCTAGACTATCAGTCCATCATGGCCTC, and cloned as an Nde I - Xba I fragment into pcDNA3/Gal4DBD (Kressler et al., 2002). The plasmid pSiERRα was generated by cloning the annealed primers GAT CCC CGA GCA TCC CAG GCT TCT CAT TCA AGA GAT GAG AAG CCT GGG ATG CTC TTT TTG GAA A (ERRα907/927-s) and AGC TTT TCC AAA AAG AGC ATC CCA GGC TTC TCA TCT CTT GAA TGA GAA GCC TGG GAT GCT CGG G (ERRα907/927-a) into pSUPER (Brummelkamp et al., 2002).Yeast expression vectors for Gal4-PGC-1 (aa 91-408, wild type or mutants) were generated by subcloning the PGC-1 cDNA fragments encoding aa 91 to 408 in the vector pGBKT7 (Clontech). Plasmid pAS2-ERRαLBD expresses the ERRαLBD fused to the Gal4 DBD and was generated by subcloning the NdeI - XbaI fragment encoding the ERRα LBD into pAS2-1 (Clontech). pGBKT7/hERα.280C expresses the human ERα LBD (starting at aa 280) fused to the Gal4 DBD. Human PPARγ (full-length), RXRα (starting at aa 10) and ERRα (starting at aa 221) fused to the Gal4 activation domain (AD) were isolated in a yeast two-hybrid screen, and were expressed from the vector pACT2 (Clontech).

Adenoviral vectors were generated by CRE-lox mediated recombination in CRE8 cells (Hardy et al., 1997). Briefly, CRE8 cells were transfected with 3 μg of purified Ψ5 adenovirus DNA and 10 μg of pAdlox DNA shuttle plasmid (Hardy et al., 1997) carrying the cDNA for human PGC-1 (wild-type or mutant) downstream of the CMV promoter. For the expression of siRNA from adenoviruses, the CMV promoter and SV40 polyadenylation sequences of pAdlox were replaced by a DNA fragment harboring the expression cassette of pSUPER (Brummelkamp et al., 2002) to generate AdSUPER. The viral vector AdSiERRα expresses the same siRNA as pSiERRα. All

viruses were plaque-isolated to obtain single clones, titered by serial dilution in CRE8 cultures that were grown under 0.6% Noble agar overlay, and used as freeze-thaw lysates.

Cell lines, Infections, and Transfections. 293, CRE8 (Hardy et al., 1997), HepG2, SAOS2-GR(+)(Rogatsky et al., 1997), and HtTA-1 [derived from HeLa; (Knutti et al., 2001)] cells were cultured in Dulbecco's modified Eagle's medium supplemented with 9% fetal calf serum. When measuring ERRα- and GR-mediated transcription, cells were grown in medium with charcoal-stripped serum. SAOS2-GR(+) and CRE8 cultures were supplemented with G418 (400 μg/ml). For infection, cells were plated at $2x10^5$ per well in a 6-well dish. The next day, viruses were added at a multiplicity of infection (moi) of 40 or 100, as indicated in figure legends, for 2 h. Cells were then washed and replenished with fresh medium. For transfections, cells were incubated with a calcium phosphate/DNA precipitate. Transfections included 0.2 μg of p6RlacZ for normalization of transfection efficiency, and 1 μg of the luciferase reporters pΔLuc, pERE-Luc or pGK1. The amounts of expression plasmids per transfection were: 0.5-1 μg of pcDNA3 or pcDNA3/HA-PGC-1; 1 μg of pSG5 or pSG5-mERRα; 1 μg of pcDNA3/Gal4 DBD or pcDNA3/Gal4-ERRαLBD;1 μg of pSUPER or pSiERRα for siRNA. Cell lysates were prepared 40 to 48 h after transfection and assayed for luciferase activity as described (Knutti et al., 2000). Luciferase values normalized to the β-gal activity are referred to as luciferase units.

RNA analysis. Total RNA was isolated using the Trizol reagent, and checked for its integrity by agarose gel electrophoresis and ethidium bromide staining. RNA was converted to cDNA and specific transcripts were quantitated by real-time PCR using the Light Cycler system (Roche Diagnostics) as described previously (Kressler et al., 2002). A melting curve from 65 to 95°C (0.05°C/sec) at the end of the reaction was used to check the purity and nature of the product. In all cases, a single PCR product was detected. The sequences of the primers and the sizes of the PCR products were as follows: AAGACAGCAGCCCCAGTGAA (exon 4)
and ACACCCAGCACCAGCACCT (exon 5) for human ERRα (product 254 bp); TGTGGAGGTCTTGGACTTGGA (exon 4/5)

and TCCTCAGTCATTCTCCCCAAA (exon 6) for MCAD (product 173 bp); CTGTGCCAGCCCAGAACACT (exon 4) and TGACCAGCCCAAAGGAGAAG (exon 5) for 36B4/ribosomal protein P0 large (product 201 bp); CGGGATGAGTTGGGAGGAG (exon 1)
and CGGCGTTTGGAGTGGTAGAA (exon 2) for p21 (product 212 bp); GGAGGACGGCAGAAGTACAAA (exon 4) and GCGACACCAGAGCGTTCAC (exon 5) for mouse ERRα (product 130 bp); primers for mouse PGC-1 and actin have been described in (Kressler et al., 2002).

Western analysis. Cells were lysed in 100 mM Tris pH 7.5, 1% NP40, 250 mM NaCl, 1 mM EDTA buffer. Cell lysates were subjected to western analysis using antibodies against the HA epitope (HA.11, BAbCO), ERRα (Johnston et al., 1997), or PGC-1 (sera from rabbits immunized with a PGC-1 fragment bearing aa 1-293).

Yeast two-hybrid interaction assays. Yeast carrying Gal4-responsive β-gal reporters (CG1945xY187, Clontech) were transformed by the lithium acetate transformation method with expression plasmids for Gal4 DBD and Gal4-AD fusion proteins. Single transformants were grown to stationary phase, diluted 1:20 in selective media, grown for an additional 14 h at 30°C in 96-well plates, and assayed for β-gal activity as described (Kressler et al., 2002)

Results

PGC-1 induces ERRα expression. To identify genes that are induced by PGC-1 and that could execute the cellular processes activated by PGC-1, we have compared the RNA profiles of SAOS2-GR(+) cells infected with adenoviral vectors expressing PGC-1, to those of cells infected with control vectors expressing β-gal or GFP. Analysis of the RNA profiles after hybridization to high density oligonucleotide arrays (data not shown) identified the orphan nuclear receptor ERRα as a gene that is induced strongly by PGC-1. Expression of PGC-1 led to the induction of ERRα at the RNA and protein level in SAOS2-GR(+) cells, as well as in HtTA-1, HepG2, and 293 cells (Fig. 1A, and data not shown). Evaluation of protein levels by immunoblotting showed that the increase in the levels of ERRα protein followed closely the appearance of PGC-1 protein at different times after infection, suggesting that ERRα induction is an early event upon PGC-1 expression (Fig. 1B).

ERRα mRNA levels have been reported to be high in PGC-1 expressing tissues, such as kidney, heart, muscle and brown adipose tissue (Esterbauer et al., 1999; Knutti et al., 2000; Larrouy et al., 1999; Puigserver et al., 1998; Shi et al., 1997; Shigeta et al., 1997; Sladek et al., 1997; Vanacker et al., 1998; Vega and Kelly, 1997). Analysis of mRNA expression levels in tissues of adult mice shows that indeed ERRα levels correlate with PGC-1 mRNA levels (Fig. 1C). PGC-1 expression in some of these tissues is known to be induced in response to physiological signals, such as exposure to cold (Puigserver et al., 1998). Thus, to test the ability of PGC-1 to induce ERRα *in vivo*, we determined PGC-1 and ERRα mRNA levels in the brown fat and muscle of mice that were exposed to cold for 6 hours. As seen in Fig. 1D, the increase in PGC-1 expression was accompanied by an increase in ERRα mRNA levels, suggesting that PGC-1 can also induce ERRα expression *in vivo*.

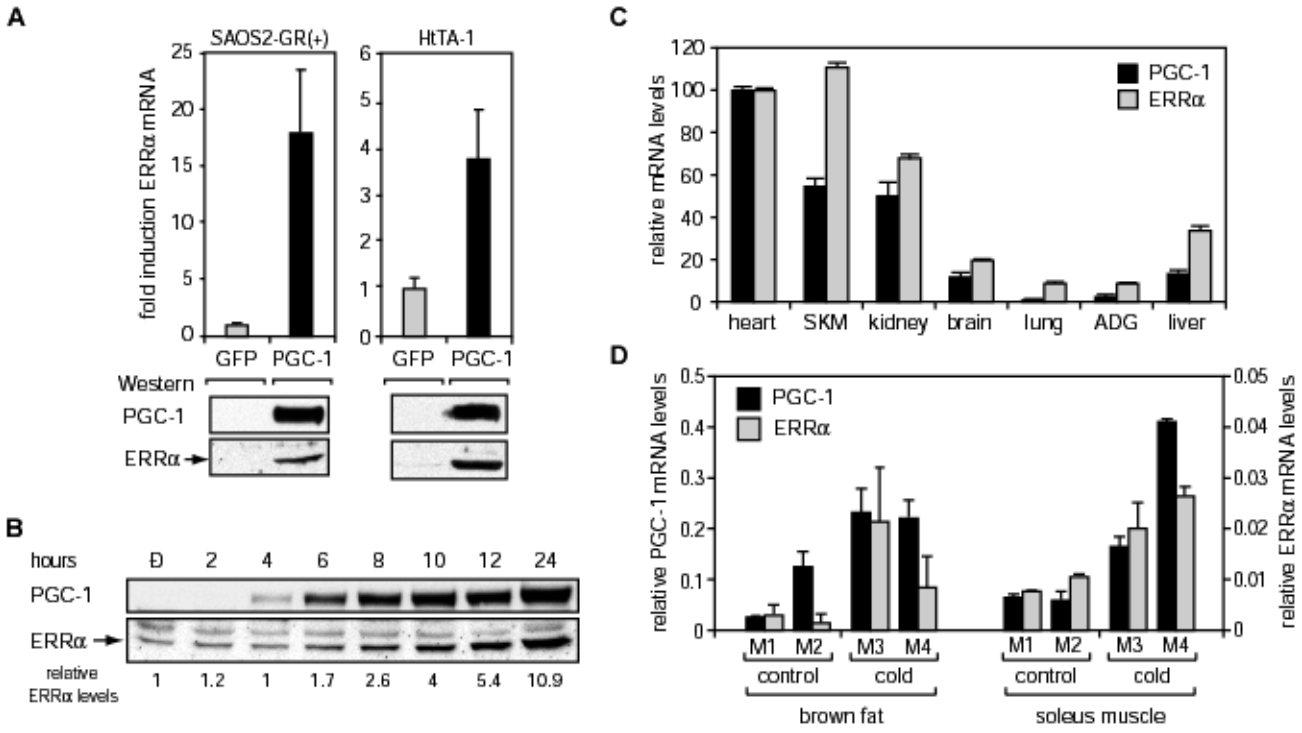

Figure 1. PGC-1 induces ERRα at the mRNA and protein level.

(A, B) Cells were infected with either GFP (control) or PGC-1 expressing adenoviruses at a moi of 40 [SAOS2-GR(+)] or 100 (HtTA-1). **(A)** RNA was isolated 24 h (HtTA-1) or 48 h [SAOS2-GR(+)] after infection. Levels of ERRα mRNA were determined by quantitative RT-PCR, normalized to 36B4 levels, and expressed relative to levels in control cells. Data represent the mean ± standard deviation of four experiments performed in duplicates. Cell extracts prepared 24 h after infection were analyzed by immunoblotting with antibodies against PGC-1 (upper panel) or ERRα (lower panel). (**B**) Cell extracts prepared at the indicated times after infection of HtTA-1 cells with a PGC-1 expressing adenovirus were analyzed by immunoblotting with antibodies against PGC-1 (upper panel) or ERRα (lower panel). (-), extracts from uninfected HtTA-1 cells. (**C, D**) Levels of PGC-1 and ERRα mRNA in the indicated mouse tissues were determined by quantitative RT-PCR, and normalized to β-actin levels. (**C**) Relative mRNA levels in tissues of a ~ 6 week-old female mouse. Levels in heart were set equal to 100 for each transcript. Data represent mRNA levels relative to expression in heart, and are the mean ± range of duplicate PCR reactions. SKM, skeletal muscle; ADG, adrenal gland. (**D**) Relative mRNA levels in brown fat and soleus muscle of four ~8-week-old male siblings, kept at 23 ^{o}C (M1, M2; control) or exposed to 4 ^{o}C for 6 hours (M3, M4; cold). Data shown are the mean ± range of PGC-1 and ERRα mRNA levels normalized to β-actin levels in each RNA sample.

PGC-1 induces strongly ERRα-mediated transcription. The finding that PGC-1 induces the expression of ERRα suggests that PGC-1 enhances also the activity of ERRα-regulated promoters. To test this, we transfected 293 cells with a PGC-1 expression vector and a reporter that carries the luciferase gene under the control of the minimal ADH promoter with or without binding sites for ERRα (pERE-Luc and pΔLuc, respectively). PGC-1 enhanced strongly expression from the pERE-Luc reporter, in a manner dependent on the presence of the binding sites for ERRα (Fig. 2A). Estradiol, tamoxifen or hydroxytamoxifen did not affect the enhancement by PGC-1 (data not shown), suggesting that it was not mediated by receptors that are regulated by these ligands and can recognize the same DNA binding site (e.g. ERα, ERβ, ERRβ or ERRγ). To confirm that endogenous, PGC-1-induced ERRα was mediating the effect of PGC-1 on the pERE-Luc reporter, we determined the effect of inhibiting the expression of ERRα. For this, cells were transfected with a vector expressing a small interfering (si) RNA specific for ERRα (pSiERRα) (Brummelkamp et al., 2002). Expression of the ERRα-specific siRNA led to a decrease in ERRα mRNA levels (Fig. 2B), and a decrease in the PGC-1 – mediated induction of the luciferase reporter, demonstrating that endogenous ERRα was required for the PGC-1 effect (Fig. 2C). In the absence of PGC-1, pSiERRα decreased ERRα expression (Fig. 2B) but had no effect on the pERE-Luc reporter (Fig. 2C), suggesting that in this context ERRα was not transcriptionally active.

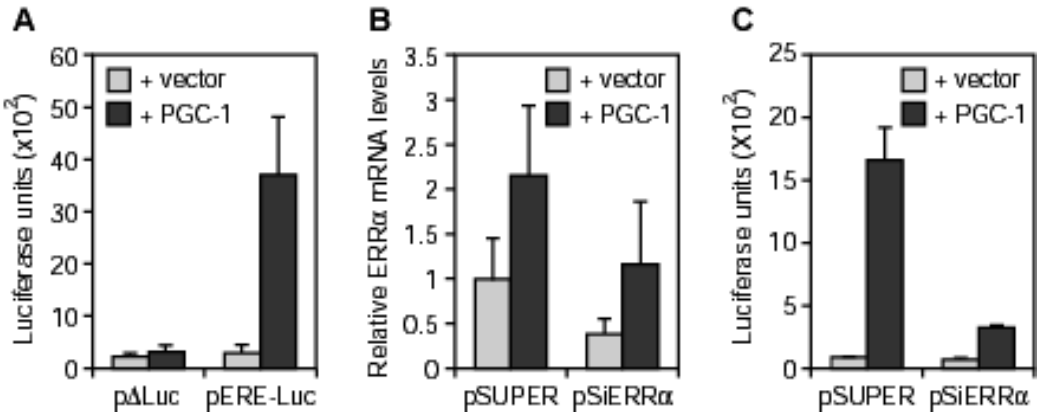

Figure 2. PGC-1 induces ERRα-mediated transcription.

(A) 293 cells were transfected with a luciferase reporter driven by either just the minimal ADH promoter (pΔLuc) or 2 EREs upstream of the minimal ADH promoter (pERE-Luc), and either the control vector pcDNA3 or a PGC-1 expression vector. Data are the mean ± standard deviation of luciferase activities from three experiments performed in duplicates. **(B)** 293 cells were transfected with the empty vector pSUPER (Brummelkamp et al., 2002) or the vector expressing siRNA for ERRα (pSiERRα), and either pcDNA3 (+vector) or the PGC-1 expression vector pcDNA3/HA-PGC-1 (+PGC-1). Transfection efficiency was 40-50%. RNA was prepared 48 h later. ERRα mRNA levels were determined by quantitative RT-PCR and normalized to levels of 36B4. Data are the average of two experiments performed in duplicates. **(C)** 293 cells were transfected with the pERE-Luc reporter, a control or PGC-1 expression vector as indicated, and either the control pSUPER or the siRNA expressing pSiERRα. Data represent the mean ± standard deviation of luciferase activities from two experiments performed in duplicates.

PGC-1 activates ERRα at the protein level. PGC-1 interacts physically with many nuclear receptors and enhances their transcriptional activity [reviewed in (Knutti and Kralli, 2001)]. Thus, PGC-1 could also interact with ERRα. In this case, the increased ERRα-mediated transcription could be the combined result of PGC-1 inducing ERRα levels, and enhancing ERRα activity. To address this, we first asked if overexpression of ERRα would lead to the same phenotype as PGC-1 expression. If the only function of PGC-1 were to increase ERRα levels, we would expect that exogenous ERRα expression would mimic the PGC-1 effect. Surprisingly, overexpression of ERRα had very little effect on pERE-Luc (< 2-fold), suggesting that ERRα alone was not sufficient for the transcriptional activation of this reporter (Fig. 3A). Coexpression of PGC-1 with ERRα led to an increase in luciferase expression that was stronger than that seen with just endogenous ERRα, indicating that PGC-1 activated the exogenously introduced ERRα (Fig. 3A).

To determine the effect of PGC-1 on the activity of ERRα directly, we evaluated the consequence of PGC-1 expression on the activity of a Gal4 DNA binding domain (DBD) - ERRα LBD chimera, using a Gal4-responsive luciferase reporter. In this context, endogenous ERRα does not interfere with the luciferase readout. As seen in Fig. 3B, Gal4-ERRα LBD by itself activated transcription modestly, ~2-fold, suggesting that the LBD of ERRα carries only a weak transcriptional activation function. Addition of PGC-1 converted the Gal4-ERRαLBD fusion to a strong activator of transcription, indicating that PGC-1 enables the transcriptional function of ERRα (Fig. 3B).

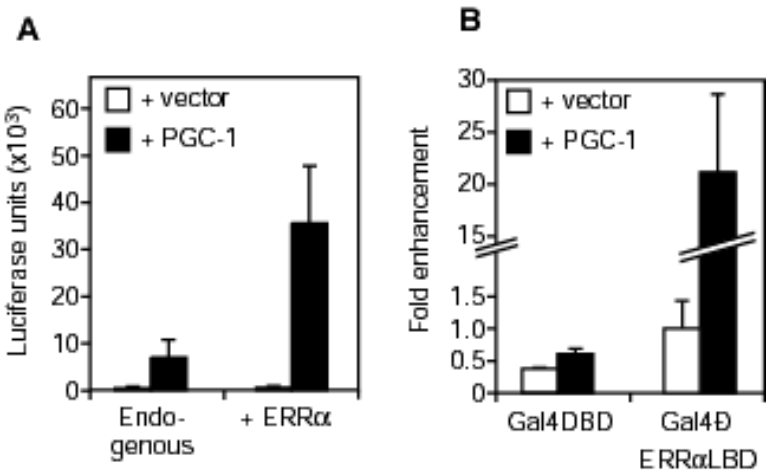

Figure 3. PGC-1 activates ERRα, which by itself is a weak activator of transcription.

(A) 293 cells were transfected with the pERE-Luc reporter, a control or PGC-1 expression vector as indicated, and either the pSG5 vector (endogenous) or the pSG5-mERRα expression vector (+ERRα) (Vanacker et al., 1999b). Data are the mean ± standard deviation of luciferase activities from three experiments performed in duplicates. (**B)** 293 cells were transfected with the Gal4 responsive luciferase reporter pGK1, an expression vector for either the Gal4 DBD or a fusion of the ERRα LBD to the Gal4 DBD, and either vector alone or PGC-1 expression vector. Data are from one representative experiment performed in triplicates, and are expressed relative to the activity of Gal4-ERRαLBD in the absence of PGC-1.

ERRα interacts with PGC-1 via an atypical Leu-rich box. PGC-1 harbors three Leu-rich motifs, (L1, L2, and L3), one of which, (L2), bears the consensus LxxLL sequence present in many proteins that interact with the LBD of nuclear receptors. The L2 motif serves as the major binding site for many nuclear receptors, and mutations in L2 disrupt the interactions of PGC-1 with nuclear receptors tested so far (Knutti et al., 2001; Tcherepanova et al., 2000; Vega et al., 2000). Surprisingly, PGC-1 harboring a mutant L2 (L2A) was still capable of interacting with ERRα in a yeast two-hybrid assay; in the same context, the L2A mutant was severely compromised for interaction with PPARγ, RXRα, and ERα (Fig. 4A,B). In previous studies, we had noted that the L3 site can mediate a weak interaction with the glucocorticoid receptor (Knutti et al., 2001). We thus tested the contribution of the L3 site to the PGC-1/ERRα interaction. As seen in Fig. 4A and B, PGC-1 bearing a disruption of just L3 (L3A) was also capable of interacting with ERRα, while the double L2/3A mutation abolished the interaction. Mutations in motif L1, alone or in combination with L2, had no effect on the physical interaction of PGC-1 with ERRα (data not shown). Thus, we concluded that motifs L2 and L3 can be used equivalently for physical interactions between PGC-1 and ERRα, while L2 is the preferred site for most other receptors (Fig. 4A & B).

Next, we determined the requirement of the physical interaction between PGC-1 and ERRα for the activation of the ERRα LBD in mammalian cells, using the context of the Gal4-ERRαLBD chimera. Single mutations in either L2 or L3 did not compromise the PGC-1 effect (Fig. 4C), suggesting that interaction via either site is sufficient for activation of ERRα by PGC-1. The double L2/3A mutation abolished the activation, indicating that the physical interaction between the two proteins is necessary for the effect of PGC-1 on the ERRα LBD (Fig. 4C).

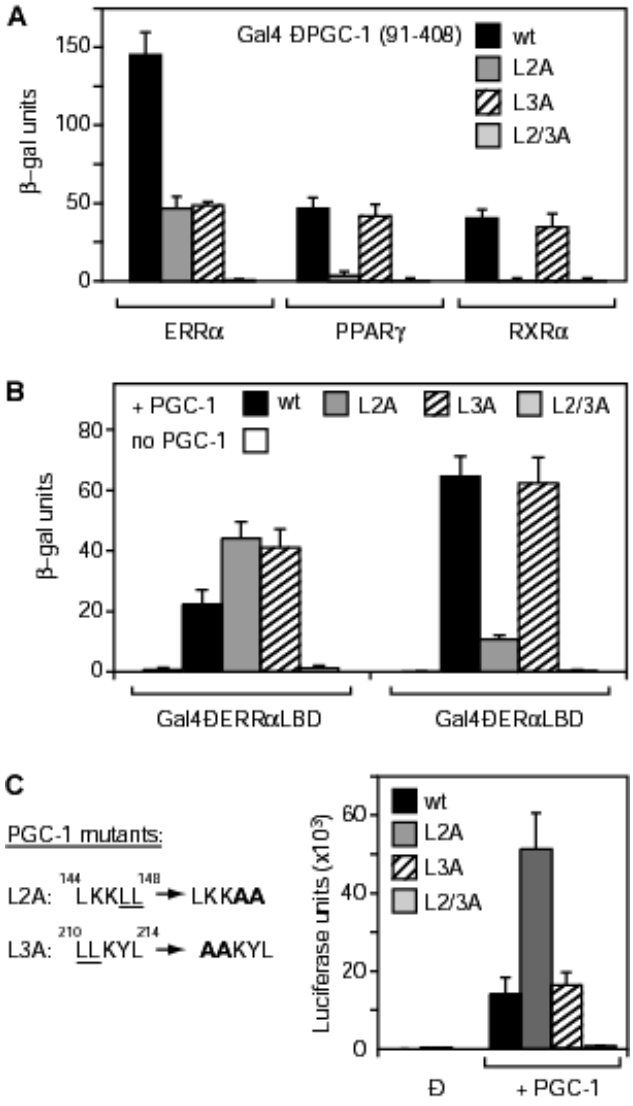

Figure 4. ERRα interacts with the L3 as well as the L2 site of PGC-1.

(**A, B**) Yeast two-hybrid assay. (**A**) Interactions between the L2/L3 containing PGC-1 fragment (aa 91-408) fused to the Gal4 DBD, and the indicated receptors fused to the Gal4 AD. Data are the mean ± standard deviation of β̃gal activities from four independent transformants. (**B)** Interactions between the LBD of ERRα or ERαto the Gal4 DBD (Gal4-ERRα and Gal4-ERα), and full-length PGC-1 [wild type (wt) or L2/L3 mutants] fused to the Gal4 AD. Interaction with ERα was assayed in the presence of 10 μM 17β-estradiol. Data are the mean ± standard deviation of β̃gal activities from twelve yeast transformants. (**C**) Activation of the ERRα LBD in mammalian cells. 293 cells were transfected with the Gal4 responsive luciferase reporter pGK1, the vector expressing the Gal4-ERRα LBD fusion, and either vector alone (–), PGC-1 wild type (wt), or the indicated PGC-1 mutants. Data are the mean ± standard deviation of luciferase activities from at least four experiments performed in duplicates.

PGC-1 can induce the expression of the endogenous gene MCAD in an ERRα - dependent manner. The ability of PGC-1 to induce ERRα expression and activity predicts that PGC-1 should also induce the expression of ERRα target genes. To test this, we determined the effect of PGC-1 on the RNA levels of a proposed ERRα target, the medium chain acyl-coenzyme A dehydrogenase (MCAD), an enzyme in fatty acid oxidation (Sladek et al., 1997; Vega and Kelly, 1997). As seen in Fig. 5, PGC-1 expression led to the induction of MCAD in HtTA-1 and SAOS2-GR(+) cells. To address whether the induction was mediated by ERRα, we asked if suppression of ERRα expression affected the response of MCAD to PGC-1. Infection of HtTA-1 cells with adenoviruses that express ERRα-specific siRNA led to a decrease in ERRα mRNA levels (Fig. 5A), and a reduced induction of MCAD (Fig. 5B), consistent with ERRα mediating the PGC-1 effect at the MCAD promoter.

The distinct utilization of the L3 site of PGC-1 for interaction with ERRα and not other receptors like GR, suggests that mutations in the L2 and L3 sites could be used to diagnose the type of nuclear receptors that mediate specific functions of PGC-1. Functions that are mediated by receptors utilizing the L2 site should be abrogated by the single PGC-1 mutation L2A, while functions that rely on ERRα should be disrupted only by the double L2/3A and not the single L2A mutation. To test this, we infected SAOS2-GR(+) cells that express GR from a stably-integrated locus, with adenoviruses expressing PGC-1, wild-type or mutant variants. As predicted, the glucocorticoid-dependent induction of the endogenous GR target p21 (Rogatsky et al., 1997) was enhanced by both wild-type PGC-1 and the L3A mutant, but not by the L2A mutant (Fig. 5C). In contrast, induction of MCAD in the same cells was not affected by the L2A mutation and was only abolished by the double L2/3A mutation (Fig. 5D). These findings indicate that the L2 and L3 sites of PGC-1 are indeed used selectively by different nuclear receptors to recruit PGC-1 at their respective endogenous target genes.

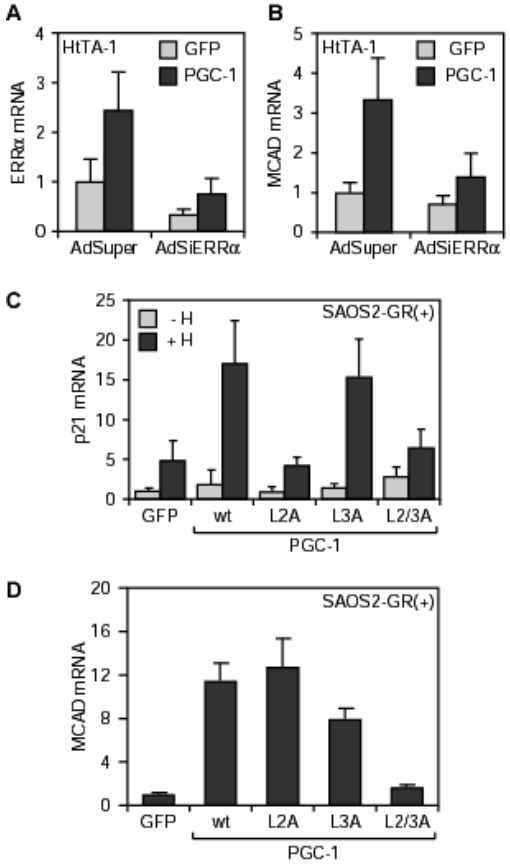

Figure 5. PGC-1 induces the endogenous MCAD gene in an ERRα.

(A, B) HtTA-1 cells were infected with either control (AdSUPER) or siERRα expressing (AdSiERRα) adenoviruses on day 1, and either GFP- or PGC-1-expressing adenoviruses on day 2. RNA was harvested on day 3, and mRNA levels for ERRα and MCAD were analyzed by quantitative RT-PCR, normalized to 36B4 levels, and expressed relative to levels in cells infected with AdSUPER/GFP viruses. Data represent the mean ± standard deviation of three experiments performed in duplicates. **(C, D)** SAOS2-GR(+) cells were infected with adenoviruses expressing either GFP or PGC-1 [wild type (wt) or mutants L2A, L3A, or double L2/3A]. (**C**) 24 h after infection cells were treated with either 50 nM corticosterone (+H) or just vehicle ethanol (–). RNA was harvested 8 h after hormone addition, and p21 mRNA levels were determined by quantitative RT-PCR, normalized to 36B4 levels, and expressed relative to levels in cells infected with GFP virus and treated with just ethanol. Data represent the mean ± range of duplicates of one experiment. (**D**) RNA was harvested 48 h after infection, and MCAD mRNA levels were determined by quantitative RT-PCR, normalized to 36B4 levels, and expressed relative to levels in cells infected with GFP virus. Data represent the mean ± standard deviation of two experiments performed in duplicates. Wild-type, L2A, L3A and L2/3A mutants were expressed at similar levels, as determined by western blot analysis.

Discussion

Many members of the nuclear receptor superfamily are still orphan receptors, with no known physiological ligands. The mechanisms that regulate the activity of these receptors are not fully understood. The results presented here provide evidence that the transcriptional coactivator PGC-1 is a key regulator of the orphan nuclear receptor ERRα. PGC-1 acts at two levels. First, it induces ERRα expression; second, it associates with ERRα and enables the transcriptional activation of ERRα target genes. PGC-1 expression is known to be regulated in a tissue-selective manner by physiological signals that relay metabolic needs (Goto et al., 2000; Herzig et al., 2001; Lehman et al., 2000; Puigserver et al., 1998; Yoon et al., 2001). Accordingly, PGC-1 function has been implicated in the regulation of energy metabolism [(Lehman et al., 2000; Vega et al., 2000; Wu et al., 1999), reviewed in (Knutti and Kralli, 2001)]. Our findings suggest that ERRα functions in PGC-1 - regulated pathways, where it may contribute to the transcriptional activation of genes important for energy homeostasis.

The activity of several orphan nuclear receptors is restricted by expression of the receptors in specific tissues or at particular times [reviewed in (Giguere, 1999)].The mechanisms that control the selective expression of these receptors are often not clear. The observation that PGC-1 induces ERRα mRNA levels, provides a molecular explanation for the high ERRα expression in heart, kidney, muscle and brown fat, i.e. tissues that express PGC-1. Moreover, it suggests physiological signals that are likely to control ERRα expression, as shown here for exposure to cold in brown fat and muscle. In support of these findings, Ichida et al. have recently shown that fasting, which is known to induce PGC-1 expression in the liver (Herzig et al., 2001; Yoon et al., 2001), also increases ERRα mRNA levels (Ichida et al., 2002).The spatial and temporal correlation of PGC-1 and ERRα expression implies that ERRα induction is an early, and possibly direct outcome of PGC-1 action. Future studies must address if PGC-1 acts directly at the ERRα promoter. Additional regulatory mechanisms may restrict or enhance ERRα induction by PGC-1, in a tissue- or physiological state-dependent manner.

Interestingly, we find that in the absence of PGC-1, ERRα is a very weak activator of transcription. Coexpression of PGC-1 enables potent transcriptional activation by ERRα. These findings suggest that ERRα is not a constitutively active receptor, and that transformation into an active form is favored by binding to protein ligands, such as PGC-1, rather than to small lipophilic ligands. Expression levels of PGC-1 may explain why ERRα has been reported as an efficient transcriptional activator in some cells (e.g. ROS 17.2/8) and a poor activator in others (Bonnelye et al., 1997b; Johnston et al., 1997; Kraus et al., 2002; Lu et al., 2001; Sladek et al., 1997; Vanacker et al., 1998; Vanacker et al., 1999b; Xie et al., 1999; Zhang and Teng, 2000). Many established cell lines express very low, if any, levels of PGC-1. Importantly, the ability of PGC-1 to activate ERRα at the protein level predicts that physiological signals that induce PGC-1 are likely to activate ERRα-mediated transcription, even in the absence of increased ERRα expression.

The activation of ERRα at the protein level requires the physical interaction of PGC-1 with ERRα. Surprisingly, this interaction differs from that of PGC-1 with other nuclear receptors. While PGC-1 recognizes most receptors tested until now (GR, ERα, TRα, RXRα, RARα, PPARα, PPARγ, HNF4) via the canonical LxxLL motif L2, it can interact with ERRα equally well via the L2 or the L3 site. Similar to our findings, Huss et al. have recently shown that ERRα, as well as the related receptor ERRγ, bind the L3 site of PGC-1 (Huss et al., 2002), suggesting that the L3-mediated interaction is characteristic of the ERR subfamily of receptors. Interestingly, the differential utilization of the Leu-rich motifs can be used to dissect the receptors that mediate specific PGC-1 functions, as shown by the fact that L2A mutations disrupt GR- but not ERRα-dependent effects of PGC-1. Thus, the L2 and L3 mutants of PGC-1 may provide useful tools for elucidating the types of receptors that recruit PGC-1 at distinct promoters.

The *in vivo* functions of ERRα are not yet defined. Based on its ability to bind EREs and modulate some estrogen-responsive genes, ERRα has been proposed to modulate ER signaling and possibly play a role in ER-dependent tumors (Kraus et al., 2002; Lu et al., 2001; Vanacker et al., 1999b). A function of ERRα in bone development is supported by the high levels of ERRα at sites of ossification during embryogenesis,

and the ability of ERRα to promote osteoblast differentiation *in vitro* and to activate the promoter of the bone matrix protein osteopontin (Bonnelye et al., 2001; Bonnelye et al., 1997b). Finally, the strong expression of ERRα in tissues with high capacity for fatty acid oxidation, and its ability to bind the promoter of the MCAD gene, suggest a role in the mitochondrial β-oxidation of fatty acids (Sladek et al., 1997; Vega and Kelly, 1997). Our findings support a function of ERRα in PGC-1 – stimulated cellular processes, such as fatty acid oxidation (Vega et al., 2000), and possibly other aspects of energy homeostasis. Interestingly, the close relationship of PGC-1 and ERRα activity may reflect not only an involvement of ERRα in known PGC-1-regulated functions, but also of PGC-1 in processes where ERRα roles have been suggested, such as bone development and homeostasis, or breast cancer.

Acknowledgements. We thank M. Senften and U. Muller for Ψ5 adenoviral DNA; L. Dolfini and M. Meyer for technical help; J.-M. Vanacker and R. Agami for plasmids; J. Mertz for the anti-ERRα serum; I. Rogatsky and M.J. Garabedian for the SAOS-GR(+) cells; R. Emter, D. Kressler, and U. Muller for discussions and comments on the manuscript. The work was supported by the Swiss National Science Foundation, the University of Basel, the Novartis Stiftung (S.N.S.), Roche (D.K.), and the Max Cloëtta Foundation (A.K.).

Chapter II: supplementary data

Results and Discussion

Mechanism of ERRα induction

In our published study, we demonstrated that the mRNA and protein levels of ERRα were induced by PGC-1α. Since coactivators do not bind DNA directly, we wanted also to determine which transcription factor mediates the PGC-1α effect on the expression of the ERRα gene. As described earlier, PGC-1α interacts with most nuclear receptors via the L2 motif, but with ERRs via either the L2 or the L3 motif. To find if a nuclear receptor with characteristic of either of ERRs or of other NRs was required for the PGC-1α mediated induction, we tested what effect mutations in the L2/L3 motifs of PGC-1α would have on the induction of ERRα expression. As seen in figure 6 A, mutation of the L2 motif had no effect, while mutation of the L3 motif caused a small drop in ERRα induction. When both motifs were mutated, the upregulation of ERRα expression levels by PGC-1α was mostly abolished, suggesting that it was mediated by a nuclear receptor that could recruit PGC-1α via L2 as well as L3 (as predicted for ERRα, ERRβ, and ERRγ).

To further address the hypothesis that ERRα is involved in the regulation of its own expression, we used a dominant negative version of ERRα. Nuclear receptor activity depends on the integrity of the AF-2 domain in the LBD (reviewed in Warnmark et al., 2003). Deletion of the AF-2 domain of ERRα abolishes the ability of ERRα to recruit PGC-1α, and to activate transcription (Bonnelye et al., 1997a), but does not affect its ability to bind DNA. Consequently, this mutant is able to compete with the endogenous ERRα for binding to DNA, and blocks ERRα signaling (Bonnelye et al., 1997a). Therefore, we constructed adenoviral vectors expressing the dominant negative ERRα. As shown in figure 6 B, expression of the dominant negative ERRα decreased the levels of ERRα in the absence of PGC-1α similar to the effect of

specific siRNA for ERRα. Furthermore, PGC-1α induced the expression of ERRα in the absence, but not anymore in the presence of the dominant negative ERRα.ΔAF2, consistent with the idea that endogenous ERRα is required for the induction.

To further address the mechanism by which PGC-1α and ERRα regulate ERRα expression, we analyzed the sequence of the ERRα promoter and identified three putative ERREs (figure 6 C). These results strongly suggested us to analyze the ERRα effect on the ERRα promoter. We therefore cloned base pairs –537 to –829 of the ERRα upstream regulatory sequence in a luciferase reporter construct, and tested its response to PGC-1α and ERRα in transient transfection assays. Figure 6 D shows that expression of PGC-1α led to the induction of the ERRα promoter reporter construct activity. This induction required the expression of endogenous ERRα, since specific siRNA for ERRα completely inhibited the effect of PGC-1α. Inhibition of ERRα expression had no influence on the basal activity of the promoter, in the absence of PGC-1α. To confirm that the decrease in activity caused by the siRNA resulted from the specific loss of ERRα, we cotransfected a plasmid expressing ERRα to overcome the effect of the siRNA. As seen in figure 6 E, we detected a dose-dependent increase in the ability of PGC-1α to induce the ERRα promoter, with 1-20 ng of DNA of the plasmid expressing ERRα. Interestingly, higher levels of ERRα expression showed an inhibitory effect.

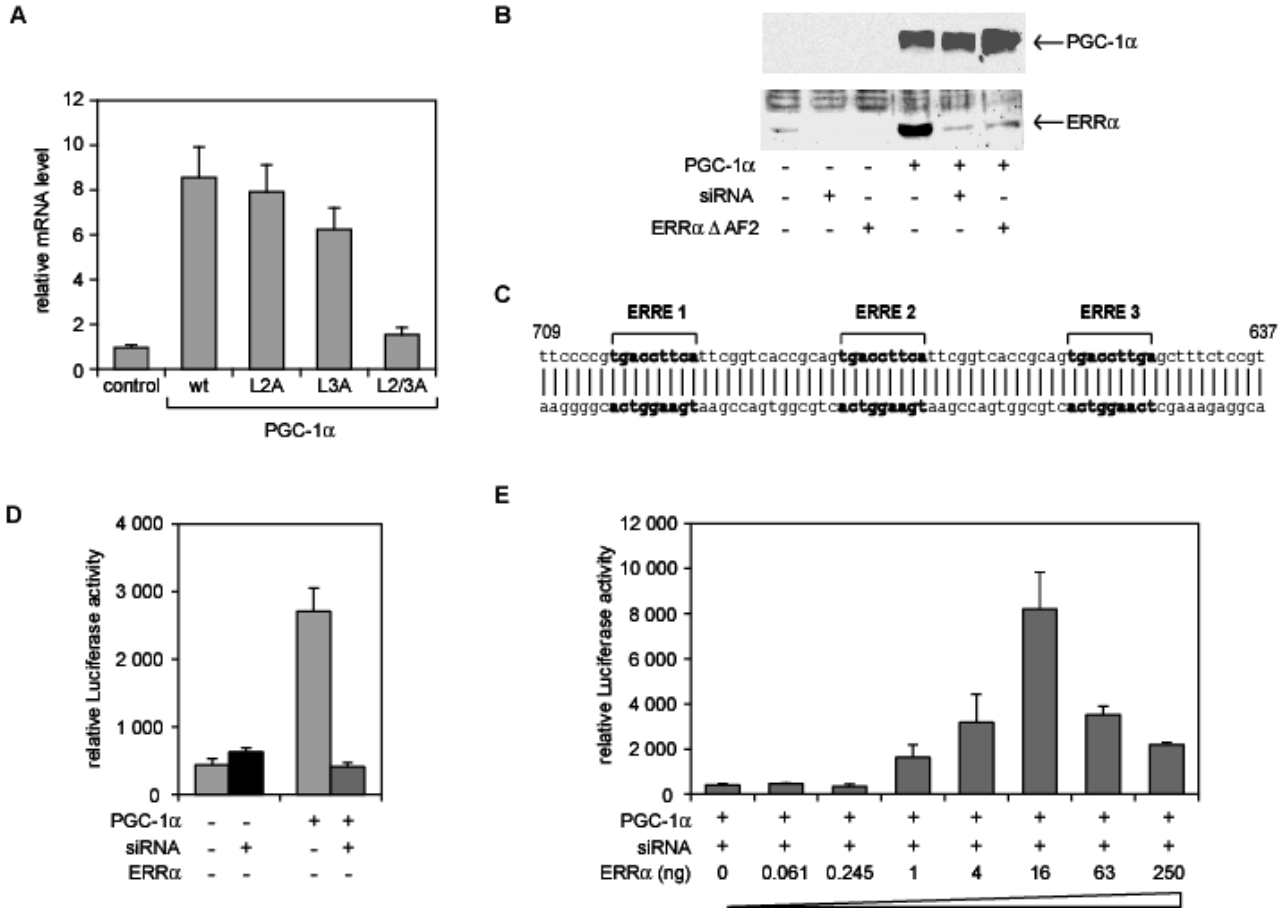

Figure 6. ERR α induces its own expression by acting at the ERR α promoter.

(A) SAOS2 cells were infected with adenoviruses expressing GFP (control), wt PGC-1 α or mutants L2A, L3A and L2/3A (MOI 40); RNA was isolated at 46 h after infection, and analyzed by quantitative RT-PCR. Values for ERR α mRNA were normalized to values of 36B4 mRNA and expressed in relation to the control, which was set as 1. Values represent the mean ± SEM of two experiments with duplicates. **(B)** Cells were infected with adenoviruses expressing control (pSuper), siRNA for ERR α, or the dominant negative ERR α.ΔAF-2 mutant. After three days, cells were re-infected with control (GFP) or PGC-1 α adenoviruses. Cell extracts were isolated 24 hours later and analyzed with polyclonal PGC-1 α or ERRa antibodies. **(C)** Promoter sequence of ERR α with three putative ERR α binding sites. **(D, E)** SAOS2 cells were infected with adenoviruses expressing siRNA for ERR α on day 1. On day 4, cells were transfected with the ERR α -promoter luciferase reporter and either pcDNA3 (control vector) or the expression plasmid pcDNA3-PGC-1 α. Data are from duplicates of 1 of 2 representative experiments. In **(D),** increasing amounts of the ERR α expression vector pcDNA3.ERR α (as indicated) were cotransfected with the PGC-1 α expression plasmid.

Our results imply that the induction of ERRα by PGC-1α is executed by a positive feedback-loop. The fact that high expression levels of ERRα have an inhibitory effect, suggests that the role of ERRα in the regulation of its own promoter may be more complex than a simple activation mechanism. While low levels of ERRα are required for the induction of its expression by PGC-1α, at high levels ERRα may represses its promoter. The physiological significance of this finding is currently not clear. Figure 7 shows a possible model for the positive feedback loop. Physiological signals induce the expression of PGC-1α. PGC-1α and ERRα proteins bind to the promoter of ERRα and induce the expression of more ERRα. After the synthesis of new ERRα protein, PGC-1α and ERRα act as a transcriptional couple to activate other genes in response to the physiological signals.

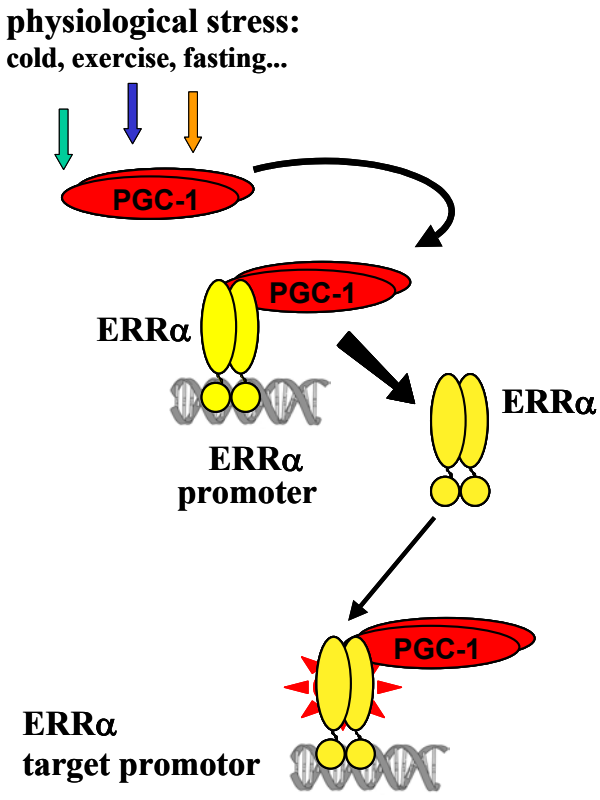

Figure 7. Model of the positive feedback loop by which PGC-1α induces ERRα

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Chapter III:

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

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Summary

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, 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.

Introduction

Estrogen-related receptor alpha (ERRα, NR3B1) was identified on the basis of its sequence similarity to classical, hormone-regulated steroid receptors (Giguere et al., 1988). Based on its ability to recognize similar DNA sequences as the estrogen receptors, ERRα has been proposed to modulate estrogen signaling (Giguere, 2002; Johnston et al., 1997; Vanacker et al., 1999; Yang et al., 1996). ERRα may also regulate bone formation, as it is highly expressed at ossification sites, promotes osteoblast differentiation *in vitro*, and activates the promoter of the bone matrix protein osteopontin (Bonnelye et al., 2001; Bonnelye et al., 1997). 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-coenzyme A dehydrogenase gene (Sladek et al., 1997; Vega and Kelly, 1997).

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 if ERRα activity is regulated by small lipophilic ligands. Compounds that inhibit ERRα-dependent transcription, such as toxaphene, chlordane and diethylstilbestrol, have been described (Tremblay et al., 2001; Yang and Chen, 1999). However, these compounds are not specific enough for ERRα to facilitate studies of its cellular function. Recently, we demonstrated that the transcriptional coactivator PGC-1α regulates ERRα function (Schreiber et al., 2003). PGC-1α induces the expression of ERRα and interacts physically with ERRα, enabling it to activate transcription (Huss et al., 2002; Schreiber et al., 2003). 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 (Knutti and Kralli, 2001; Puigserver and Spiegelman, 2003; Puigserver et al., 1998). Its expression is most prominent in tissues with high energy demands, similar to the expression pattern of ERRα (Puigserver and Spiegelman, 2003; Schreiber et al., 2003). 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 (Knutti and Kralli, 2001; Puigserver and Spiegelman, 2003)]. 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 (Lehman et al., 2000; Puigserver et al., 1998; Vega et al., 2000; Wu et al., 1999). This is accomplished via 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, as well as members of other transcription factor families, such as NRF-1, which controls the expression of mitochondrial proteins, and MEF2C/D (Knutti and Kralli, 2001; Puigserver and Spiegelman, 2003).

The recent identification of ERRα as a protein that is coexpressed with, as well as 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, siRNA for ERRα, and control AdSUPER have been described (Schreiber et al., 2003). Adenoviruses expressing ERRα or VP16-ERRα were constructed using the insert of pSG5-mERRα or pSG5-mΔAB.ERRα respectively (Bonnelye et al., 1997). For the reporter plasmids, human genomic DNA and gene-specific oligonucleotides (Supplement 2) were used to amplify the sequences -385 to +90 and -686 to +55 (relative to transcription initiation site) of the ATPsynβ and 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 (Knutti et al., 2001). The 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 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 (Schreiber et al., 2003)] 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 (Knutti et al., 2000). The amounts of plasmids per transfection were 100 ng of the reporters pCytc/-686Luc or pATPsynβ/-385Luc, 100 ng pcDNA3/HA-PGC-1α (Knutti et al., 2000), and 50 ng pcDNA3/ERRα(Coward et al., 2001).

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

Microarray analysis. Data from 3 experiments were analysed using the Affymetrix Microarray Suite v5 and GeneSpring 5.1 (Silicon Genetics). Changes in gene expression were assessed by looking for concordant changes between replicates 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 1-way ANOVA test ($p<0.05$) using 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 NCBI PubMed, and the OXPHOS and human_mitoDB_6_2002 lists curated at WICGR (Mootha et al., 2003b).

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, using primers specific to the COX II (mitochondrial) and β actin (nuclear) genes (Supplement 2), 1 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 (Kressler et al., 2002).

RNA analysis. Isolation of RNA, conversion to cDNA and quantification of transcripts by real-time PCR using the Light Cycler system (Roche Diagnostics) and gene-specific primers (Supplement 2) have been described (Kressler et al., 2002).

Western analysis. Cell lysates were subjected to western analysis using antibodies against PGC-1α (Schreiber et al., 2003) and ERRα (Johnston et al., 1997).

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 then 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, Beckton Dickinson), using the software WinMDI 2.8.

In silico analysis for ERREs. 35 sequences reported to bind ERRα (Johnston et al., 1997; Sladek et al., 1997; Vanacker et al., 1998; Vanacker et al., 1999) were aligned using ClustalW and used to compile a position-weighted nucleotide distribution matrix. Cross-validation 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, using a variant of the NUBIScan algorithm (Podvinec et al., 2002).

Electrophoretic mobility shift assay. 0.5 µl of *in vitro* translated ERRα [T7 Coupled Reticulocyte system (Promega)] or of unprogrammed lysate was incubated in 20 µl buffer (10 mM Hepes pH 7.5, 2.5 mM $MgCl_2$, 50 mM EDTA, 1mM DTT, 6% glycerol) with 1ng ^{32}P end-labeled oligonucleotide probe and 1 µg poly dI:dC, in the absence or presence of 100 ng unlabeled oligonucleotide competitor (Supplement 2). Complexes were resolved in 6% native polyacrylamide gels.

Results

PGC-1α induces mitochondrial biogenesis in SAOS2 cells, via 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. 17 hours after infection with a PGC-1 expressing adenovirus, 151 of the upregulated transcripts were classified as nuclear genes encoding mitochondrial proteins (Supplement 1, Lists A, B). These genes define "mitochondrial functions" that are upregulated in the early phase of the PGC-1α-induced response (~12 hours 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 (TCA) cycle (17 genes), and oxidative phosphorylation (55 genes) (Supplement 1). An additional 23 of the upregulated transcripts represent genes that do not encode known mitochondrial proteins but have been reported as co-regulated with "mitochondrial genes", and proposed to carry functions relevant to mitochondrial biology (Mootha et al., 2003a) (Supplement 1). PGC-1α also induced the expression of the mitochondrial transcription and translation factor mtTFA. Interestingly, it did not affect the expression of NRF-1 or NRF-2 (Supplement 1), the transcription factors that regulate the expression of many nuclear genes encoding mitochondrial proteins, and that are induced by PGC-1α in C2C12 cells (Wu et al., 1999). 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 (Wu et al., 1999).

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 non-infected cells (Fig. 1A 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 (Gm 151) compared to control cells (Gm 90) was consistent with an increase in mitochondrial content (Fig. 1B). 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 DNA content per cell, by 1.7- and 2-fold, at 48 and 60 hrs, respectively (Fig. 1C).

PGC-1α interacts with nuclear receptors via two leucine rich motifs. Leucine motif 2 (L2) mediates interaction with most nuclear receptors, including ERRα, while motif 3 (L3) recognizes specifically ERRα and the related receptors ERRβ and ERRγ (Huss et al., 2002; Ichida et al., 2002; Schreiber et al., 2003). Mutation of motifs L2 and L3 (L2/3A) disrupts interactions with nuclear receptors, without affecting the interaction domains for other factors like NRF-1 and MEF2C (Knutti and Kralli, 2001; Puigserver and Spiegelman, 2003). 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. 1A-C, the PGC-1α variant L2/3A showed a reduced ability to induce mitochondria, when compared to wild type PGC-1α. The L2/3A PGC-1α was also deficient in inducing the expression of nuclear genes encoding mitochondrial proteins, and of ERRα (Fig. 1D,E). The single L2A mutant, which is defective for interactions with PPARs, GR, and TR but retains interactions with ERRα (Huss et al., 2002; Schreiber et al., 2003; Vega et al., 2000; Wang et al., 2003; Wu et al., 2003), was as active as wt PGC-1 in inducing the expression of target genes (Fig. 1D 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.

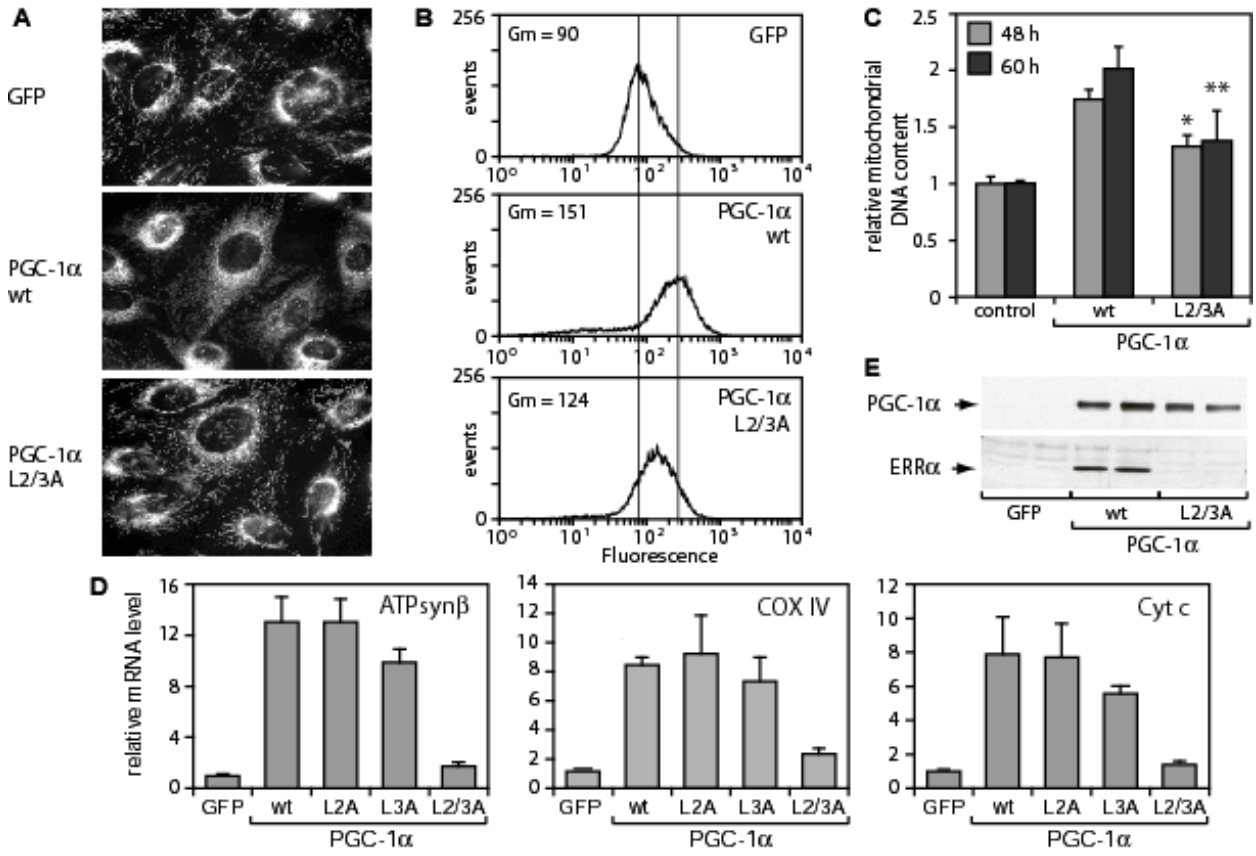

Figure 1. PGC-1α induces mitochondrial biogenesis in SAOS2 cells, dependent on interaction with nuclear receptors.

(A-E) Cells were infected with GFP- or PGC-1α- [wild type (wt) or mutant L2/3A] expressing adenoviruses at an moi of 40. (**A)** Mitochondria in cells labelled 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 mean fluorescence intensity of 20,000 cells. **(C)** Mitochondrial (COX2) DNA levels, normalised to nuclear (β actin) DNA levels, are expressed relative to levels in control cells expressing GFP, which were set to 1, at 48 h and 60 h after infection. Data are the mean ± SEM of three experiments performed in duplicates. *, $p < 0.0001$ vs. PGC-1α wt at 48 h; **, $p < 0.001$ vs. PGC-1α wt at 60 h, as determined by the Students t-test. **(D)** mRNA levels of ATPsynβ, Cyt c and COXIV at 48 h after infection were determined by quantitative RT-PCR, normalised 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.

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 where ERRα expression was inhibited by small interfering RNAs (siRNA). As seen in Fig. 2A, 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 mitochodrial biogenesis and function: mtTFA (mitochondrial DNA replication and transcription), Tim22 (protein import into mitochondria), isocitrate dehydrogenase alpha (IDH3A, TCA cycle), carnitine/acylcarnitine translocase (CACT, fatty acid oxidation), and cytochrome c, somatic and ATP synthase β (Cyt c, synβ, 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. 2B). Since 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. 2A), as well as by other pathways. Inhibition of ERRα did not prevent PGC-1α from inducing GR targets such as p21 (data not shown) nor affect the mRNA levels of the transcription factors NRF-1 and NRF-2 (Fig. 2B).

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. 2C). Inhibition of ERRα had no effect on mitochondrial DNA in the absence of PGC-1α, leading us to conclude that ERRα contributes to the PGC-1α mediated induction, but not the basal expression of genes important in mitochondrial biogenesis.

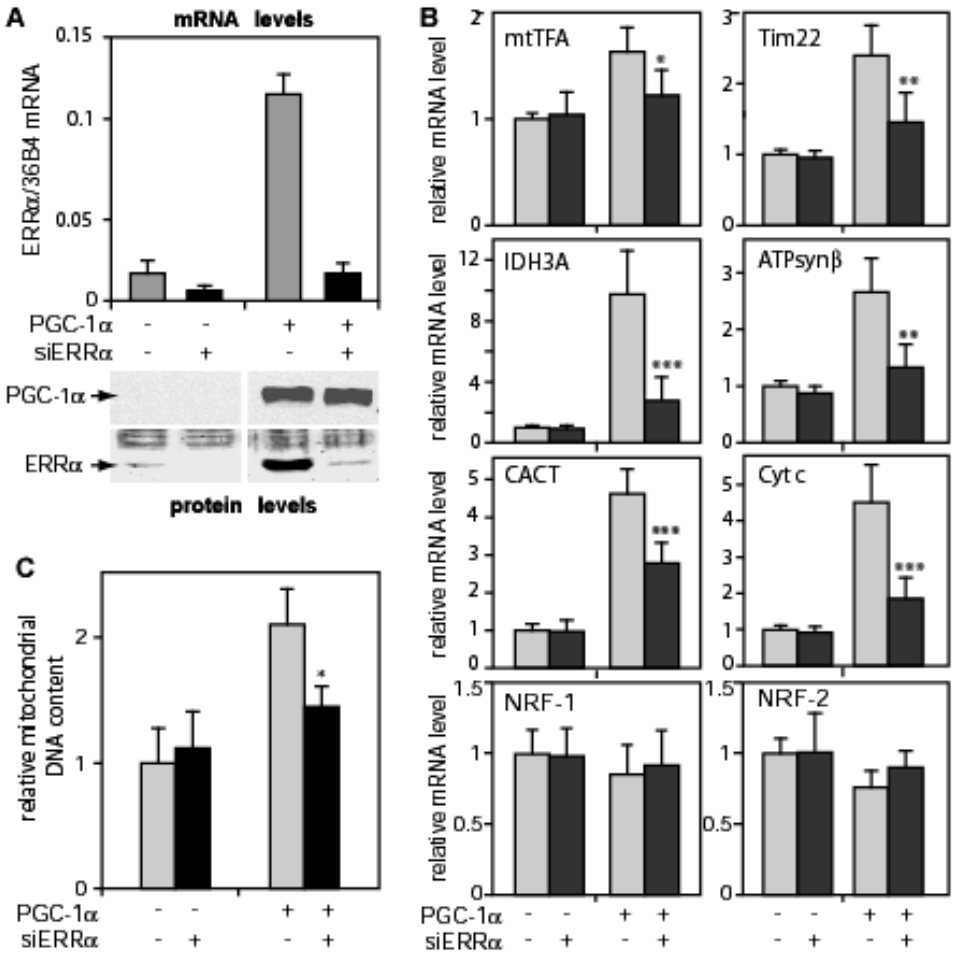

Figure 2. Inhibition of ERRα expression impairs the induction of mitochondrial biogenesis by PGC-1α.

SAOS2 cells were infected with control- (AdSUPER) or an adenovirus expressing siRNA for ERRα (siERRα) at a moi of 100, and two days later with GFP- or PGC-1α- expressing adenoviruses at a moi of 20 **(A,B)** or 40 **(C).** Cells were harvested 24 h (A,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, synβ, carnitine/acylcarnitine translocase (CACT), Cyt c, NRF-1, and NRF-2, were determined by quantitative RT-PCR, normalised 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. *, $p < 0.02$; **, $p < 0.003$; ***, $p < 0.0005$ vs. PGC-1α expressing cells in the absence of siERRα. **(C)** Mitochondrial (COX2) DNA levels were normalised to nuclear (β actin) DNA levels, and expressed relative to levels in control (AdSUPER and GFP) infected cells, which were set to 1. Data are the mean ± SEM of two experiments performed in duplicates. *, $p < 0.008$ vs. PGC-1α expressing cells in the absence of siERRα.

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. 2A), 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 fused to the VP16 activation domain (VP16-ERRα) and, as control, the VP16 activation domain alone, were expressed in SAOS2 cells 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. 3A). VP16-ERRα also led to a significant increase in the amount of cellular mitochondrial DNA (Fig. 3B), indicating that ERRα is capable of inducing mitochondrial biogenesis in the absence of PGC-1α, if activated by other means.

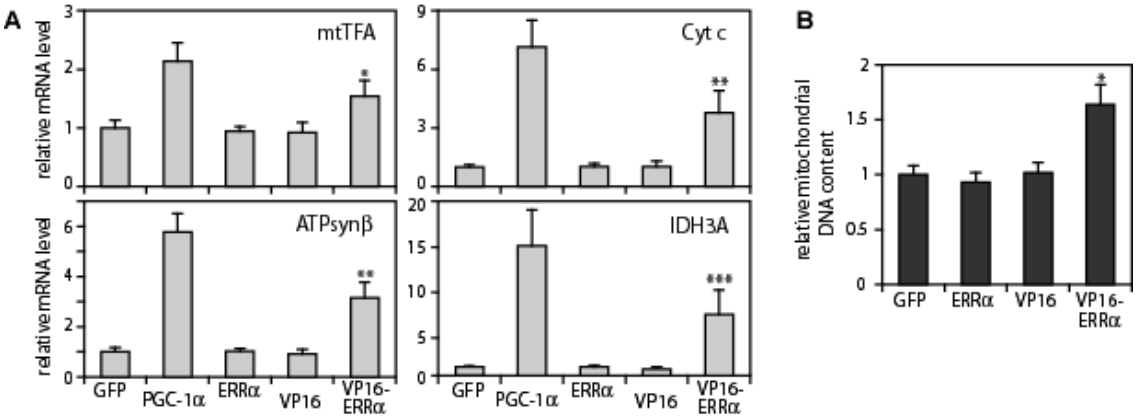

Figure 3. A constitutively active ERRα induces mitochondrial biogenesis.

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 indicates genes were determined by quantitative RT-PCR, as in Fig. 1D. Data are the mean ± SEM of three experiments performed in duplicates. *, $p< 0.002$; **, $p\leq 0.0001$; ***, $p< 0.0004$ vs. GFP infected cells. (**B**) Mitochondrial DNA content was determined as in Fig. 1C. Data are the mean ± SEM of two experiments performed in triplicates. *, $p< 0.0001$ vs. GFP infected cells.

ERRα binds to regulatory sites in the promoters of ATP synthase β and cytochrome 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 (Bonnelye et al., 1997; Johnston et al., 1997; Sladek et al., 1997; Vanacker et al., 1999). 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 (not shown). We focused on ATPsynβ and Cyt c, whose promoters have been studied (Evans and Scarpulla, 1989; Haraguchi et al., 1994; Villena et al., 1994) and where the putative ERREs are within 1 kb of the characterized transcription initiation sites (Fig. 4A). 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. 4B). The complex was inhibited by a 100-fold excess of an oligonucleotide bearing a known ERRE from the TRα promoter (Vanacker et al., 1998), and oligonucleotides representing the candidate ERREs from the Cyt c/-9, ATPsynβ/-338, and IDH3A/-4023, but not by oligonucleotides harboring a mutated TRα ERRE (M4), a random sequence, or another site of the IDH3A gene.

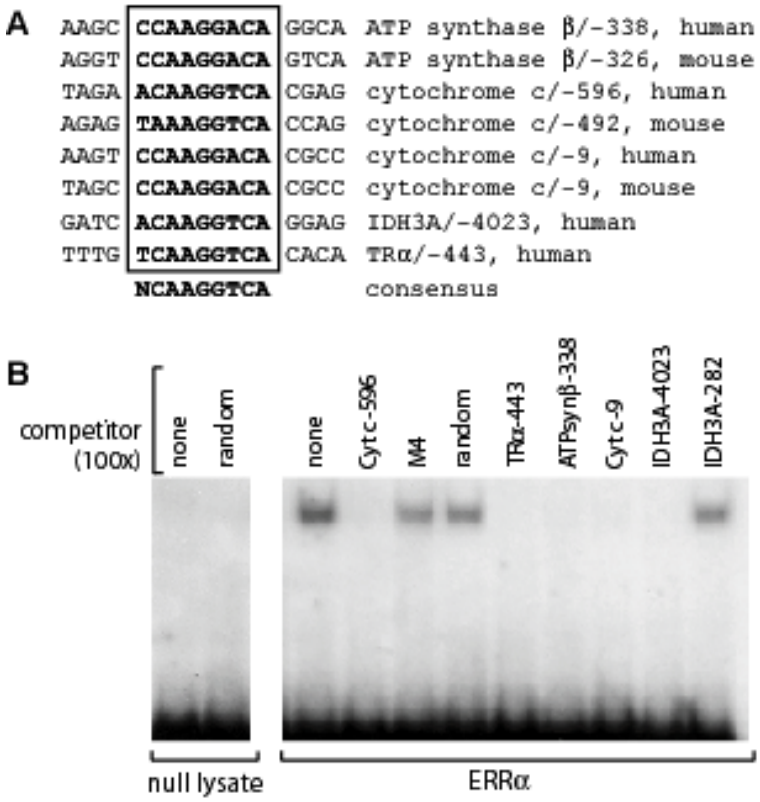

Figure 4. ERRα recognizes sites in ATPsynβ and Cyt c regulatory sequences.

(A) Sequences of candidate ERREs identified by in silico analysis. The TRα.ERRE has been described previously (Vanacker et al., 1998). **(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 two bp subsitution in the core ERRE (Vanacker et al., 1998).

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. Mutations in the ERREs decreased the response to PGC-1α and to PGC-1α/ERRα by 40-50% (Fig. 4C), without affecting the basal levels of expression in the absence of PGC-1α (not shown). The ATPsynβ and Cyt c promoters harbor also binding sites for NRF-2 and NRF-1, respectively (Evans and Scarpulla, 1989; Villena et al., 1994). 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. 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.

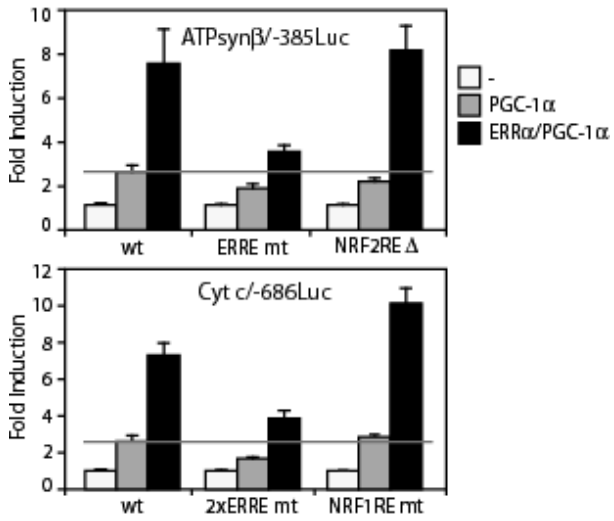

Figure 5. The ERREs of ATPsynβ and Cyt c contribute to the transcriptional response to PGC-1α.

COS7 cells were transfected with reporters pATPsynβ/-385Luc or pCytc/-686Luc, [wild type (wt) and bearing mutations (mt) or deletions (Δ) at the ERREs and NRF-1/-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.

Discussion

PGC-1α has been shown previously to induce mitochondrial biogenesis and oxidative metabolism in muscle cells, adipocytes and cardiomyocytes (Lehman et al., 2000; Puigserver et al., 1998; Wu et al., 1999). 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 (Wu et al., 1999). We now show that PGC-1α expression in SAOS2 cells, osteoblast progenitors with adipocyte differentiation capacity (Diascro et al., 1998), 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 for 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 (Mootha et al., 2003a).

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 (Harper and Himms-Hagen, 2001; Moyes and Hood, 2003). 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 (Moyes and Hood, 2003; Scarpulla, 2002). Since not all genes encoding mitochondrial proteins have binding sites for NRF-1 and NRF-2, additional factors must contribute to the response (Lenka et al., 1998; Scarpulla, 2002). 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 mitochondrial levels rise during brown fat development (Baar et al., 2002; Villena et al., 2002). 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 (Luo et al., 2003). Further studies will be necessary to define mitochondrial defects, and to determine if 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 (Luo et al., 2003). Together with our findings that ERRα is important for the PGC-1α-driven induction of the carnitine/acylcarnitine translocase and MCAD genes (Schreiber et al., 2003), these observations suggest that ERRα function contributes to other PGC-1α-induced pathways, such as fatty acid β-oxidation (Sladek et al., 1997; Vega et al., 2000; Vega and Kelly, 1997). 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 (Ichida et al., 2002).

Mitochondrial dysfunction and in particular decreases in oxidative capacity have been linked to insulin resistance and type 2 diabetes (Bjorntorp et al., 1967; Petersen et al., 2003). 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 (Mootha et al., 2003b; Patti et al., 2003). Supporting this notion, polymorphisms in the PGC-1α gene have been associated to an increased risk of diabetes (Ek et al., 2001; Hara et al., 2002), while mice overexpressing PGC-1β show

increased levels of ERRα and resistance to high-fat induced obesity (Kamei et al., 2003). Strategies aiming in enhancing ERRα activity may thus have therapeutic applications in diseases associated with reduced mitochondrial function, such as diabetes.

Acknowledgements. We thank D. Kressler, M. Meyer, P. Coward, J-M. Vanacker, and J. Mertz for plasmids and antibodies; A. McLachlan and U. Mueller for discussions; the Swiss National Science Foundation, the University of Basel, the Max Cloëtta Foundation, and the Roche Research Foundation for their support.

Supplement 1.

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 1-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 ± 167.6	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 conferring protei	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 ± 248.6	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)	BF978872
	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-glutarate comp	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 hydratase (trif	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 hydratase (trif	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	A1024450.1
	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 1-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 2 of the 3 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 3 experiments and the 1-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 have been classified as "co-regulated with mitochondrial genes" even though they do not encode mitochondrial proteins, [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 1-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	lipin 1	D80010.1
	212274_at	260 ± 23.6	430 ± 25.7	0.014	differentiation	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

Gene	Probe set	Expression , control	Expression , +PGC-1α	Significance	Classification	Description	RefSeq
NRF1	204652_s_at	124 ± 5.5	94 ± 5.5	not determined	transcription	nuclear respiratory factor 1	NM_005011.1
	211279_at	109 ± 5.4	104 ± 6.3	not determined	transcription	nuclear respiratory factor 1	L22454.1
	211280_s_at	56 ± 3.1	57 ± 3.0	not determined	transcription	nuclear respiratory factor 1	L22454.1
GABPB1	204618_s_at	204 ± 16.0	185 ± 12.1	not determined	transcription	GA binding protein transcription factor, beta subunit 1, 53kDa	NM_005254.2
GABPB2	206173_x_at	117 ± 5.6	99 ± 7.0	not determined	transcription	GA binding protein transcription factor, beta subunit 2, 47kDa	NM_002041.2
GABPA	210188_at	70 ± 6.9	107 ± 18.3	not determined	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 1-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

Supplement 2

A. Oligonucleotides used to determine gene-specific mRNA levels by 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
COX II	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 ATPsynB 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**

Chapter III: Supplementary data

Results and discussion

Regulation of mitochondrial biogenesis by PGC-1β

PGC-1β has a similar expression pattern as PGC-1α (Kressler et al., 2002), and has also been reported to induce mitochondrial biogenesis (Meirhaeghe et al., 2003; St-Pierre et al., 2003). Given that our work established a role for ERRα in mediating the PGC-1α effects in mitochondrial biogenesis, we were interested to test if ERRα could be acting also downstream of PGC-1β to increase the mitochondrial content in SAOS2 cells. To answer this question, we first infected SAOS2 cells with adenoviruses expressing GFP or PGC-1β and analyzed the mitochondrial DNA content of the cells (figure 6A). We observed that PGC-1β induced mitochondrial biogenesis as potently as PGC-1α, around 2 fold after 48 and 60 hours.

PGC-1β can interact with and coactivate ERRα (Kressler, unpublished data; (Kamei et al, 2003)). We would therefore expect that PGC-1β, like PGC-1α, induces the expression of endogenous ERRα in SAOS2 cells. As can be seen in figure 6 B, PGC-1β upregulates ERRα mRNA levels strongly. Both basal and PGC-1β-induced expression of ERRα could be suppressed by siRNA specific for ERRα, enabling us to ask whether the induction and expression of ERRα is important for the effects of PGC-1β on mitochondrial functions. First, we looked at the expression of mtTFA, the mitochondrial replication and transcription factor, and isocitrate dehydrogenase 3, a key enzyme of the TCA cycle. As results in figure 6 C show, PGC-1β induces both genes, and this induction depends on endogenous ERRα, as it is not seen when ERRα expression is blocked via siRNA. Nevertheless, when we tested the importance of ERRα on the induction of mitochondrial density by PGC-1β (figure 6 D), we observed only a small, not significant inhibition of the effect of PGC-1β. Although these data are very preliminary and more work should be done, our results suggest

that PGC-1β requires the function of the endogenous ERRα to induce at least some genes important for mitochondrial biogenesis. It seems as though ERRα is not as important for PGC-1β as for PGC-1α for the final increase of the mitochondrial content in the cell, although more careful analysis would be required to conclude this. It would be interesting to test, if the PGC-1α homologues and ERRα act as a complex, or if PGC-1β induces mitochondrial biogenesis under different physiological conditions distinct from PGC-1α. In particular the role of ERRα in PGC-1β signaling should be further studied.

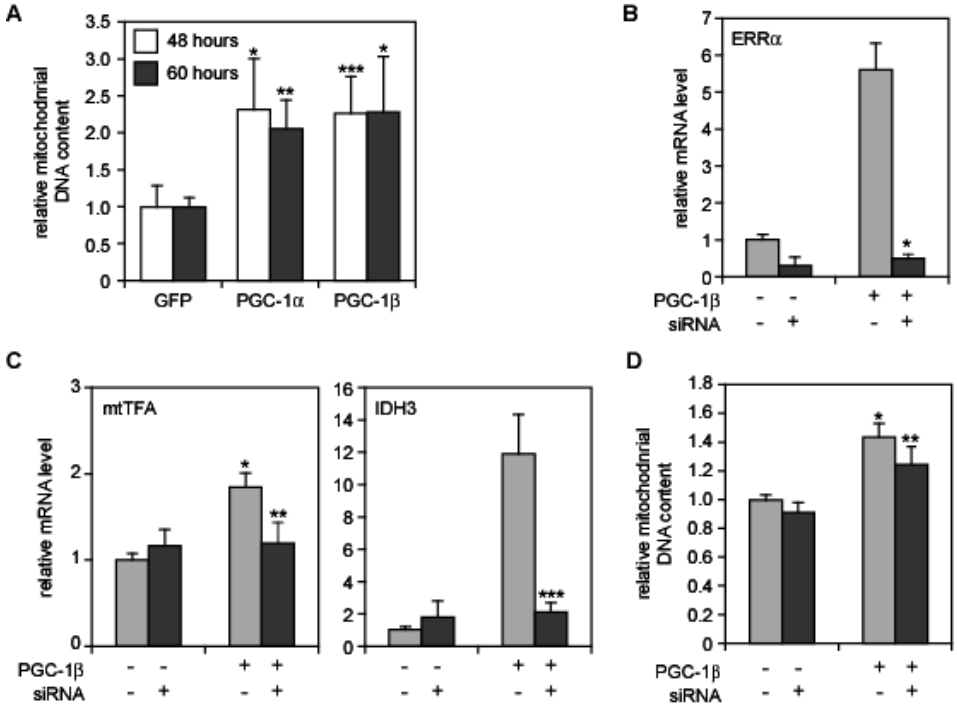

Figure 6. PGC-1β induces mitochondrial biogenesis through ERRα.

(A) SAOS2 cells were infected with adenoviruses expressing GFP, PGC-1α or PGC-1β (MOI40). 48 and 60 hours later, total cellular DNA was isolated and analyzed by quantitative real-time PCR. Levels for COX2 (mitochondrial DNA) were normalized to β-actin (nuclear DNA) levels and expressed relative to the GFP control with was set as one. Equal expression levels for PGC-1α and PGC-1β were controlled by western blot analysis (not shown). Values are the mean ± SEM of two experiments with duplicates. Asterisks indicate that values with PGC-1α or PGC-1β are significantly induced compared to GFP infected cells. *< 0.02, **< 0.003, ***< 0.005. **(B, C, D)** Cells were infected with adenoviruses expressing control (pSUPER) or siRNA for ERRα, and 3 days later re-infected with adenoviruses expressing GFP or PGC-1β at an MOI of 20 **(B,C),** or 40 **(D). (B,C)** RNA was isolated at 24 hours after infection with PGC-1α viruses, and analyzed by real-time RT-PCR. Data shown here represent the mean ± SEM of two experiments with duplicates. The mRNA levels of ERRα, mtTFA and IDH3 were normalized to the levels of 36B4 and expressed as fold induction compared to control (GFP and pSUPER infected cells). Asterisks show that values of the PGC-1β infected cells with siRNA are significantly reduced compared to PGC-1β without siRNA (*< 0.0001, **< 0.005, *< 0.0002) and values for mtTFA are also significantly induced by PGC-1β without siRNA compared to cells without PGC-1β (*< 0.0001), as determined by students t-test. **(D)** DNA was harvested at 60 hours after GFP or PGC-1β expression, and analysed as in A. Data represent mean ± SEM from two experiments with duplicates. Asterisks indicate that values with PGC-1β are significantly induced compared to values without (no siRNA) *< 0.0002; values with PGC-1β and siRNA are not significantly reduced compared to no siRNA. **p= 0.054.

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Chapter IV: Analysis of PGC-1α and GR expression profiles

Results and discussion

PGC-1α is a coactivator that is expressed in an inducible, tissue-specific manner, and that regulates metabolism in response to stressors, such as cold, fasting or exercise. As discussed earlier, PGC-1α exerts its effects via interactions with many different nuclear receptors and other transcription factors like HNF4, PPARγ, GR, NRF1, MEF2C (Knutti et al., 2000; Michael et al., 2001; Puigserver et al., 1998) and ERRα. Furthermore, PGC-1α acts as a strong activator of GR-mediated transcription in transient transfection assays (Knutti et al., 2000). GR is activated by glucocorticoids, whose levels also increase in response to stressors, including cold, fasting or exercise, and is known to contribute to the mobilization of energy during stress. Interestingly, glucocorticoids may regulate the expression of PGC-1α in liver (Yoon et al., 2001), suggesting a complex regulatory interaction. Since the two proteins can be viewed as acting in response to stressors, it seems likely that they collaborate, at least partially, to regulate genes important in stress responses. Our goal was therefore to analyze how PGC-1α and GR would influence each other's effects on gene expression. At the same time, we could analyze how PGC-1α impacts gene expression in collaboration with other transcription factors (i.e. absence of hormone), and identify gene targets for GR, since only a few direct targets were known when this study started. The previous two chapters focused on genes induced by PGC-1α in an ERRα-dependent manner. In this chapter we will discuss the preliminary analysis of expression profiling of genes responsive to PGC-1α and/or GR:

Part 1: Introduction into the experimental settings and the in silico analysis method

Part 2: Genes or programs that are regulated by PGC-1α independent of GR

Part 3: The effect of activated GR on PGC-1α regulated expression profiles

A) genes that are induced by PGC-1α and repressed by GR

B) genes that are activated by PGC-1α in a GR-dependent manner

Part 4: Genes that are regulated by GR independent of PGC-1α

Part 1: Introduction

The cell culture system chosen should fulfill two major requirements: cells should, first, express wild type GR that can be activated by glucocorticoids, and, second, have low or possibly no detectable levels of PGC-1α expression, so that we could compare GR responses in the absence and presence of PGC-1 α. Because many established cell lines express inactive GR, we decided to test SAOS2 cells that stably express GR and that were recently characterized (Rogatsky et al., 1997). Furthermore, the osteoblast progenitor nature of SAOS2 cells was interesting, given that glucocorticoids can induce bone loss. To introduce the coactivator PGC-1α with high efficiency, we constructed adenoviral expression vectors for PGC-1α. These vectors could deliver efficient expression of PGC-1α in ~70 % of the cells, when used at a multiplicity of infection (MOI) of 50 (figure 1 A).

First, we used transient transfection assays to determine if the adenovirally expressed PGC-1α was transcriptionally active. Figure 1 B shows that adenovirally expressed PGC-1α was able to enhance GR activity at a model reporter luciferase construct, by 5- to 6-fold. Next, we analyzed if adenovirally expressed PGC-1α was able to induce endogenous target genes. For a PGC-1α target, we assayed the expression of cytochrome c (Wu et al., 1999). As seen in figure 1 C, cytochrome c was induced by the PGC-1α adenovirus already at an MOI of 5; an MOI of 50 showed a stronger induction. For a GR target, we chose the cell cycle-dependent kinase-inhibitor p21, a known target gene for GR in SAOS2 cells (Rogatsky et al., 1997), and asked how many hours of glucocorticoid treatment were required to detect its induction. Figure 1 D shows that p21 mRNA levels were already increased after ~1 hour of treatment with 50 nM corticosterone, and reached a plateau after 3 hours. Finally, we determined the expression levels of PGC-1α at different times after infection. PGC-1α expression was detectable at 4-6 hours after infection, and reached steady levels at ~ 10 hours (see figure 1B chapter 2). Based on these findings, we decided on a protocol where cells were infected with either control or PGC- 1α expressing viruses (MOI 50) for 14 hrs, and treated with either vehicle (ethanol) or hormone (50 nM

corticosterone in ethanol) for the next 3 hours. At 17 hours after infection, cells were harvested and RNA was prepared.

After RNA isolation of the following four conditions, the expression profiles were analyzed on the Affymetrix U133A arrays:

Cells infected with control βgal adenovirus, treated with vehicle (1)
control βgal adenovirus, treated with glucocorticoids (2)
PGC-1α expressing adenovirus, incubated with vehicle (3)
PGC-1α, incubated with glucocorticoids (4)

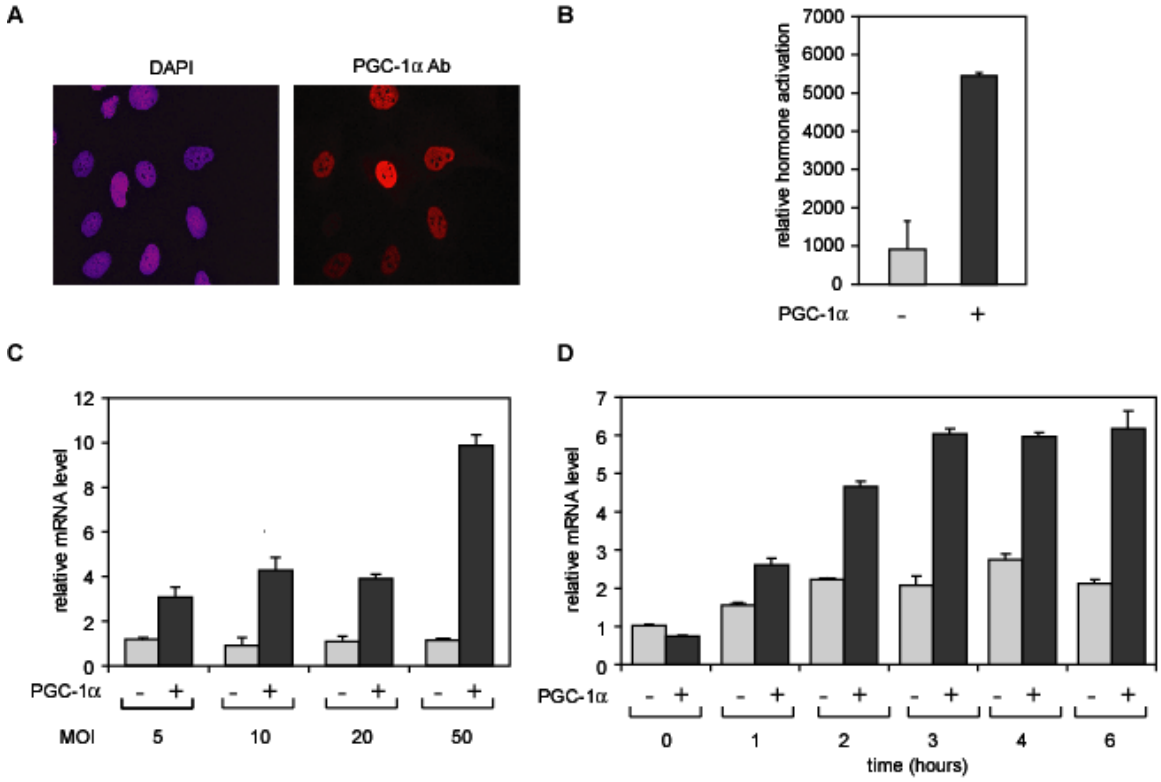


Figure 1. Titration of the experimental settings.

(A) Immunofluorescence staining with a polyclonal antibody against PGC-1α after the infection with an adenovirus expressing PGC-1α a for around 48 hours. Left picture represents a nuclear DAPI staining. **(B)** SAOS2 cells were infected with adenoviruses expressing control (βgal) or PGC-1 α for 48 hours. Transfection of an MMTV-Luc reporter was performed at the same time and then luciferase activity was measured. **(C)** Cells were infected with different amounts of PGC-1α expressing adenovirus for around 48 hours. RNA was harvested and analyzed by quantitative RT-PCR. Cytochrome c mRNA levels were normalized to 36B4 levels and expressed relative to the control levels without PGC-1α and in the absence of glucocorticoids. One representative experiment with duplicates**. (D)** After infection with the PGC1α expessing adenovirus for around 48 hours, cells were treated with 50 nM corticosterole for the indicated amount of time. Isolated RNA was analyzed by quantitative RT-PCR and values for p21 were normalized to levels of 36B4 and expressed as fold of the control. Data show one experiment with duplicates. The same results were obtained with another GR target gene.

To determine the genes that were differentially regulated from one condition to the other, we used the Microarray Suite program v5 (Affymetrix). Comparison of the genes expressed under the different conditions led us to generate four groups of lists, each with a list of up- and a list of down-regulated genes (figure 2). These lists served as the starting point for the subsequent analysis. In the following chapters, we will present some first validations of these results and furthermore discuss some functional groups with respect on their relevance for PGC-1α or GR signaling, followed by the discussion of selective interesting examples of genes that were regulated on the gene chips (**examples marked in bold**). At the current state of the analysis, we have verified that PGC-1α regulates the program of mitochondrial biogenesis, and the expression of many 'mitochondrial genes' in an ERRα -dependent manner (Chapter 3). Furthermore, we have verified by real-time PCR the expresion levels of 23 additional genes that are regulated by PGC-1α and/or GR.

The human U133A array contains approximately 22,500 probe sets, representing around 14,500 well-characterized genes or 18,400 transcripts and variants. As the numbers suggest, some genes are represented more than once on the gene chip array. Only a small percentage of the genes are represented several times, whereas most genes appear only once on the U133A chip. Due to this fact, in the subsequent analysis I will use the terms 'probe set', when genes represented multiple times have not been eliminated, and "genes", when the actual number of genes has been determined. Our experience from the analyzed lists suggests that the number of regulated genes is around 10 to 20 % less than the number of probe sets.

Several different layers of statistical analysis have been incorporated into the analysis. Besides the cutoff lines set by the Affymetrix software, the data was normalized with the robust multichip analysis (Irizarry et al., 2003), and only genes that were consistently regulated in all three experiments were taken into account. Notably, the three repeats of the experiments were done on different days, as far as more than 6 months from each other. Further statistical analysis was provided using the 1-way anova test, and setting a threshold of significance of 0.05. All values above were designated as "not significantly regulated". While the use of this cutoff has reduced the large number of genes to be analyzed, it has also excluded some genes that fell

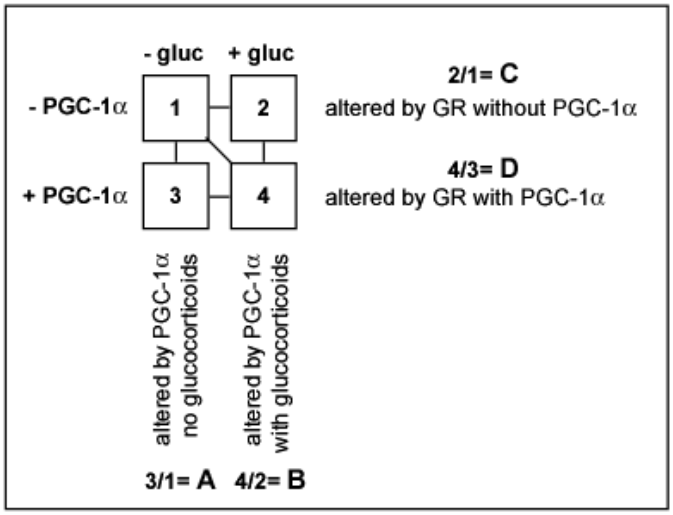
below the significance level, using these stringent conditions, but were found to be significantly regulated by quantitative RT-PCR analysis. In the following sections, only "significantly regulated" genes will be presented or discussed.

Figure 2B presents the numbers of probe sets that were differentially regulated by PGC-1α and GR, classified into four groups. Two points appeared to be interesting at first glance:

First, more probe sets were induced by PGC-1α than by GR. This could be explained by the fact that PGC-1α exerts its transcriptional coactivator function with several different endogenous nuclear receptors and other transcription factors. Furthermore, PGC-1α expression could be detected after around 4-6 hours (figure 1 B chapter 2). Around eleven hours of PGC-1α expression could possibly lead to indirect effects on the expression of some genes. The conditions for GR were more stringent (3 hrs of activation) and could have led to fewer indirect effects.

The second point was that a smaller percentage of probe sets was significantly repressed by PGC-1α alone compared to with GR alone (group A and C). Whereas PGC-1α a repressed significantly 40 % of the probe sets in the absence of GR, the latter repressed 74 % significantly. These findings indicate that the role of PGC-1α in transcriptional repression is smaller than in activation. This observation is supported by the absence of any data for PGC-1α as a negative regulator of transcription.
Even more striking is the situation if both factors are present and activated (group B and D). PGC-1α only represses 6 out of 340 probe sets significantly, but GR represses 47 % significantly. This clearly demonstrates that PGC-1α does not exert a function in GR mediated repression and even influences GR signaling positively, since a lower percentage of GR targets were significantly repressed in the presence of PGC-1α.

A The comparison of the 4 different conditions as base for our analysis



B All probe sets that were regulated in the four different groups

		all	significantly changed
Group A	PGC-1 induced no glucocorticoids	673	547
	PGC-1 repressed no glucocorticoids	401	162
Group B	PGC-1 induced with glucocorticoids	624	490
	PGC-1 repressed with glucocorticoids	340	6
Group C	GR induced no PGC-1α	236	198
	GR repressed no PGC-1α	156	116
Group D	GR induced with PGC-1α	391	323
	GR repressed with PGC-1α	203	95

Figure 2. Transcriptional profiles of PGC-1α and GR in SAOS2 cells

(A) The comparison of genes expressed in the different conditions, using the Microarray Suite program, led to four groups of probe sets, each with two lists: one of up- and one of down-regulated genes. **(B)** Numbers of probe sets that were regulated, in each of the 8 lists. The "significantly" regulated probe sets were determined using the 1-way anova test and a cut-off value of 0.05.

Part 2: Genes that are regulated by PGC-1α in the absence of glucocorticoids

PGC-1α exerts its functions in metabolism by interacting with several different nuclear receptors and other transcripton factors (reviewed in Knutti and Kralli, 2001; Puigserver and Spiegelman, 2003). Importantly, PGC-1α is expressed at significant levels in some tissues, such as heart and muscle, in the absence of any stress, i.e. at a state when glucocorticoid levels are low (Knutti and Kralli, 2001; Puigserver and Spiegelman, 2003). It is therefore expected that many of the effects of PGC-1α on gene expression are GR-independent. Our first goal was therefore to identify genes and pathways that were regulated by PGC-1α in the absence of any glucocorticoids.

Classification of all genes significantly induced by PGC-1α

The classification of all genes induced significantly by PGC-1α in the absence of glucocorticoids shows an interesting picture (figure 3). As discussed in chapter 3, around 28 % of all induced genes are nuclear encoded with a function in mitochondrial biogenesis. Assuming that there are around 700 to 800 genes with functions in mitochondria, as proposed recently by Mootha and coworkers (Mootha et al., 2003), ~23-26 % of all 'mitochondrial genes' are induced by PGC-1α, already at 17 hours after infection with the PGC-1α adenovirus. Since this function of PGC-1α was already discussed extensively, the main focus of this chapter will be on the genes that have no predicted function in mitochondrial biogenesis.

As can be seen in figure 3 and table 1 of the Appendix I, all PGC-1α induced genes were classified into functional groups. Interestingly, the second largest group of genes seems to be involved in cell signaling processes, such as cytoskeletal organization, cell growth and viability, and others. Followed by this, there is a group of genes involved in protein homeostasis, with roles in protein synthesis, transport, and ubiquitination, and, next, a group of genes encoding transcriptional regulators, including ERRα. The wide variety of genes regulated by PGC-1α suggests that PGC-1α expression influences many cellular processes; some of them will be discussed in the following paragraphs.

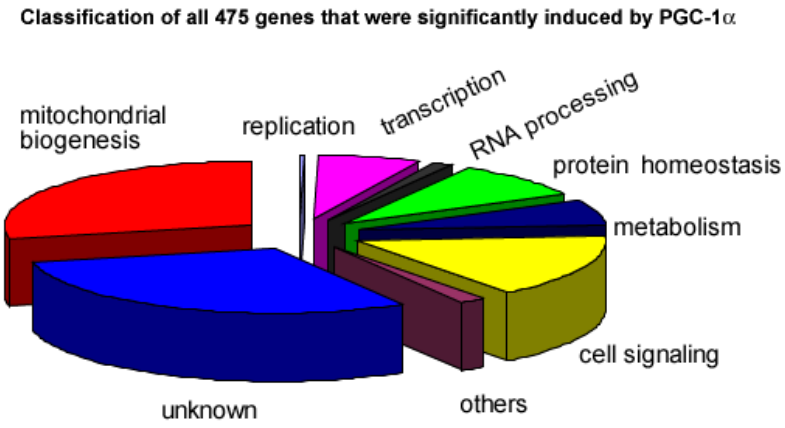


Figure 3. Classification of all genes that were significantly induced by PGC-1α in the absence of glucocorticoids (475 genes, represented by 547 probe sets).

The graph illustrates the portion of each functional group. The most prominent function was chosen for the classification. Cell signaling includes: cytoskeleton organization, cell adhesion, apoptosis, cell cycle, differentiation, immune response and signaling pathways. Protein homeostasis includes protein synthesis, posttranslational modification, protein folding, trafficking and ubiquitination. The group of metabolism includes genes involved in energy homeostasis, oxidative stress, lipid, cholesterol, nucleotide metabolism and some others. (The list of the gene, together with their classification, can be found in table 1 of the Appendix I)

RNA processing

Monsalve and coworkers have proposed a function for PGC-1α in the regulation of RNA processing (Monsalve et al., 2000). In their study, PGC-1α was able to interact with different splicing factors, and influence the splicing pattern of a fibronectin minigene, presumably as a result of the interaction between the C-terminus of PGC-1α (RS and RRM domains) with the splicing factors. Interestingly, PGC-1α expression in SAOS2 cells led to the induction of six proteins involved in mRNA splicing (one **cdk-like kinase** known to affect splicing, **two RNA-binding motif factors**, a **homologue of a drosophila splicing factor** and more importantly, a **component of the spliceosome**), as well as **ribonuclease P**, which is involved in tRNA splicing, Although we have not tested yet the significance of these factors, these observations raise the possibility that the reported effect of PGC-1α on splicing are due not to direct interactions with splicing regulators, but rather to indirect effects of PGC-1α on the expression of some splicing components. It would be interesting to test if PGC-1α influences splicing in general or perhaps only the processing of some specific mRNAs.

Transcription

Several transcription factors and coregulators were induced by PGC-1α, suggesting that some of the genes regulated in the arrays may be indirect, rather than direct targets of PGC-1α.

EAR-2 (erb A related 2, NR2F6) is a member of the orphan nuclear receptor family that was identified as TRα -related gene in 1988 (Miyajima et al., 1988). The function of EAR-2 is not fully understood, though it seems to act as a transcriptional repressor. High expression levels in the liver have suggested a role in the repression of apolipoproteins, lipid transporters with an important role in lipid and cholesterol metabolism (Ladias et al., 1992; Vorgia et al., 1998). Other studies have suggested a role for EAR-2 together with COUP-TFII in the repression of the estrogen activity on the oxytocin gene promoter (Chu et al., 1998; Chu and Zingg, 1997). Interestingly,

ERRα and EAR-2 were identified in the same study to both regulate aromatase, the key enzyme of estrogen synthesis, albeit in opposite directions (Chen et al., 2001; Yang et al., 2002; Yang et al., 1998).

Interestingly, we also detected the regulation of other orphan nuclear receptors by PGC-1α, such as **LXRβ** and **LRH-1**, two receptors involved in the regulation of lipid metabolism. Furthermore, PGC-1α expression led to the repression of COUP-TF II, a nuclear receptor with repressor activities, (Pereira, 2000) and RXRα, the common heterodimer partner for many nuclear receptors (reviewed in Rastinejad, 2001). Since not all of these genes were regulated significantly, they will not be discussed further, though, it could be important to verify their regulation by PGC-1α, and to test a potential role of PGC-1α in the regulation of lipid metabolism.

SRC-1 was the first nuclear receptor coactivator identified (Kamei et al., 1996; Onate et al., 1995) and belongs to the class of histone modifying coactivators. Early studies showed the assembly of SRC-1 together with PGC-1α and CBP (Puigserver et al., 1999) in a complex that exerts HAT activity. Later studies by Surapureddi et al (Surapureddi et al., 2002) confirmed these data by the identification of a putative PPARα -interacting cofactor complex (PRIC) from rat liver, which contained CBP, SRC-1, PGC-1α and others. Besides SRC-1, several other coactivators were induced by PGC-1α, for example **TRAP80**, a member of the mediator complex, which was also proposed to interact with PGC-1α (Surapureddi et al., 2002; Wallberg et al., 2003). Interestingly, the regulation of these genes by PGC-1α suggests a much higher complexity in transcriptional regulation than expected. It could imply that increases in levels of PGC-1α in response to specific metabolic signals alter the expression of specific coactivators, as well as recruit them to PGC- 1α complexes. Such complex mechanisms may explain the potency with which PGC-1α induces specific pathways.

Cell signaling

Our early studies, before the gene expression profiling experiments, had already suggested that PGC-1α expression in SAOS2 cells influences cell shape, cytoskeleton organization and possibly cell growth or viability. As can be seen in figure 4, both the actin (A) and the tubulin staining (B) change dramatically after PGC-1α expression for around 48 hours. Interestingly, one of the PGC-1α repressed genes was a member of the tubulin family, suggesting that changes in the pattern of cytoskeletal proteins expressed could underlie the observed changes in cell shape.

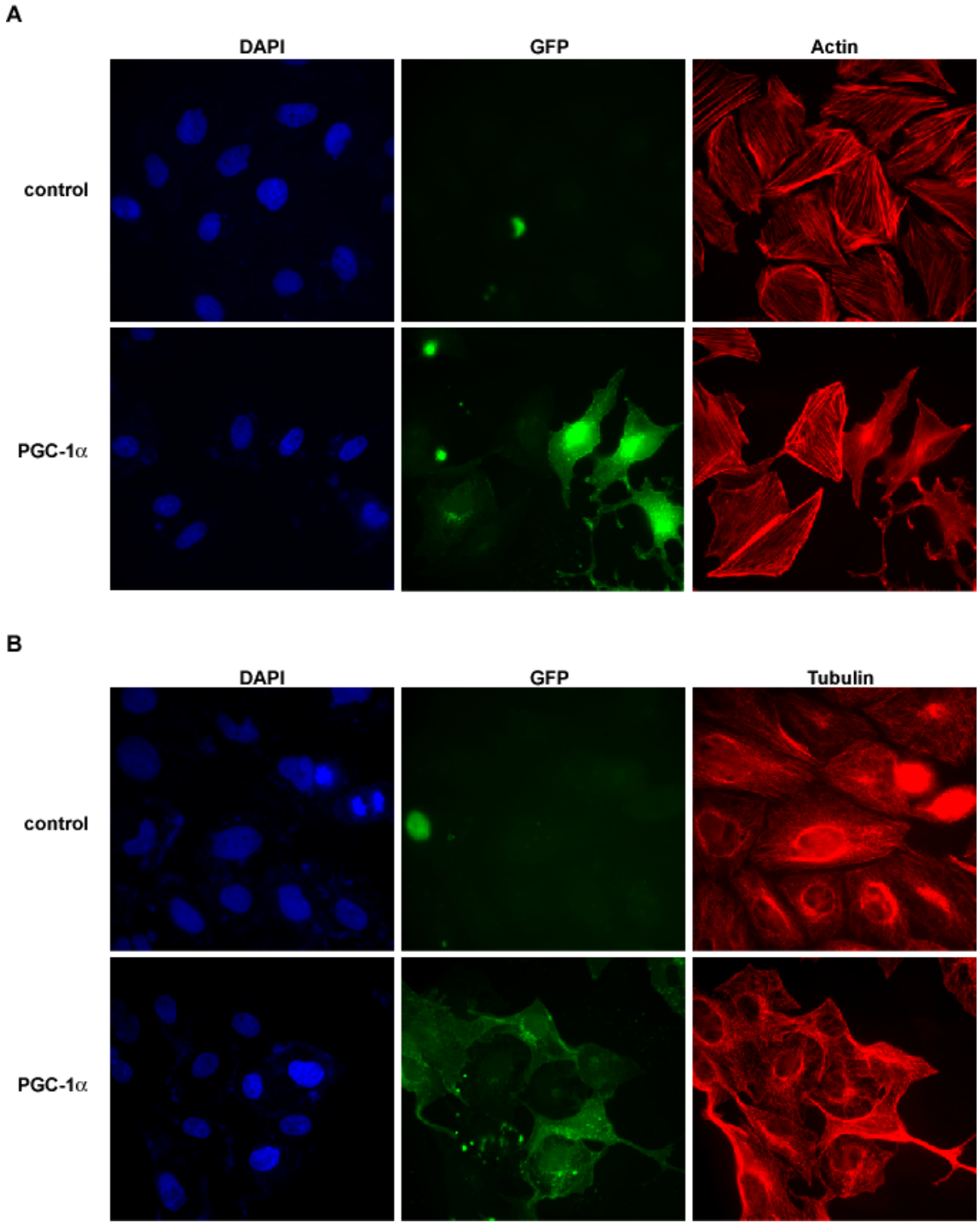


Figure 4. PGC-1α expression changes the morphology of SAOS2 cells.

SAOS2 cells were infected with an adenovirus expressing PGC-1α or the control βgal for around 48 hours (MOI 50). The GFP expressing cells represent PGC-1α positive cells, since PGC-1α and GFP were expressed from the same bicistronic message of this vector. **(A)** Actin staining. **(B)** Tubulin staining.

The role of PGC-1α in differentiation. The regulation of many genes involved in cell signaling, cytoskeleton organization, cell growth, but also the influcence of PGC-1α on the cell shape, suggested to us that PGC-1α might alter the differentiation state of the cells. SAOS2 cells are osteoprogenitor cells that posses the ability to differentiate into osteoblasts or adipocytes, (Diascro et al., 1998). While ERRα has been shown to promote bone formation and induce osteopontin, ERRα expression also parallels adipocyte differentiation and leads to the induction of the β-fatty acid oxidation enzyme MCAD (Bonnelye et al., 2001; Bonnelye et al., 1997; Sladek et al., 1997; Vanacker et al., 1998; Vega and Kelly, 1997). Interestingly, neither osteopontin, nor alkaline phosphatase, osteocalcin, collagens or other classical osteoblast markers were upregulated by PGC-1α. However, these may be genes induced in later stages of development. PGC-1α did induce the expression of **C/EBPδ**, a transcription factor carrying roles in both adipocyte and osteoblast differentiation (Guerra et al., 1994; Gutierrez et al., 2002; Lane et al., 1999).

Three more differentiation factors, **BMP-2**, **VEGF** and **lipin** were upregulated by PGC-1α. BMP-2 induces the differentiation of mesenchymal cells into osteoblasts, chondrocytes or adipocytes (Ahrens et al., 1993). VEGF was identified as a BMP-2 target and could also be involved in myoblast, osteoblast or adipocyte differentiation (Claffey et al., 1992; Marrony et al., 2003; Midy and Plouet, 1994). Finally, lipin was suggested as an important factor for adipocyte differentiation, and mutations in its gene cause lipodistrophy (Peterfy, 2001). However, it has also been identified as a marker of muscle atrophy in fasted mice and rats with renal failure, cancer or diabetes (Lecker et al., 2004).

The induction of these factors by PGC-1α could imply that SAOS2 cells differentiate into adipocytes, rather than osteoblasts. This would be consistent with the induction of other genes in these cells, such as the ones encoding enzymes of the fatty acid oxidation pathway. However, it is not possible at this state of analysis to tell which, if any, differentiation program is induced by PGC-1α. Differentiation studies over a longer period of time, and possibly in different cell types, could give some insight into the role of PGC-1α and possibly ERR α in driving differentiation programs.

Metabolism

Oxidative stress. ROS (reactive oxygen species) are byproducts of several metabolic reactions in the cell and primarily in mitochondria. An excess of ROS in mitochondria is able to cause oxidative stress. To prevent the oxidative damage of cellular machineries and in particular of the DNA, cells have developed a complex antioxidant defense system consisting of superoxide dismutases and glutathione transferring enzymes. PGC-1α, a potent regulator of mitochondrial biogenesis has been proposed to be involved in the cellular response to oxidative stress. The significant induction of three glutathione transferases, as seen in our gene chip experiment, and of SOD2 (verified by real time RT-PCR), is consistent with this theory. Interestingly, at 17 hours after infection with the PGC-1α adenovirus, cells have not yet made new mitochondria. Thus, the enhancement of enzymes fighting oxidative stress seems to fit a program induced by PGC-1α in preparation for the oxidative stress that is expected from the increased mitochondrial activity at later times, rather than as a response to the upregulated oxidative phosphorylation and oxidative stress.

Glucose catabolism in SAOS2 cells. During fasting, in the fasting hyperglycemia situation of diabetes type II and during physiological exercise, glycogen serves as the major energy storage for the body. By the time glycogen reserves are exhausted, the body has to seek for new alternatives. Non-carbohydrates like lipids, provided from adipocytes and mainly muscle proteins are catabolized and glycerol, lactate and amino acids can be used for gluconeogenesis in the liver, which is regulated by PGC-1α (Yoon et al., 2001). Peripheral tissues as adipocytes, bone or muscle are not gluconeogenic, and therefore depend on glycogenolysis, uptake of glucose from the circulation, and glycolysis.

The following metabolic enzymes were induced in our experiments (see figure 5):

1) We detected an increase in the **amylo-1, 6-glucosidase, 4-α-glucanotransferase**, the glycogen debranching enzyme, which is activated during glycogenolysis.

2) An increase in **phosphoglucomutase**, could convert the resulting glucose 1-phosphate into glucose 6-phosphate, thereby making it accessible for glycolysis.

3) Furthermore, we saw an upregulation of **phosphofructokinase**, the key enzymatic step in glycolysis.

4) The glycerol 3-phosphate dehydrogenase reaction in glycolysis forms $NADH+H^+$, which is not able to enter the mitochondria to be oxidized by the respiratory chain. Therefore, upregulation of the **malate-aspartate shuttle** tries to overcome this by reducing oxaloactetate to malate and transporting the latter into the mitochondrion. There, malate is oxidized back to oxaloacetate, "producing" one $NADH+H^+$, which can then be used by the respiratory chain.

In conclusion, assuming that the increased expression of genes also leads to an increased production of the enzymes described here, PGC-1α seems to induce catabolic pathways in the SAOS2 cells, by activating glycogenolysis and glycolysis. These processes could provide the necessary substrates for entry into the mitochondrial oxidative pathways, and the production of ATP.

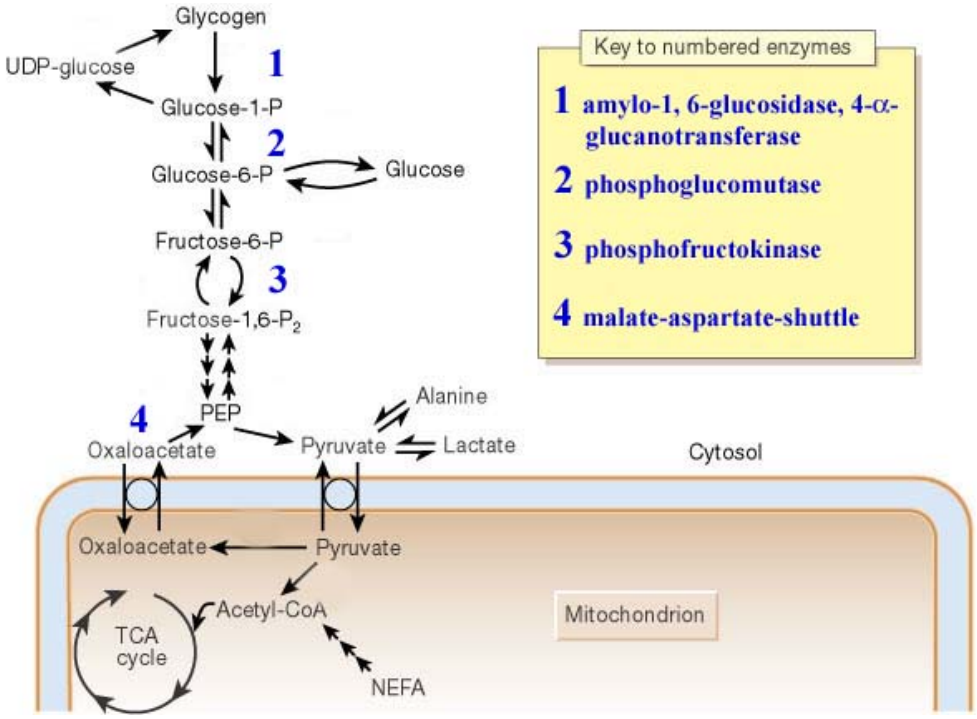


Figure 5. PGC-1α influences glycogen breakdown and glycolysis in SAOS2 cells.

Catabolic glucose pathway, numbers indicate proteins that were induced in our gene chip experiments. (Adapted from Moller, 2001).

Part 3: Genes regulated by PGC-1α and glucocorticoids

GR exerts at least two types of effects on stress responses: 1) suppressive functions that either protect the cells from a metabolic overshoot or help prepare for new stress responses, and 2) permissive and stimulating effects that are part of the stress response (reviewed in Sapolsky et al., 2000). Based on this, we decided to analyze:
A) if any of the PGC-1α induced genes in the absence of glucoorticoids were inhibited by glucocorticoids, suggesting a suppressive function for GR in PGC-1α signaling, and B) genes whose induction was dependent on both PGC-1α and glucocorticoids

A) Genes that were induced by PGC-1α but repressed by GR

24 probe sets (21 genes) were significantly induced by PGC-1α and repressed by GR (figure 6). To examine the possible programs that could be induced by PGC-1α and repressed by GR, we classified the genes according to their proposed functions (Table 2 of Appendix I).

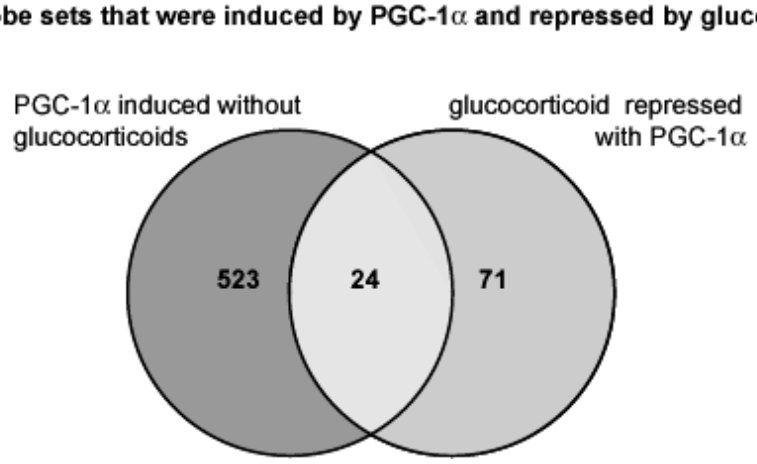


Figure 6. Venn Diagrams were genereated using GeneSpring. 24 probe sets were significantly induced by PGC-1α but repressed by GR.

Strikingly, only 3 genes with mitochondrial function were repressed by GR, suggesting that GR does not play a role in mitochondrial biogenesis in our system, as will be discussed later. A few of the other genes could be classified into one functional group, the BMP-2 signaling pathway: **BMP-2**, **VEGF** and **G0/G1**. The TGFβ superfamily member BMP-2 and VEGF, and their possible function in osteoblast or adipocyte differentiation were discussed before. The putative lymphocyte G0/G1 switch gene is highly expressed in adipocytes and stimulated by BMP-2 in late adipogenic differentiation (Bachner et al., 1998; Russell and Forsdyke, 1991). Our data could suggest that PGC-1α induces a differentiation program in SAOS2 cells, but GR inhibits it. This is of course speculative, since it is not clear at this point if BMP-2 induces adipocyte or osteoblast differentiation in SAOS2 cells.

Metabolism

GR suppresses the metabolic function of PGC-1α in SAOS2 cells in two ways. Phosphoenolpyruvate carboxykinase 1 (**PEPCK1**) is known as a key enzyme for gluconeogenesis in liver and kidney. In adipocytes, PEPCK is important not for gluconeogenesis, but rather for glyceroneogenesis, which leads to the reesterification of fatty acids and to lipid synthesis (reviewed in Hanson and Reshef, 2003). Glyceroneogensis seems to be essential for storing the fatty acids, when they are not required for energy. PGC-1α induced the expression of PEPCK1, suggesting that it could promote glyceroneogenesis in our cell system. GR repressed PEPCK1, as it has been shown before to do in adipocytes, in vivo and in vitro, and consistent with its role in promoting lipolysis and inhibiting lipid synthesis in adipose tissue (Nechushtan et al., 1987). Interestingly, this regulation of PEPCK1 would further suggest that the SAOS2 cells display adipocyte-like characteristics.

An interesting question arises, which transcription factor mediates the PGC-1α upregulation of PEPCK1. In liver, HNF4, GR and FOXO1 have been implicated in the PGC-1α induction of PEPCK1 (Herzig et al., 2001; Yoon et al., 2001). In adipocytes, PPARγ has been proposed to play an important role in the regulation of PEPCK1 expression (Tontonoz et al., 1995). Furthermore, PPARγ is a major regulator

of adipocyte differentiation and was proposed to be upregulated by BMP-2 (Grimaldi, 2001; Hata et al., 2003). Although, PPARγ expression was not altered in our experiments profiles, it could be activated by fatty acids and coactivated by PGC-1α in our cell system.

Pyruvate dehydrogenase kinase 4 (**PDK4**) was induced, not repressed by glucocorticoids. However, because this regulation may fit the suppressive effects of glucocorticoids on PGC-1α induced pathways, it is discussed here. PDK4 inhibits the activity of the pyruvate dehydrogenase complex by phosphorylation and prevents pyruvate from entering the TCA cycle. The induction of PDK4 by GR has been reported earlier. Interestingly, it seems to be dependent on binding to FOXO1, a factor also shown to be required for PGC-1α and HNF4 regulation of PEPCK1 in liver (Furuyama et al., 2003; Puigserver et al., 2003). As figure 7 A shows, the expression values of the gene chips suggested that the induction of PDK4 was stimulated by PGC-1α. However, analysis of the mRNA levels by quantitative real-time PCR in figure 7 B suggests no role for PGC-1α. Further analysis is necessary to determine if PGC-1α coactivates GR on the PDK4 promoter. The physiological implications of the PDK4 regulation by GR is that it would block the entry of energy substrates (whose levels would be increased by PGC-1α -induced glycogenolysis and glycolysis), into the TCA cycle and the respiratory chain. This suppressive effect on the PGC-1α induced program could be to protect cells from an overload with energy in form of ATP, or, quite likely, to divert energy substrates away from the cell's oxidative system and back to the liver, where they could be used for gluconeogenesis.

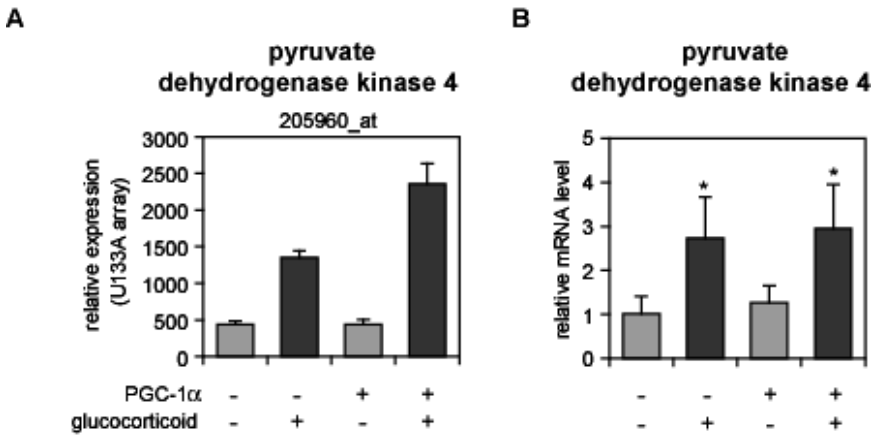


Figure 7. Pyruvate dehydrogenase kinase 4 is induced by GR

(A) Expression values represent the mean ± SEM from the three experiments analyzed on the U133A chip. **(B)** SAOS2 cells were infected with adenoviruses expressing βgal or PGC-1α (MOI 50). At 14 hours, cells were treated with 50 nM of corticosterone or vehicle for 3 hours. RNA was then isolated and analyzed by real-time RT-PCR. The mRNA levels for pyruvate dehydrogenase 4 were normalized to the mRNA levels of 36B4 and expressed relative to the βgal infected cells treated with vehicle. Results shown are the mean ± SEM of 4 experiments with one value. Asterisks indicate that values with and without PGC-1α are significantly induced by glucocorticoids, *< 0.02

B) Genes that were induced by PGC-1α dependent on GR

We compared the PGC-1α -induced probe sets, in the absence and presence of glucocorticoids. As we show in figure 8 A, many new genes appeared to be regulated by PGC-1α in the presence of hormone. In addition, more than half of the PGC-1α induced probe sets in the absence of glucocorticoids were also regulated in their presence. This represents a group (319 probe sets) that seems to be induced independently of GR (although, as exception, 6 of these probe sets may also be responsive to glucocorticoids). More interestingly, 171 probe sets responded to PGC-1α only in the presence of glucocorticoids. As this represented around a third of all PGC-1α upregulated genes in both conditions, it would suggest an important function for GR in PGC-1α signaling. To obtain a subgroup of these probe sets that were induced significantly by both PGC-1α and GR, we compared the lists of probe sets induced in response to PGC-1α in presence of glucocorticoids, and GR-induced genes in the presence of PGC-1α (figure 8 B). This revealed a consistent group of 55 probe sets (49 genes) that were induced by PGC-1α and GR (shown as Table 3 in Appendix I). Strikingly, this represented only a small subgoup of the 171 probe sets that were induced by PGC-1α only in the presence of glucocorticoids. We do not exclude that the rest are not dependent on both PGC-1α and GR. Experimental validation of members from the different groups will be required to gain some insights on the significance of the regulated genes. Our current analysis focused on the group of the 55 probe sets.

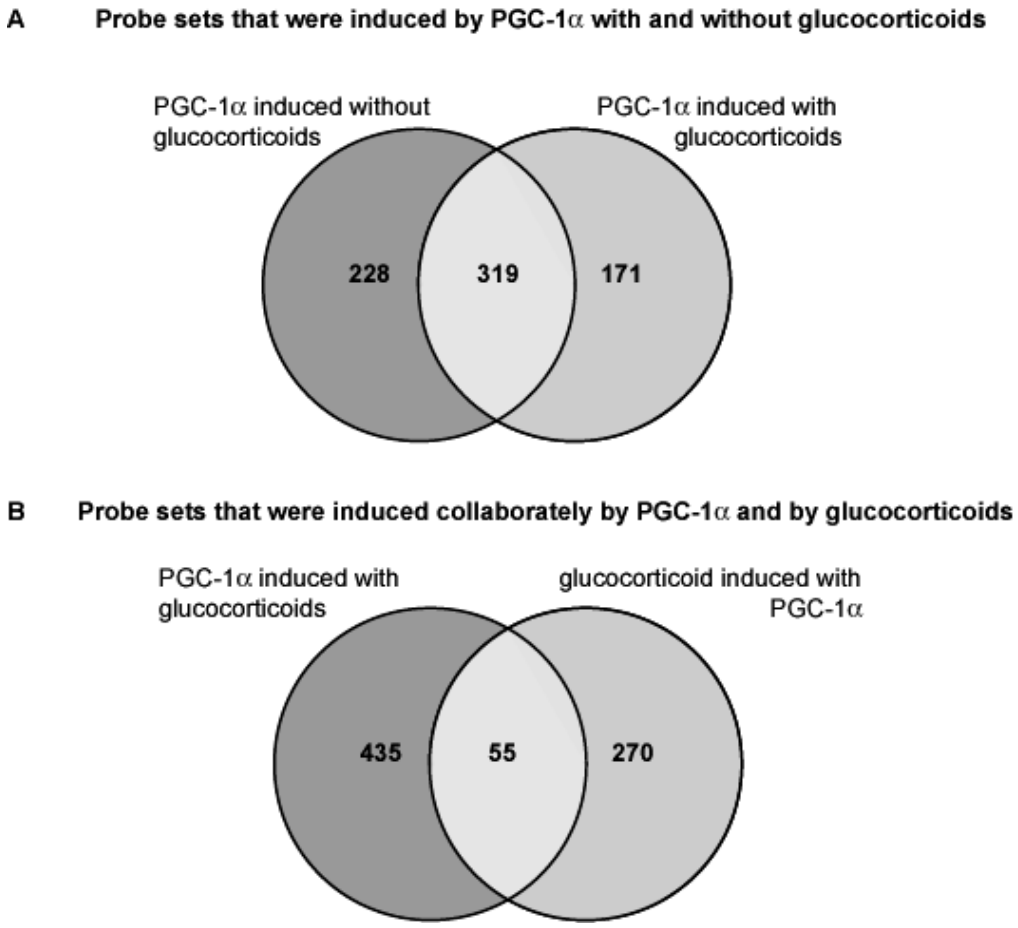


Figure 8. Comparison of the probe sets that were regulated by PGC-1α and GR. (**A**) GR activation shifts the PGC-1α responsive probe sets. (**B**) Overlap of PGC-1α and GR induced genes.

To validate some of our findings, we analyzed the mRNA levels of three examples by quantitative real-time PCR. Figure 9 A and B shows the U133A expression values next to the results of the quantitative real-time PCR for Bardet Biedl syndrome 1 (BBS1), solute carrier 19 member 2 (thiamine transporter) and interleukin receptor 22 (IL22R). As could be observed, all three were upregulated by GR, with an additional effect of PGC-1α.

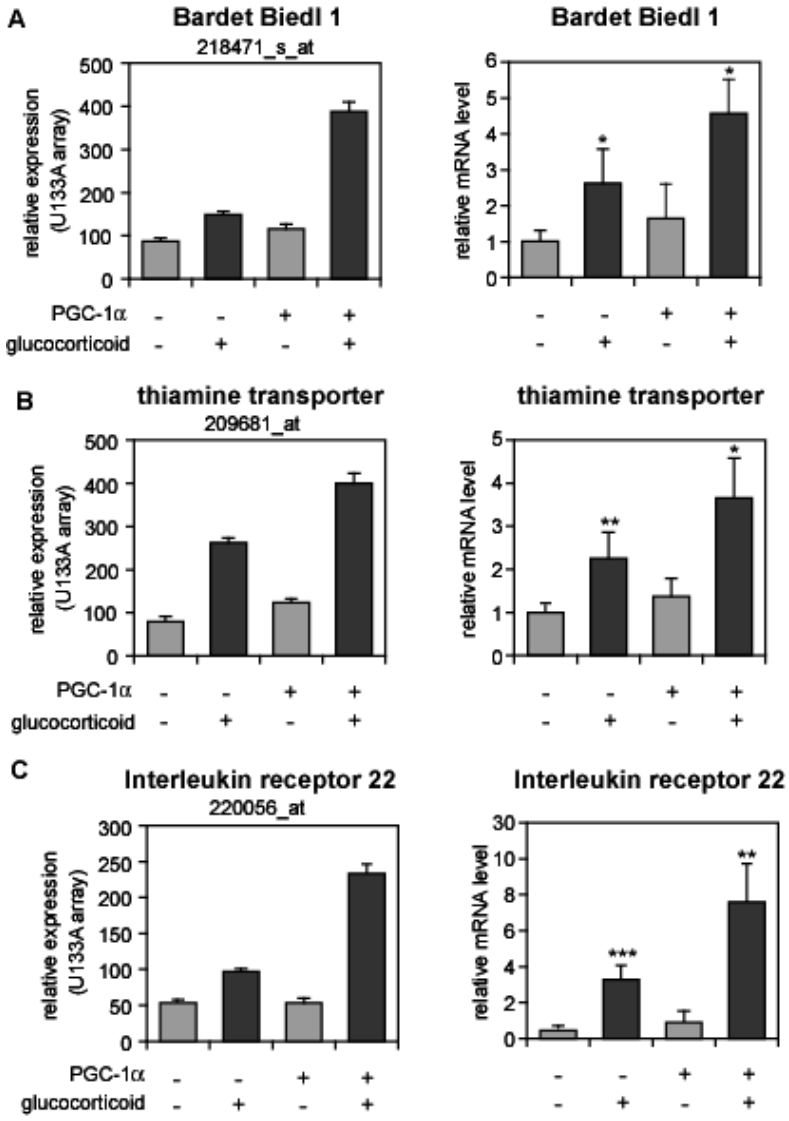


Figure 9. Genes that are regulated by PGC-1α and GR

(A) Values represent the mean ± SEM of three different expression profile experiments with the human 133A chips. **(B)** Cells were infected with either the controls (βgal) or PGC-1α expressing adenovirus for 14 hours. Then, cells were treated with glucocorticoids for 3 additional hours and RNA was isolated. The mRNA levels were analyzed by quantitative PCR. Values for Bardet Biedl syndrome 1, the thiamine transporter and interleukin receptor 22 were normalized to the levels of 36B4 and expressed relative to the βgal control and vehicle. Results represent the mean ± SEM of four to five different experiments. Asterisks indicate that values with glucocorticoids and without PGC-1α were significantly induced (*< 0.05, ** < 0.006, *** < 0.0001) or values with PGC-1α and glucocorticoids showed a significant enhancement to the ones without PGC-1α (* < 0.05, ** < 0.006).

These results showed that both GR and PGC-1α regulate the expression of these genes. We cannot yet tell if this is due to direct regulation by PGC-1α and GR. Further studies are necessary to determine if GR binds directly the promoters of IL22R, thiamine transporter and BBS1 and if PGC-1α coactivates GR on these genes.

Classification of genes in this group according to proposed functions, implied a regulation of the cell cycle and cell viability by GR and PGC-1α, since we detected an induction of the cycle inhibitor p21, which was already identified as GR target in SAOS2 cells and used as a control for the experimental design ((Rogatsky et al., 1997), figure 1), and a few more genes involved in cell cycle arrest or apoptosis (Appendix I table 3). We also observed the induction of transcriptional regulators, signaling molecules and several other cellular pathways. More experiments are necessary to verify these preliminary data. In the following, some examples will be discussed.

Metabolism

PGC-1α and GR regulate together important metabolic factors. Bardet Biedl syndrome (BBS) is a heterogeneous, autosomal recessive disorder, which is characterised by mental retardation, obesity, hypogonadism, short stature, retinitis pigmentosa and polydactyly, and secondary disorders like diabetes, hypertension and renal and cardiac anomalies (reviewed in Katsanis et al., 2001). At least five gene loci seem to be involved in the manifestation of this syndrome, but the most important was allocated to **BBS1**. BBS4 could play a role in signaling, and BBS6 seems to act as molecular chaperone; the function of BBS1 is not yet known. Interestingly, not only BBS1 was induced in our experiments, but also **BBS6**, also known as McKusick-Kaufman gene (MKKS), which was significantly upregulated by PGC-1α with some GR responsiveness (see Appendix I table 1 and 3 respectively).

Humans lack biosynthesis pathways for several micro-nutrients like thiamine, also known as vitamine B1. Uptake through nutrients and in particular the transport of those vitamins via the **thiamine transporter** are essential. The active form of

thiamine serves as cofactor for several metabolic enzymes involved in carbohydrate catabolism (reviewed in Schorken and Sprenger, 1998), the biosynthesis of neurotransmitters, and the production of reducing equivalents used in the oxidative stress defenses (Gibson and Zhang, 2002). For example, thiamine is essential for the pyruvate dehydrogenase complex, the key enzyme in glucose metabolism that provides acetyl-CoA and NADH for the TCA cycle. Defects in the thiamine transporter THTR1 lead to diabetes mellitus, sensoneural deafness, cardiovascular diseases, neurosensory defects and defects in the haematopoietic system (Beri-beri, Wernicke encephalopathy (Singleton and Martin, 2001)).

We have verified the regulation of BBS1 and the thiamine transporter by PGC-1α and GR, as seen in figure 9. It remains to be further analyzed if GR directly binds to the promoters of those genes and what could be the function of BBS1. Regulation of these target genes supports previous findings that defects in PGC-1α signaling, but also prolonged glucocorticoid levels, could be involved in insulin-resistance, hyperglycaemia and diabetes (Ek et al., 2001; Hammarstedt et al., 2003; Hara et al., 2002; Lane et al., 1999; Rosmond, 2002).

Signaling

GR possibly regulates PGC-1α activity. The MAP kinases p38 and the stress-activated protein kinase/c-Jun N-terminal kinases (SAPK/JNK) are activated by stress generators such as toxins, proinflammatory cytokines and also by reactive oxygen species (ROS) during oxidative stress. Work of our and other labs have shown that the activity of PGC-1α is induced through p38 phosphorylation, in response to cytokines, and through the displacement of a repressor (Fan et al., 2004; Knutti et al., 2001; Puigserver et al., 2001). PGC-1α and GR induced the kinase MAP3K7, better known as **TAK1**. Strikingly, TAK1 is the upstream MAP kinase for MKK6, which in turn phosphorylates and activates p38 (Moriguchi et al., 1996). These data would suggest a feedback mechanism of PGC-1α for its own activation and more importantly, another mechanism by which GR could regulate PGC-1α activity.

Although MAP kinases show some specificity, they exert influence on many different functions in cell signaling. TAK1 was also shown to act in response to the PGC-1α target BMP-2, a growth factor involved in differentiation and discussed before (Shibuya et al., 1998). However, the fact that GR repressed BMP-2 but induced TAK1, would suggest that TAK1 does not respond to BMP-2 in this system. Promoter binding studies with TAK1 and phosphorylation studies with PGC-1α in the absence and presence of glucocorticoids could provide support for this theory.

PGC-1α regulates GR through a negative feedback mechanism. "Prereceptor" or "intracrine" regulatory mechanisms have been described for several steroid hormones and involve target tissue activation or inactivation of the circulating hormone. Glucocorticoid metabolism is regulated by two isoenzymes of 11ß-hydroxysteroid dehydrogenase 11ß-HSD (Stewart and Krozowski, 1999). The type 1 isozyme (11ß-HSD1) is involved in the generation of the active glucocorticoid, cortisol, in tissues with high GR expression like liver, gonads or adipose tissue (Tannin et al., 1991). In contrast, 11ß-HSD2 converts active cortisol to inactive cortisone. In tissues with high MR expression, like kidney and colon, 11ß-HSD2 acts to protect the MR from cortisol, which binds to GR and MR (Albiston et al., 1995). Deficiency of 11ß-HSD2 leads to hyperactive MR, natrium retention and hypertension (reviewed in Stewart, 1999; Wilson et al., 2001). Surprisingly, studies have also suggested a high expression for 11ß-HSD2 in osteoblast cells (Bland et al., 1999; Eyre et al., 2001), not known to have functional MR, suggesting an alternative role for this enzyme in the regulation of bone homeostasis or cell proliferation. Experiments revealed insensitivity to glucocorticoids in cells that show a high 11ß-HSD2 activity (Bland et al., 1999; Eyre et al., 2001). This suggests an important role for 11ß-HSD2 in modulating ligand availability for GR. The regulation of 11ß-HSD2 by GR and PGC-1α implies an interesting, up to date unidentified negative feedback regulation of GR activity. Interestingly, the expression levels were not altered in the presence of one factor alone, but induced around 1.8 fold in the presence of both. This could imply an essential function for PGC-1α in this regulation. Further studies should address whether GR acts on 11ß-HSD2 directly, and the requirement of PGC-1α instead of other coactivators.

PGC-1α and GR influence the expression of the vitamin D receptor. Vitamin D receptor (**VDR**) functions as a ligand-induced nuclear receptor regulating the expression of genes involved not only in the control of calcium homeostasis, phosphate homeostasis and bone remodeling, but also in immunomodulation, inhibition of cell growth and induction of cell differentiation (Lin and White, 2004). Polymorphisms in the VDR gene, besides the ERα gene, were proposed by many studies as candidates in contributing to osteoporosis in menopausal women. Strikingly, osteoporosis appears as a negative side effect of long-term glucocorticoid treatment in patients with autoimmune diseases. In our cell system, VDR was one of the GR targets, where PGC-1α was definitively required for the induction. Interestingly, the cytochrome P450 enzyme (**CYP24A1**), which inactivates cycling vitamine D, was significantly upregulated by PGC-1α and repressed by GR. Although many other examples that were discussed before implied a non-osteoblast phenotype for the SAOS2 cells and VDR does not exclusively function in bone, the regulation of the VDR could imply a role for PGC-1α as a coactivator for GR in the bone. Consistent with this J. Miner and his group recently identified a new synthetic compound, which activates GR and reduces the ability to interact with PGC-1α, but still allows to interact with other coactivators (Coghlan et al., 2003). Interestingly, this compound showed similar antiinflamatory effects as glucocorticoids, but less side effects in bone.

Part 4: Genes that were regulated by GR independent of PGC-1α

Our data suggested that PGC-1α and GR could exert some collaborative functions in SAOS2 cells. However, not all GR -induced genes were regulated by PGC-1α. This led us to further analyze the GR induced expression profiles. Figure 10 shows that the expression of PGC-1α dramatically influenced the expression profiles of GR. Interestingly, three times more probe sets were induced by GR only in the presence than only in the absence of PGC-1α, supporting the idea that PGC-1α plays an important role in the regulation of GR activity. Still, 139 probe sets were significantly induced irrespective of PGC-1α expression. We focused on this group of genes in our first analysis. As can be noted in table 4 of the Appendix I, assignment of proposed functions to this group of induced genes suggests an effect of glucocorticoids on many cellular processes, including effects on a large number of transcriptional regulators.

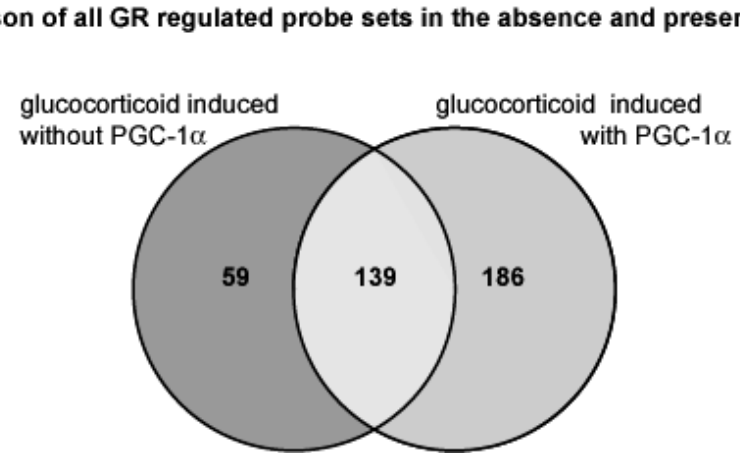


Figure 10. Ven Diagragram compares all probe sets significantly induced by GR, in the absence and presence of PGC-1α.

Furthermore, only three probe sets were induced by GR independent on PGC-1α and significantly repressed by latter. One example will be discussed later.

To verify that the regulation of some of these genes does not depend on PGC-1α, we analyzed the mRNA levels of two examples: lipase protein and zinc finger protein 145. Lipase is a protein with unknown function, but sequence analysis revealed some similarity with acetyltransferases, which could suggest a function in metabolism. The zinc finger protein 145 or promyelocytic leukemia zinc finger (PLZF) protein gene is disrupted in therapy-resistant acute promyelocytic leukemia (APL) (Chen et al., 1993). This Kruppel-like transcription factor seems to be involved in apoptosis, growth suppression (Shaknovich et al., 1998) and differentiation of different tissues, for example in adipocyte differentiation (Sekiya, 2004). As seen on the U133A expression values and the mRNA levels tested by quantitative RT-PCR in figure 11, both genes responded to glucocorticoids, whereas expression of PGC-1α did not show any effect.

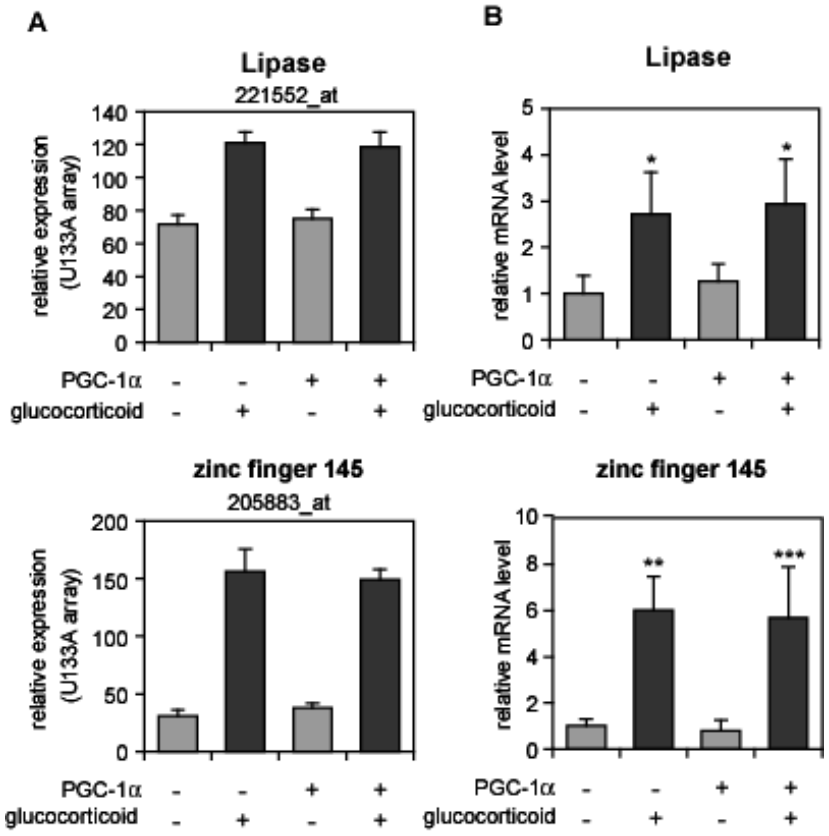


Figure 11. Genes induced by GR independent of PGC-1α

(A) Affymetrix U133A expression values represent the mean ± SEM from three experiments. **(B)** SAOS2 cells were infected with adenoviruses expressing βgal or PGC-1α (MOI 50) for 14 hours, followed by a 3 hour treatment with 50 nM of corticosterone or vehicle. Then, RNA was isolated and analyzed by quantitiative RT-PCR. The mRNA levels for lipase and the zinc finger protein 145 were normalized to the mRNA levels of 36B4 and expressed relative to the βgal infected cells treated with vehicle. Results shown are the mean ± SEM from 4 experiments with one value. As the asterisks show, were the values with glucocorticoids significantly induced to the values without. *< 0.02, ** 0.0004, *** < 0.005

Role of GR in mitochondrial biogenesis. Since early results suggested a regulation of mitochondrial biogenesis by glucocorticoids, several studies have provided support for these findings (Allan et al., 1983; Wakat and Haynes, 1977). Characteristics of long-term stress situations are enhanced catabolism of muscle protein and stimulation of hepatic gluconeogenesis. Eventually, insulin resistance followed by diabetes and increased energy expenditure can occur. Several studies have shown an increased expression of genes with functions in mitochondria after glucocorticoid treatment in liver (Kadowaki and Kitagawa, 1988; Morris and Kepka-Lenhart, 2002), colon (Rachamim et al., 1995) and in muscle (Weber et al., 2002). In contrary, the decrease of several mitochondrial enzymes was observed after glucocorticoid treatment in kidney and pancreas (Fabregat et al., 1999; Simon et al., 1998). Finally some evidence exists for the localization of GR in mitochondria (Demonacos et al., 1995; Demonacos et al., 1993; Demonacos et al., 1996; Moutsatsou et al., 2001; Tsiriyotis et al., 1997) or for the association with the mitochondrial outer membrane (Koufali et al., 2003). However, the mechanism of this glucocorticoid action, is still not clear, since no regulation of the mitochondrial transcription and replication factor mtTFA could be seen (Weber et al., 2002).

Based on the parallel activation of PGC-1α and GR by some stressors, the role of both proteins in the regulation of energy pathways, and the ability of PGC-1α to regulate GR activity, it appeared possible that PGC-1α and GR regulate mitochondrial biogenesis coordinately. However, the analysis of glucocorticoid induced or repressed genes revealed only a small percentage of genes with mitochondrial function. This does not rule out the potential collaboration between GR and PGC-1α in mitochondrial biogenesis completely, since glucocorticoids may function in this program in a cell-type specific manner, and not in SAOS2 cells. Recent results suggested an important role for the regulation of mitochondrial biogenesis by glucococrticoids in muscle (Weber et al., 2002). It would be therefore interesting to establish a muscle cell system to study the role of GR/PGC-1α in mitochondrial biogenesis.

Transcription

GR might affect DNA repair through the regulation of forhead box transcription factor 3A. In the following, one example will be discussed, which is one of the three genes that were induced by GR but repressed by PGC-1α.

Forkhead box O 3A (**FOXO3A**) was represented by four probe sets on the U133A chip, and three of them were significantly induced in all three experiments by glucocorticoids. PGC-1α significantly repressed FOXO3A expression in the absence of hormone, whereas it did not show an effect in the presence. FOXO transcription factors are regulated by protein kinase B in response to insulin signaling (Kops et al., 1999). Studies have implicated a role for them in the regulation of cell cycle and cell viability (Dijkers et al., 2000a; Dijkers et al., 2000b; Medema et al., 2000). Insulin activates PI3kinase, which in response activates PKB by phosphorylation. The subsequent phosphorylation of FOXO3A by PKB leads to the shuttling of FOXO3A out of the nucleus, and to its inactivation (Brunet et al., 2002). FOXO3A possibly regulates cyclin-dependent-kinase inhibitor p27 expression through the regulation of ubiquitin-mediated degradation and causes cell cycle arrest in the G1 phase (Medema et al., 2000). Since the insulin-PI3K pathway seems to be involved in the regulation of life span in Caenorhabditis elegans (Lin et al., 1997; Ogg et al., 1997), Greenberg and colleagues were wondering if a similar mechanism could be identified in mammals. Interestingly, their studies suggested a FOXO3A dependent induction of G2-M cell cycle arrest and DNA repair (Tran et al., 2002). Their DNA microarray analysis identified two factors that were induced by FOXO3A and involved in DNA repair, **GADD45** and **PA26**. Strikingly, both of these genes were also induced by GR, independent of PGC-1α in our experiments.

PGC-1α influenced the cell morphology of SAOS2 cells (figure 4), and also induced several genes involved in the induction of cell cycle arrest and apoptosis. Some factors, which generate cell cycle arrest, like p21, were induced by both PGC-1α and GR. However, whereas PGC-1α overexpression seems to induce stress to the cells, GR might counteract this effect and tries to protect the cells from DNA damage through the regulation of FOXO3A.

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Chapter V: Discussion

Numerous different coactivators and coactivator complexes have been identified to modulate nuclear receptor signaling. Increasing amount of evidence gained with many studies in the last years have proposed that coactivators define the specificity of transcriptional regulation in a promoter- and tissue-specific manner. The goal of my thesis was to analyze if the tissue-specific and signal-inducible coactivator PGC-1α would provide specificity to GR signaling and the signaling of other nuclear receptors. We designed a cellular model system with either undetectable levels of PGC-1α or overexpressed PGC-1α, and with inactive or actived GR. Our results gave us new information about the role of the orphan nuclear receptor ERR α in PGC-1α signaling. Furthermore, we asked if PGC-1α would be able to redirect GR signaling to a specific subset of genes, thereby altering the specificity of gluccocorticoid signaling.

Part 1: The function of ERRα in PGC-1α signaling

PGC-1α regulates the orphan nuclear receptor ERRα.

The identification of ligands for orphan nuclear receptors in the last years opened a new view on the regulation of metabolism by nuclear receptors, which can function as direct metabolite sensors, and respond immediately and specifically to the metabolic requirement of the cell (reviewed in (Giguere, 1999). Still, some receptors like ERRα remain 'orphan'. ERRα expression is high in tissues with high β-fatty acid oxidation and seems to regulate a key enzyme in this program, MCAD (Luo et al., 1997; Sladek et al., 1997; Vega and Kelly, 1997). Interestingly, ERRα knockout mice show reduced adipose tissue and the altered expression of several enzymes involved in lipid metabolism (Luo et al., 2003). We demonstrated that the expression of ERRα paralleledd the specific expression of PGC-1α, a sensor and integrator of metabolic signals, in tissues with high energy demands (Schreiber et al., 2003). Moreover, our data suggested that PGC-1α is important for ERα function, by regulating both its expression and activity, thereby serving as a protein ligand for this orphan receptor.

Finally, we have shown that PGC-1α induces the expression of ERRα through an autoregulatory loop, in which low levels of ERRα enable PGC-1α to induce the ERRα promoter.

PGC-1α as a regulator of several orphan nuclear receptors.

Our expression analysis revealed that PGC-1α also induced other orphan nuclear receptors, like LXRβ and LRH-1, which seem to play an important role in the regulation of lipid metabolism (Francis et al., 2003; Repa and Mangelsdorf, 2000) and EAR-2 (erb A like 2), a less known family member with proposed functions in lipid metabolism and estrogen signalling (Ladias et al., 1992; Yang et al., 1998). Strikingly, besides ERRα, also other orphan nuclear receptors like LXRα or RAR are regulated through autoregulatory loops (Laffitte et al., 2003; Leroy et al., 1991; Li et al., 2002). Interestingly, PGC-1α was recently reported to induce FXR expression in liver, in response to fasting (Zhang et al., 2004). PGC-1α coactivates PPARγ or HNF4 at the FXR promoter, leading to enhanced FXR levels and the regulation of lipid metabolism. Our findings, together with these recent studies, suggest that PGC-1α may serve as a regulator of several orphan nuclear receptors, orchestrating the regulation of metabolism in response to metabolic demands.

PGC-1α regulates mitochondrial biogenesis in collaboration with ERRα.

Mitochondrial biogenesis describes the processes required for the formation and homeostasis of mitochondria. The efficiency of mitochondrial respiration is under the strict control of physiological activity, temperature changes, hormones, growth factors, cell cycle or developmental aspects (Moyes and Hood, 2003; Scarpulla, 2002). One of the key factors controlling the rapid adaptation to enhanced respiratory demands seems to be PGC-1α, which is regulated by exercise, fasting or thermogenic processes (reviewed in Knutti and Kralli, 2001; Puigserver and Spiegelman, 2003). Ectopic expression of PGC-1α led to the induction of mitochondrial biogenesis in muscle, heart and adipose tissue. In adipocytes and perhaps myocytes, PGC-1α seems to regulate the expression of genes encoding mitochondrial proteins, especially the

mitochondrial replication and transcription factor mtTFA through the induction and interaction with NRF-1 and NRF-2 (Baar et al., 2002; Wu et al., 1999). In SAOS2 cells, we observed that the increase in the mitochondrial content by PGC-1α was highly dependent on ERRα, and that NRF-1 and NRF-2 levels did not change. Our results suggest that ERRα does not influence the basic, constitutive activity of mitochondria, which could be exerted by NRF-1, NRF-2, Sp1, YY1, CREB, MEF-2 proteins and others that have been proposed before (reviewed Scarpulla, 2002). In contrast, PGC-1α modulates mitochondrial activity in collaboration with ERRα, possibly in response to physiological signals like exposure to cold or fasting. Our findings are supported by an integrative genomic and proteomic approach by Mootha and coworkers, who recently identified ERRα as a transcription factor that is coregulated with mitochondrial biogenesis (Mootha et al., 2003). Furthermore, ERRα knockout mice show reduced cytochrome c levels (Luo et al., 2003). Since we propose that ERRα regulates the mitochondrial content of the cell in response to metabolic requirements, it would be interesting to analyze the response of ERRα-deficient mice to exercise or cold, and determine the effects on mitochondrial functions. Experiments with isolated primary tissues like adipose cells or muscle could also test if PGC-1α is still able to induce mitochondrial biogenesis in ERRα-deficient cells.

The possible role for ERRγ in PGC-1α regulated pathways.

Interestingly, PGC-1α has also been shown to coactivate the other two members of the ERR family, ERRβ and ERRγ (Hentschke et al., 2002; Huss et al., 2002; Kamei et al., 2003) Although ERRβ is mainly expressd during early embryonic development with only low levels in the adult, ERRγ seems likely to be coexrpessed with ERRα in several tissues (Bonnelye et al., 1997b; Heard et al., 2000; Hong et al., 1999; Sladek et al., 1997; Susens et al., 2000). All three family members show high similarities in the DBD and seem capable of binding the same response elements; still they may exert different specificities in the regulation of target promoters, as for example SHP (Sanyal et al., 2002). Our lab furthermore observed that all three receptors bind to the same interaction surface in PGC-1α (Knutti, unpublished). These data suggest some

potentially overlapping functions for ERRα, ERRβ and ERRγ. Some differences must exist, since the ERRβ knockout mouse is embryonic lethal (Luo et al., 1997), while disruption of ERRα leads to viable mice (Luo et al., 2003).

Interestingly, ERRα mice displayed decreased ERRβ expression, which could suggest the regulation of ERRβ by ERRα. Recent studies also proposed a regulation of ERRα by ERRγ or vice versa, since the interaction of both family members *in vitro* inhibited their transcriptional activity (Huppunen and Aarnisalo, 2004). Although this interaction remains to be verified *in vivo,* these observations suggest that some regulatory network exists between ERRα, ERRβ and ERRγ. We did not detect any ERRγ in our system, suggesting that even if ERRγ could compensate for the loss of ERRα function, this would not be evident in the SAOS2 cells. Analysis of ERRγ overexpression or disruption by siRNA and the effects on mitochondrial biogenesis in another cell line could address the question of the involvement of ERRγ in ERRα pathways, and in particular mitochondrial biogenesis. At least four scenarios seem possible: (1) ERRα and ERRγ could bind as heterodimers to the promoters of mitochondrial genes and play a role in PGC-1α pathways. (2) ERRγ could replace ERRα and mediates PGC-1α programs in different tissues or in response to different physiological requirements. (3) ERRγ regulates ERRα activity through competition for binding sites or by direct protein-protein interaction, or by binding to the ERRα promoter. (4) ERRγ cannot replace ERRα; instead it mediates distinct PGC-1α functions.

As observed before, ERRα is able to not only activate, but also inhibit transcription (Sladek et al., 1997; Zuo and Mertz, 1995). Our experiments with the ERRα promoter suggested a dependency on the concentration: in low amounts ERRα activates transcription from its own promoter, and at high levels it represses it. The effect of the cellular concentration on ERRα function was already described for the interaction with ERα (Kraus et al., 2002). Interestingly, overexpression studies have proposed that ERRα acts as a repressor of PGC-1α function on the PEPCK promoter (Ichida et al., 2002). Given our findings that ERRα can activate and repress at the same time the ERRα promoter, it seems important that any conclusion on the role of ERRα at any promoter are based on loss of function, as well as gain of function experiments. Our

findings demonstrate that ERRα functions are complex, and imply that ERRα function may depends on the cell type, promoter and especially the available cofactors.

Role of PGC-1β in mitochondrial biogenesis.

Our results suggest that both PGC-1α and PGC-1β require the transcriptional activity of ERRα to induce the expression of 'mitochondrial function' genes. Although our data are still preliminary, PGC-1β shows less dependency on ERRα to increase mitochonddrial density. Strikingly, also PRC, the third PGC-1α homologue, increased the cellular content of mitochondria, although the role of ERRα in this function has not yet been analyzed. Whereas PGC-1α and ERRα were induced by metabolic signals, PGC-1β and PRC are not. In contrast, PRC was regulated during proliferation (Andersson and Scarpulla, 2001), whereas PGC-1β is possibly controlled by the differentiating state of the cell (Kamei et al., 2003; Lin et al., 2002). It is still an open question, whether there is a redundancy among the three PGC-1 family members, and if so, why. In particular the structural differences of the three family members suggest that they should carry distinct functions. It is possible that PGC-1α, PGC-1β and PRC regulate mitochondrial biogenesis through the integration of different cellular requirements like cell cycle, differentiation, proliferation and metabolism. The question remains, which place is taken by ERRα in the induction of mitochondrial biogenesis by PGC-1β. As suggested by others, PGC-1β shows some differences in the regulation of mitochondrial processes to PGC-1α. Studies observed that PGC-1β, but not PGC-1α expression led to the regulation of specific enzymes that protect from oxidative stress (St-Pierre et al., 2003). This could propose that ERRα together with PGC-1α regulates different mitochondrial pathways than with PGC-1β. However, our data is still preliminary and more experiments are necessary to define the role of ERRα in PGC-1β signalling.

Part 2: Analysis of the PGC-1α and GR expression profiles

PGC-1α induced specific metabolic pathways in SAOS2 cells.

GR plays an important role in bone homeostasis and long-term treatment with glucocorticoids leads to osteoporosis. Although no studies with PGC-1α function in bone are available, an interesting study identified a new GR ligand, which prevented the interaction with PGC-1α and were proposed to have less side effects on glucose metabolism and bone homeostasis (Coghlan et al., 2003). These findings suggest that PGC-1α may play a role in regulating GR functions in bone.

In the osteoprogenitor SAOS2 cell line, PGC-1α activated specific metabolic programs. We saw the promotion of catabolic pathways as the glycogenolysis and glycolysis, the induction of new mitochondria and the upregulation of enzymatic pathways in mitochondria like the β-fatty acid oxidation, the TCA cycle and the respiratory chain. Furthermore, our results demonstrated the induction of several transcription factors like ERRα, EAR-2, CEBPδ and others, which possibly mediate a part of the PGC-1α response. Interestingly, we found that a significant group of the PGC-1α induced genes, which were exclusively regulated by PGC-1α and not by GR, were dependent on ERRα, namely genes involved in the regulation of mitochondrial pathways. We also provided some evidence that at least some of those genes were regulated directly by ERRα, through binding to the promoters of these genes. This implies ERR α as an important mediator of PGC-1α functions. Further studies with overexpressed or reduced levels of ERRα could provide more indications about the participation of ERRα to other PGC-1α regulated pathways in SAOS2 cells.

PGC-1α function was strongly altered by glucocorticoids.

PGC-1α regulates cellular metabolism and energy balance in response to several stressors and glucocorticoids could possibly modulate PGC-1α action. Furthermore, both PGC-1α and GR are involved in diseases like diabetes. We observed that PGC-1α dramatically influenced the profile of genes induced by glucocorticoids, and vice versa. The major difference between the PGC-1α regulated pathways that were

independent of, and dependent on GR was seen in the mitochondrial biogenesis, which was induced by PGC-1α alone, and was only minimally influenced by GR. The role of glucocorticoids in PGC-1α mediated programs appeared to be both "permissive/stimulative" and "suppressive". On the one hand, we observed the collaborate induction of interesting metabolic factors involved in diabetes and obesity, such as the thiamine transporter or the Bardet Biedl syndrome 1. On the other hand, GR repressed the activity of the pyruvate dehydrogenase complex (via PDK4) and the expression of PEPCK1, which were induced by PGC-1α. These results suggest that PGC-1α does not act as a general transcriptional coactivator of GR, which would enhance the activity of GR on all target genes. To the contrary, PGC-1α seems to play a specific role in GR signaling, inducing specific cellular programs. However, at this state of the analysis and with not much experimental validation of the array results, not many conclusions can be drawn about the common or opposing cellular programs. The quantitative analysis of mRNA levels of more genes, coupled to in silico promoter studies, cellular assays, and experimental validation of the resulting data could give more information.

The nature of SAOS2 cells.

Myocytes, adipocytes, chondrocytes and osteoblasts all derive from the same mesenchymal progenitor cell. Previous studies mostly referred to SAOS2 cells as osteoblast cells. In contrast, our analysis of the PGC-1α expression profiles suggested that SAOS2 cells are osteoprogenitor cells rather that osteoblast cells. These findings are supported by the previous observation that SAOS2 cells are able to differentiate into adipocytes (Diascro et al., 1998). We could not detect a regulation of osteopontin by PGC-1α, although it has been proposed as an ERRα target gene (Bonnelye et al., 1997a; Vanacker et al., 1998), or of any other bone marker. In contrast, we observed adipocyte features, such as the induction of the β-fatty acid oxidation genes by PGC-1α. Surprisingly, we also observed the induction of myocyte-specific genes, such as the genes encoding myoglobin and the cardiac specific Hsp27, which were induced by PGC-1α. The induction of BMP-2 and CEBPδ, which are able to promote both osteoblast and adipocyte differentiation, suggest that PGC-1α and GR could play a role in the differeniation of the SAOS2 cells. Furthermore, PGC-1α and GR seem to

exert antiproliferative effects in our system by the induction of p21 and other factors, which could be involved in differentiation. On account of these observations it would be interesting to perform differentiation studies in the SAOS2 cells with overexpressed PGC-1α, and in particular analyse the possible role of ERRα.

Can PGC-1α be replaced by other coactivators?

Our results suggest that PGC-1α exerts distinct programs with ERRα and influences the activity of GR on specific target genes. However, the question arises, if only PGC-1α can exert these functions, or if other coactivators like SRC-1 could induce similar responses, for example mitochondrial biogenesis. In principle, this was one question I would have liked to answer during my thesis. Due to several reasons I decided to focus on other aspects. Therefore I would like to discuss this possibility at this point.

If the induction of SRC-1 levels by PGC-1α can be verified, it would be indeed interesting to test the activity of SRC-1 on some of the identified targets. Furthermore, I would suggest an experiment with specific downregulation of SRC-1 by siRNA to reveal if PGC-1α is still able to induce mitochondrial biogenesis or if SRC-1 is required for PGC-1α functions. The disruption of SRC-1 (Picard et al., 2002) suggested at least some dependency in the regulation of energy metabolism by PGC-1α. However, the function of PGC-1α shows high complexity and several requirements would have to be fulfilled by SRC-1. For example, PGC-1α induced the expression of other coactivators, like TRIP 80, a component of the mediator complex. Earlier studies suggested already that PGC-1α interacts with SRC-1 to recruit chromatin-modifying coactivators like CBP and with the mediator complex as bridge to the basal transcription machinery. Interestingly, SRC-1 was also shown to interact with the mediator and may therefore replace PGC-1α. Importantly, PGC-1α seems to represent the regulatory element in this complex, responding to several signals like the cAMP cascade activated by glucagon, glucocorticoids or phosphorylation by p38.

An important argument against SRC-1 being able to substiture PGC-1α function may stem from studies on the orphan receptor ERRα. Expression and activation of ERRα seems important for the induction of the mitochondrial biogenesis program. Unpublished work from our group shows that coactivators like SRC-1 are very poor in activating ERRα, suggesting that they could not substitute for all PGC-1α functions.

In conclusion, the specific function of PGC-1α seems to be a sum of several pieces, such as the expression at the right time and the right place, the ability to interact with specific nuclear receptors like ERRα, the induction of specific target genes, and finally the post-translational regulation by specific signaling pathways.

Summary and conclusions

In the last years, increasing numbers of coactivators that regulate the transcriptional activity of nuclear receptors have been identified. Many of the coactivators seem to act as complexes, although redundancy has also been observed. Coactivators exert different functions like bridging to the basal transcription machinery or modification of chromatin structures. PGC-1α seems to take a special place in this scenery of transcriptional regulation. It was observed that PGC-1α levels were induced under certain physiological conditions, which signal increased metabolic demands. Ectopic expression of PGC-1α is sufficient to increase the mitochondrial density of cells representing different tissues, thereby leading to the proposal that PGC-1α is a key regulator of mitochondrial biogenesis. Furthermore, PGC-1α expression was connected to the misregulation of metabolism in obesity and diabetes. It is astonishing that the overexpression of only one component leads to the induction of so many programs; one would think that this makes the system vulnerable. To execute its metabolic functions, PGC-1α interacts with several different nuclear receptors like HNF4, PPARγ, ERRα, GR and more. Interestingly, also the expression of these receptors is controlled by metabolic means, as observed for HNF4, PPARγ, and ERRα for example, which respond to fasting. Possibly, it is the induction of both the specific coactivator and the nuclear receptor, controlled by different systems, that leads to the activation of the specific downstream programs.

We observed that PGC-1α is controlling the expression of the orphan nuclear receptor ERRα by an autoregulatory loop. This induction of ERRα seems to be essential for PGC-1α to regulate mitochondrial biogenesis, (as shown by siRNA for ERRα experiments), and ERRα fused to the VP16 activation domain is sufficient to induce this program. The identification of binding sites for ERRα in genes encoding mitochondrial proteins and our in vitro studies suggest that ERRα regulates these genes directly, coactivated by PGC-1α. However, although PGC-1α and ERRα were induced by the same physiological programs, it remains to be determined if ERRα and PGC-1α regulate mitochondrial biogenesis in response to specific metabolic

signals *in vivo*. Moreover, the question remains if ERRα is required for the induction of mitochondrial biogenesis by PGC-1α in all tissues and situations. The analysis of ERRα levels during exercise and muscle could give more information. In particular, it would be interesting to determine ERRα levels in liver and muscle of diabetes patients, since the increase of PGC-1α expression in the one and the decrease in the other tissue seems to be connected to the misregulation of metabolic processes. In this case, ERRα could possibly provide a starting point for the development of new drugs against diabetes.

Glucocorticoid levels are increased during organismal stress situations, similar to PGC-1α expression levels. Upon fasting, GR even regulates the expression of PGC-1α. Whereas GR does not play a role in the regulation of mitochondrial processes by PGC-1α in SAOS2 cells, in remains to be further analysed in other tissues. However, as the previous studies suggested and our data supported, PGC-1α could play a role in several GR regulated pathways in vivo, besides the gluconeogenesis in liver. We observed a high number of genes that was regulated by PGC-1α only in the presence of glucocorticoids. The data also suggest that GR regulates PGC-1α function in metabolism positively and negatively. Furthermore, both influenced the cell growth and the expression of "differentiation-inducing" genes in SAOS2 cells. At this state of the analysis it is not clear, if PGC-1α induces adipocyte differentiation in SAOS2 cells and if GR could influence this program. Finally and importantly, our data provide further support to the hypothesis that PGC-1α serves as a key component that confers specificity to nuclear receptor signaling.

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Appendix I

Table 1: Genes induced by PGC-1α without glucocorticoids

Table 2: Genes induced by PGC-1α and repressed by GR

Table 3: Genes induced by PGC-1α and GR

Table 4: Genes induced by GR in the absence and presence of PGC-1α

Table 1

Genes that were induced significantly by PGC-1α in the absence of glucocorticoids with no function in mitochondrial biogenesis.

Product	Probe set ID	control value	± SEM	PGC-1α value	± SEM	significance value	classification 1	classification 2	accession number	Description
ORC4L	203351_s_at	318	21.4	509	26.2	0.005	replication		AF047598.1	origin recognition complex, subunit 4-like (yeast)
GTF3C3	218343_s_at	258	16.2	375	30.6	0.036	transcription factor		NM_012086.1	general transcription factor IIIC, polypeptide 3, 102kDa
RRN3	222204_s_at	47	3.5	95	7.3	0.013	transcription factor	ribosome biogenesis	AL110238.1	RNA polymerase I transcription factor RRN3
SNAPC1	205443_at	496	48.4	922	105.3	0.037	transcription factor		NM_003082.1	small nuclear RNA activating complex, polypeptide 1, 43kDa
SNAPC5	213203_at	214	21.8	308	17.7	0.031	transcription factor		AI633709	small nuclear RNA activating complex, polypeptide 5, 19kDa
CEBPD	203973_s_at	268	49.5	501	57.6	0.021	transcription factor		NM_005195.1	CCAAT/enhancer binding protein (C/EBP), delta
ARNTL	209824_s_at	285	23.2	395	26.0	0.041	transcription factor		AB000812.1	aryl hydrocarbon receptor nuclear translocator-like
	210971_s_at	83	7.0	137	8.4	0.020	transcription factor		AB000815.1	aryl hydrocarbon receptor nuclear translocator-like
ESRRA	1487_at	214	20.6	658	36.8	0.003	transcription factor		L38487	estrogen-related receptor alpha
NR2F6	209262_s_at	332	32.5	607	30.1	0.014	transcription factor		BC002669.1	nuclear receptor subfamily 2, group F, member 6
AIM1L	220289_s_at	41	3.1	61	4.4	0.034	transcription factor, putative		NM_017977.1	absent in melanoma 1-like
HMG20A	218152_at	74	5.4	110	7.7	0.040	transcription factor, putative		NM_018200.1	high-mobility group 20A
HUMPPA	213230_at	290	34.5	631	83.9	0.044	transcription factor, putative		AI422335	paraneoplastic antigen
LOC55893	221123_x_at	126	11.6	291	17.3	0.009	transcription factor, putative		NM_018660.1	papillomavirus regulatory factor PRF-1
MGC4161	219904_at	41	2.8	62	2.8	0.008	transcription factor, putative		NM_024303.1	hypothetical protein MGC4161
ZDHHC7	218606_at	479	29.3	826	40.2	0.005	transcription factor, putative		NM_017740.1	zinc finger, DHHC domain containing 7
ZNF193	205181_at	173	11.7	271	24.2	0.039	transcription factor, putative		NM_006299.1	zinc finger protein 193
ZNF211	205437_at	149	10.1	201	12.3	0.026	transcription factor, putative		NM_006385.1	zinc finger protein 211
ZNF23	213934_s_at	35	4.0	62	4.8	0.045	transcription factor, putative		AL567808	zinc finger protein 23 (KOX 16)
ZNF306	211773_s_at	156	12.9	230	10.2	0.013	transcription factor, putative		BC006118.1	zinc finger protein 306
ZNF44	215359_x_at	79	9.4	142	14.1	0.033	transcription factor, putative		AI758888	zinc finger protein 44 (KOX 7)
TFEB	50221_at	87	5.8	136	7.9	0.013	transcription factor, putative		AI524138	transcription factor EB
HSA275986	218508_at	279	22.2	482	50.0	0.034	transcriptional coregulator		NM_018403.1	transcription factor SMIF
RNF4	212696_s_at	584	37.3	844	37.7	0.015	transcriptional coregulator		BF968633	ring finger protein 4
CIAO1	203536_s_at	410	32.1	757	34.0	0.007	transcriptional coregulator		NM_004804.1	WD40 protein Ciao1
NCOA1	209105_at	117	9.6	184	15.0	0.021	transcriptional coregulator		U19179.1	nuclear receptor coactivator 1
	209106_at	147	10.8	293	13.2	0.005	transcriptional coregulator		U19179.1	nuclear receptor coactivator 1
	209107_x_at	394	25.5	637	42.3	0.008	transcriptional coregulator		U19179.1	nuclear receptor coactivator 1
CRSP6	221517_s_at	271	18.5	405	29.9	0.036	transcriptional coregulator		AF105421.1	cofactor required for Sp1 transcriptional activation, subunit 6, 77kDa
RNF14	201823_s_at	150	18.6	467	32.7	0.005	transcriptional coregulator	ubiquitination	NM_004290.1	ring finger protein 14
	201824_at	71	9.7	217	9.6	0.005	transcriptional coregulator	ubiquitination	AB022663.1	ring finger protein 14
NCOA4	210774_s_at	1296	79.1	2399	97.3	0.003	transcriptional coregulator		AL162047.1	nuclear receptor coactivator 4
CABIN1	37652_at	237	13.7	309	15.5	0.007	transcriptional coregulator		AB002328	calcineurin binding protein 1
REA	201600_at	3432	216.2	4873	241.2	0.007	transcriptional coregulator		NM_007273.1	repressor of estrogen receptor activity
NRIP1	202599_s_at	189	24.6	679	18.6	0.007	transcriptional coregulator		NM_003489.1	nuclear receptor interacting protein 1
STAF65(gamm	201836_s_at	116	9.7	203	9.4	0.009	transcriptional regulator?		AU154740	SPTF-associated factor 65 gamma
	201837_s_at	430	31.1	631	38.9	0.019	transcriptional regulator?		AF197954.1	SPTF-associated factor 65 gamma
NFKBIE	203927_at	133	8.3	214	14.8	0.014	transcriptional regulator?		NM_004556.1	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, eps

PRPF4B	202126_at	280 ±	25.3	408 ±	16.0	0.048	RNA processing		AA156948	PRP4 pre-mRNA processing factor 4 homolog B (yeast)
RPP14	204245_s_at	232 ±	16.1	382 ±	19.5	0.006	RNA processing	protein synthesis	AW242755	ribonuclease P (14kD)
CRNKL1	219913_s_at	251 ±	17.1	419 ±	21.5	0.006	RNA processing		NM_016652.2	Crn, crooked neck-like 1 (Drosophila)
RBM7	218379_at	292 ±	22.6	486 ±	34.7	0.018	RNA processing		NM_016090.1	RNA binding motif protein 7
RBM8A	213852_at	232 ±	18.0	356 ±	21.1	0.011	RNA processing		BG289199	RNA binding motif protein 8A
SF3A3	203818_s_at	162 ±	17.3	254 ±	10.8	0.043	RNA processing		NM_006802.1	splicing factor 3a, subunit 3, 60kDa
RPS6KA1	203379_at	238 ±	15.2	488 ±	48.0	0.017	protein synthesis	signaling	NM_002953.1	ribosomal protein S6 kinase, 90kDa, polypeptide 1
XPO4	218479_s_at	76 ±	5.0	143 ±	15.6	0.028	protein synthesis	transport	NM_022459.1	exportin 4
NARS	200027_at	1495 ±	92.2	2062 ±	110.4	0.006	protein synthesis		NM_004539.2	asparaginyl-tRNA synthetase
QARS	217846_at	1911 ±	162.5	3934 ±	171.9	0.006	protein synthesis		NM_005051.1	glutaminyl-tRNA synthetase
SUI1	202021_x_at	2551 ±	152.4	3777 ±	158.4	0.005	protein synthesis		AF083441.1	putative translation initiation factor
	212130_x_at	2611 ±	148.0	3794 ±	183.2	0.004	protein synthesis		AL537707	putative translation initiation factor
	212227_x_at	2702 ±	161.8	3979 ±	210.1	0.005	protein synthesis		AL516854	putative translation initiation factor
SIAT7D	221551_x_at	241 ±	18.1	361 ±	27.3	0.044	posttranslational protein modifiaction		AB035172.1	sialyltransferase 7D ((alpha-N-acetylneuraminyl-2,3-beta-galactosyl-1,3)-N-ace
POMT1	218476_at	91 ±	5.8	127 ±	8.1	0.018	posttranslational protein modifiaction		NM_007171.1	protein-O-mannosyltransferase 1
MKKS	218138_at	233 ±	26.7	434 ±	33.9	0.037	posttranslational protein modifiaction	development, heart	NM_018848.1	McKusick-Kaufman syndrome
B4GALT5	221484_at	921 ±	84.6	1683 ±	167.5	0.025	posttranslational protein modifiaction		NM_004776.1	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 5
	221485_at	435 ±	24.7	855 ±	25.6	0.002	posttranslational protein modifiaction		NM_004776.1	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 5
SIAT9	203217_s_at	204 ±	16.3	498 ±	41.8	0.010	posttranslational protein modifiaction		NM_003896.1	sialyltransferase 9 (CMP-NeuAc:lactosylceramide alpha-2,3-sialyltransferase;
HDS	218547_at	334 ±	22.7	599 ±	24.5	0.005	posttranslational protein modifiaction		NM_024887.1	dehydrodolichyl diphosphate synthase
GORASP1	215749_s_at	296 ±	27.3	455 ±	29.2	0.039	posttranslational protein modifiaction		AK001574.1	golgi reassembly stacking protein 1, 65kDa
DPM1	202673_at	614 ±	31.7	774 ±	44.4	0.023	posttranslational protein modifiaction		NM_003859.1	dolichyl-phosphate mannosyltransferase polypeptide 1, catalytic subunit
CGI-31	201175_at	964 ±	68.2	1528 ±	84.5	0.014	protein folding/chaperone	oxidative stress	NM_015959.1	CGI-31 protein
PPIC	204518_s_at	370 ±	21.2	460 ±	27.9	0.027	protein folding/chaperone		NM_000943.1	peptidylprolyl isomerase C (cyclophilin C)
HSPB7	218934_s_at	296 ±	25.0	1055 ±	44.9	0.002	protein folding/chaperone	stress response	NM_014424.1	heat shock 27kDa protein family, member 7 (cardiovascular)
DNAJA2	209157_at	211 ±	17.9	371 ±	23.3	0.019	protein folding/chaperone	cell cycle	AF011793.1	DnaJ (Hsp40) homolog, subfamily A, member 2
DNAJA3	205963_s_at	844 ±	46.3	1134 ±	48.9	0.008	protein folding/chaperone		NM_005147.1	DnaJ (Hsp40) homolog, subfamily A, member 3
HSPBP1	202415_s_at	349 ±	27.5	571 ±	39.4	0.020	protein folding/chaperone		NM_012267.1	hsp70-interacting protein
GBAS	201816_s_at	120 ±	9.5	204 ±	15.0	0.015	protein trafficking, vesicle transport?		NM_001483.1	glioblastoma amplified sequence
GOLGA2	35436_at	127 ±	7.8	187 ±	13.4	0.023	protein trafficking, vesicle transport?		L06147	golgi autoantigen, golgin subfamily a, 2
HSU52521	218230_at	196 ±	12.9	310 ±	21.1	0.010	protein trafficking, vesicle transport?		AL044651	arfaptin 1
TMEM1	209412_at	80 ±	8.7	136 ±	9.3	0.041	protein trafficking/vesicle transport		U61500.1	transmembrane protein 1
HERC1	218306_s_at	207 ±	14.2	358 ±	20.0	0.005	protein trafficking/vesicle transport	ubiquitination	NM_003922.1	hect (homologous to the E6-AP (UBE3A) carboxyl terminus) domain and RCC
TLOC1	208943_s_at	550 ±	41.5	782 ±	41.2	0.021	protein trafficking/vesicle transport	ubiquitination	U93239.1	translocation protein 1
FRA	74694_s_at	75 ±	11.4	152 ±	10.3	0.045	protein trafficking/vesicle transport		AA907940	Fos-related antigen
GABARAPL3	211458_s_at	474 ±	36.1	684 ±	43.3	0.042	protein trafficking/vesicle transport		AF180519.1	GABA(A) receptors associated protein like 3
ITSN1	209297_at	307 ±	16.1	373 ±	21.5	0.045	protein trafficking/vesicle transport		AF114488.1	intersectin 1 (SH3 domain protein)
PSCD1	202879_s_at	179 ±	13.8	300 ±	12.1	0.005	protein trafficking/vesicle transport		AI798823	pleckstrin homology, Sec7 and coiled/coil domains 1(cytohesin 1)
	202880_s_at	154 ±	13.5	381 ±	24.6	0.006	protein trafficking/vesicle transport		NM_004762.1	pleckstrin homology, Sec7 and coiled/coil domains 1(cytohesin 1)
RER1	202296_s_at	1058 ±	64.4	1394 ±	80.7	0.027	protein trafficking/vesicle transport		NM_007033.1	similar to S. cerevisiae RER1
VPS33B	44111_at	80 ±	4.3	117 ±	8.0	0.021	protein trafficking/vesicle transport		AI672363	vacuolar protein sorting 33B (yeast)
ARFGEF2	218098_at	261 ±	17.5	435 ±	49.8	0.038	protein trafficking/vesicle transport		NM_006420.1	ADP-ribosylation factor guanine nucleotide-exchange factor 2 (brefeldin A-inhil
BAP1	201419_at	563 ±	43.2	885 ±	43.5	0.014	ubiquitination/proteasome degradation		NM_004656.1	BRCA1 associated protein-1 (ubiquitin carboxy-terminal hydrolase)
ARIH1	201878_at	51 ±	3.3	75 ±	6.5	0.039	ubiquitination/proteasome degradation		NM_005744.2	ariadne homolog, ubiquitin-conjugating enzyme E2 binding protein, 1 (Drosoph
UBE3B	212403_at	136 ±	12.7	227 ±	12.0	0.018	ubiquitination/proteasome degradation		AL096740.1	ubiquitin protein ligase
KIAA0349	212760_at	142 ±	7.9	211 ±	8.8	0.003	ubiquitination/proteasome degradation		AI761518	ubiquitin ligase E3 alpha-II

FTS	218373_at	150 ±	10.2	223 ±	11.8	0.005	ubiquitination/proteasome degradation		NM_022476.1	fused toes homolog (mouse)
USP4	202682_s_at	237 ±	18.4	471 ±	26.3	0.004	ubiquitination/proteasome degradation		NM_003363.1	ubiquitin specific protease 4 (proto-oncogene)
	211800_s_at	353 ±	35.6	750 ±	44.7	0.011	ubiquitination/proteasome degradation		AF017306.1	ubiquitin specific protease 4 (proto-oncogene)
USP8	202745_at	109 ±	9.6	218 ±	8.1	0.007	ubiquitination/proteasome degradation		NM_005154.1	ubiquitin specific protease 8
USP16	218386_x_at	463 ±	45.2	749 ±	49.8	0.035	ubiquitination/proteasome degradation	cell cycle	NM_006447.1	ubiquitin specific protease 16
AKIP	218580_x_at	1515 ±	113.3	2204 ±	153.9	0.040	ubiquitination/proteasome degradation	cell cycle	NM_017900.1	aurora-A kinase interacting protein
RNF5	209111_at	365 ±	31.2	512 ±	32.1	0.034	ubiquitination/proteasome degradation	cell motility/ cell adhesio	BC004155.1	ring finger protein 5
WWP1	212638_s_at	214 ±	17.9	459 ±	35.3	0.008	ubiquitination/proteasome degradation		BF131791	WW domain-containing protein 1
TRC8	209510_at	557 ±	55.9	1205 ±	60.8	0.003	ubiquitination/proteasome degradation		AF064801.1	patched related protein translocated in renal cancer
AK1	202588_at	227 ±	12.7	291 ±	17.7	0.028	energy homeostasis		NM_000476.1	adenylate kinase 1
CKB	200884_at	953 ±	56.1	1252 ±	61.2	0.005	energy homeostasis		NM_001823.1	creatine kinase, brain
	204179_at	88 ±	7.7	212 ±	15.2	0.005	energy homeostasis		NM_005368.1	myoglobin
PCK1	208383_s_at	16 ±	1.0	73 ±	2.4	0.002	carbohydrate metabolism, gluconeogenesis		NM_002591.1	phosphoenolpyruvate carboxykinase 1 (soluble)
MDH1	200978_at	3149 ±	219.0	5319 ±	225.4	0.002	carbohydrate metabolism, glycogenolysis		NM_005917.1	malate dehydrogenase 1, NAD (soluble)
AGL	203566_s_at	377 ±	30.1	690 ±	30.9	0.007	carbohydrate metabolism, glycogenolysis		NM_000645.1	amylo-1, 6-glucosidase, 4-alpha-glucanotransferase (glycogen debranching er
PGM1	201968_s_at	1091 ±	68.2	1535 ±	78.7	0.007	carbohydrate metabolism, glycolysis		NM_002633.1	phosphoglucomutase 1
GK	207387_s_at	41 ±	3.1	60 ±	3.4	0.008	carbohydrate metabolism, glycolysis		NM_000167.1	glycerol kinase
PFKM	210976_s_at	1083 ±	91.6	1998 ±	120.1	0.015	carbohydrate metabolism, glycolysis		U24183.1	phosphofructokinase, muscle
LCE	204256_at	76 ±	8.4	148 ±	16.3	0.048	lipid synthesis/cholesterol synthesis		NM_024090.1	long-chain fatty-acyl elongase
DHCR7	201790_s_at	182 ±	18.7	327 ±	32.3	0.045	lipid synthesis/cholesterol synthesis		AW150953	7-dehydrocholesterol reductase
APOL2	221653_x_at	270 ±	30.8	479 ±	43.9	0.047	lipid/steroid metabolism		BC004395.1	apolipoprotein L, 2
STARD5	213820_s_at	71 ±	5.3	111 ±	5.5	0.007	lipid/steroid metabolism		T54159	START domain containing 5
PCTP	218676_s_at	112 ±	13.4	338 ±	19.2	0.005	lipid/steroid metabolism		NM_021213.1	phosphatidylcholine transfer protein
AMPD3	207992_s_at	251 ±	17.9	415 ±	33.5	0.032	nucleotide/ nucleoside metabolism		NM_000480.1	adenosine monophosphate deaminase (isoform E)
PRPSAP1	202529_at	701 ±	36.9	952 ±	45.8	0.005	nucleotide/ nucleoside metabolism		NM_002766.1	phosphoribosyl pyrophosphate synthetase-associated protein 1
PHB	200659_s_at	430 ±	28.3	599 ±	41.4	0.033	nucleotide/nucleoside metabolism	proliferation inhibitor	NM_002634.2	prohibitin
HMOX1	203665_at	366 ±	26.1	626 ±	68.7	0.032	oxidative stress/ detoxification	heme catabolism	NM_002133.1	heme oxygenase (decycling) 1
GSTM2	204418_x_at	180 ±	18.9	352 ±	19.7	0.009	oxidative stress/ detoxification		NM_000848.1	glutathione S-transferase M2 (muscle)
GSTM4	204149_s_at	53 ±	8.2	315 ±	25.6	0.004	oxidative stress/ detoxification		NM_000850.1	glutathione S-transferase M4
	210912_x_at	134 ±	16.1	358 ±	18.1	0.005	oxidative stress/ detoxification		M99422.1	glutathione S-transferase M4
GSTM1	204550_x_at	140 ±	11.1	317 ±	14.5	0.002	oxidative stress/ detoxification		NM_000561.1	glutathione S-transferase M1
	215333_x_at	178 ±	15.2	334 ±	18.0	0.006	oxidative stress/ detoxification		X08020.1	glutathione S-transferase M1
PEX14	203503_s_at	235 ±	25.8	504 ±	26.9	0.015	peroxysomal biogenesis		NM_004565.1	peroxisomal biogenesis factor 14
	33760_at	158 ±	11.2	308 ±	15.7	0.005	peroxysomal biogenesis		AB017546	peroxisomal biogenesis factor 14
SLC6A6	205920_at	85 ±	11.0	364 ±	64.1	0.011	AS transporter		NM_003043.1	solute carrier family 6 (neurotransmitter transporter, taurine), member 6
CA2	209301_at	39 ±	2.9	58 ±	3.2	0.002	carbon metabolism		M36532.1	carbonic anhydrase II
C4S-2	218927_s_at	259 ±	19.2	574 ±	28.6	0.004	chondroitin, dermatan sulfate biosynthesis		NM_018641.1	chondroitin 4-O-sulfotransferase 2
SLC31A1	203971_at	216 ±	14.7	365 ±	16.7	0.003	copper homeostasis	oxidative stress	NM_001859.1	solute carrier family 31 (copper transporters), member 1
ATP1B1	201242_s_at	564 ±	138.9	2219 ±	329.6	0.023	metabolism, nutrient transport?		BC000006.1	ATPase, Na+/K+ transporting, beta 1 polypeptide
	201243_s_at	990 ±	97.8	2469 ±	115.0	0.005	metabolism, nutrient transport?		NM_001677.1	ATPase, Na+/K+ transporting, beta 1 polypeptide
SLC20A1	201920_at	764 ±	75.4	1208 ±	75.1	0.020	phosphate homeostasis, metabolism		NM_005415.2	solute carrier family 20 (phosphate transporter), member 1
GM2A	212737_at	242 ±	13.9	350 ±	18.1	0.005	glycosphingolipid catabolism		AL513583	GM2 ganglioside activator protein
CLCNKA	207047_s_at	47 ±	4.0	124 ±	8.3	0.005	excretion		NM_004070.1	chloride channel Ka
CLCNKB	205985_x_at	76 ±	5.7	112 ±	4.9	0.010	excretion		NM_000085.1	chloride channel Kb

GAS2L1	209729_at	168 ±	14.8	405 ±	37.3	0.009	cytoskeleton	cell growth	BC001782.1	growth arrest-specific 2 like 1
	31874_at	142 ±	19.9	407 ±	24.3	0.007	cytoskeleton	cell growth	Y07846	growth arrest-specific 2 like 1
AVIL	214331_at	45 ±	3.5	69 ±	5.7	0.049	cytoskeleton	development	AI796813	advillin
KIF1B	209234_at	479 ±	28.1	660 ±	39.7	0.016	cytoskeleton	transport, organelle	BF939474	kinesin family member 1B
BICD2	213154_s_at	274 ±	14.7	411 ±	37.5	0.036	cytoskeleton	transport, organelle	AB014599.1	coiled-coil protein BICD2
ACTR10	222230_s_at	877 ±	51.1	1097 ±	62.9	0.033	cytoskeleton		AK022248.1	actin-related protein 10 homolog (S. cerevisiae)
ARHGAP12	207606_s_at	72 ±	5.3	111 ±	8.2	0.033	cytoskeleton		NM_018287.1	Rho GTPase activating protein 12
KIF3B	203943_at	133 ±	11.1	301 ±	25.2	0.009	cytoskeleton		NM_004798.1	kinesin family member 3B
PNUTL2	210657_s_at	38 ±	3.9	156 ±	23.6	0.013	cytoskeleton, cell shape		U88870.1	peanut-like 2 (Drosophila)
RGL	209568_s_at	138 ±	8.4	259 ±	10.3	0.002	cytoskeleton, cell shape		AF186779.1	RalGDS-like gene
SDC4	202071_at	686 ±	62.8	1829 ±	163.1	0.011	cytoskeleton, cell shape		NM_002999.1	syndecan 4 (amphiglycan, ryudocan)
RAGD	221523_s_at	328 ±	28.8	480 ±	28.1	0.019	cytoskeleton, GTPase		AL138717	Rag D protein
CRK	202225_at	258 ±	18.7	345 ±	24.7	0.028	cytoskeleton organisation, cell motility		NM_016823.1	v-crk sarcoma virus CT10 oncogene homolog (avian)
KRT10	210633_x_at	528 ±	34.6	754 ±	40.4	0.005	cytoskeleton, intermediate filament		M19156.1	keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris)
	213287_s_at	192 ±	16.3	301 ±	16.4	0.010	cytoskeleton, intermediate filament		X14487	keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris)
KRTHB6	215189_at	74 ±	10.2	193 ±	17.1	0.020	cytoskeleton, intermediate filament		X99142.1	keratin, hair, basic, 6 (monilethrix)
PSG1	208257_x_at	77 ±	11.7	149 ±	15.0	0.043	cell adhesion	immune response	NM_006905.1	pregnancy specific beta-1-glycoprotein 1
MAEA	207922_s_at	471 ±	37.3	977 ±	56.2	0.005	cell adhesion		NM_005882.2	macrophage erythroblast attacher
LAMB3	209270_at	75 ±	9.5	203 ±	27.3	0.035	cell adhesion/ migration	development	L25541.1	laminin, beta 3
SPARCL1	200795_at	40 ±	3.1	188 ±	11.3	0.003	cell adhesion/ migration		NM_004684.1	SPARC-like 1 (mast9, hevin)
SLK	206874_s_at	90 ±	9.1	206 ±	11.0	0.010	apoptosis, actin disassembly		AL138761	Ste20-related serine/threonine kinase
	206875_s_at	85 ±	8.4	179 ±	9.2	0.012	apoptosis, actin disassembly		NM_014720.1	Ste20-related serine/threonine kinase
EBAG9	204274_at	518 ±	34.8	729 ±	42.5	0.009	apoptosis, activator		AA812215	estrogen receptor binding site associated, antigen, 9
FEM1B	212367_at	78 ±	9.6	140 ±	8.9	0.044	apoptosis, activator		NM_015322.1	fem-1 homolog b (C. elegans)
PDCD10	210907_s_at	675 ±	45.3	1423 ±	51.3	0.002	apoptosis, activator		BC002506.1	programmed cell death 10
TNFRSF9	207536_s_at	269 ±	29.5	418 ±	17.7	0.045	apoptosis	immune response, deve	NM_001561.2	tumor necrosis factor receptor superfamily, member 9
TNFRSF10B	209295_at	132 ±	21.2	298 ±	26.5	0.043	apoptosis, activator		AF016266.1	tumor necrosis factor receptor superfamily, member 10b
BAG1	202387_at	327 ±	22.7	618 ±	33.7	0.007	apoptosis-inhibitor		NM_004323.2	BCL2-associated athanogene
	211475_s_at	475 ±	43.9	1036 ±	84.0	0.013	apoptosis-inhibitor		AF116273.1	BCL2-associated athanogene
GAK	40225_at	416 ±	31.3	626 ±	29.8	0.017	cell cycle/cell growth		D88435	cyclin G associated kinase
STK6	204092_s_at	674 ±	39.6	898 ±	44.9	0.008	cell cycle/cell growth		NM_003600.1	serine/threonine kinase 6
	208079_s_at	1245 ±	66.8	1695 ±	94.3	0.014	cell cycle/cell growth		NM_003158.1	serine/threonine kinase 6
TSG101	201758_at	679 ±	43.5	881 ±	43.9	0.028	cell cycle/cell growth inhibitor	differentiation	NM_006292.1	tumor susceptibility gene 101
CPR2	220789_s_at	203 ±	12.4	289 ±	18.7	0.024	cell cycle/cell growth inhibitor	proliferation	NM_004749.1	cell cycle progression 2 protein
TOB1	202704_at	84 ±	5.6	157 ±	11.9	0.014	cell cycle/cell growth inhibitor	proliferation	AA675892	transducer of ERBB2, 1
TOB2	222243_s_at	128 ±	8.9	173 ±	10.0	0.042	cell cycle/cell growth inhibitor	signaling	AB051450.1	transducer of ERBB2, 2
NDRG3	217286_s_at	361 ±	25.7	545 ±	42.0	0.036	cell cycle/cell growth inhibitor		BC001805.1	NDRG family member 3
G0S2	213524_s_at	48 ±	5.3	1555 ±	98.2	0.002	cell cycle/cell growth		NM_015714.1	putative lymphocyte G0/G1 switch gene
TACC2	202289_s_at	48 ±	3.3	93 ±	12.4	0.041	cell cycle/cell growth		NM_006997.1	transforming, acidic coiled-coil containing protein 2
BMP2	205289_at	66 ±	3.2	674 ±	54.9	0.002	differentiation osteoblasts ?	differentiation, cell growt	AA583044	bone morphogenetic protein 2
	205290_s_at	138 ±	7.6	1192 ±	112.9	0.003	differentiation osteoblasts ?	differentiation, cell growt	NM_001200.1	bone morphogenetic protein 2
VEGF	210512_s_at	365 ±	28.5	662 ±	24.1	0.007	differentiation osteoblasts ?	proliferation, cell growth	AF022375.1	vascular endothelial growth factor
	211527_x_at	197 ±	16.1	286 ±	15.4	0.020	differentiation osteoblasts ?	proliferation, cell growth	M27281.1	vascular endothelial growth factor
MPP1	202974_at	553 ±	33.0	848 ±	55.3	0.018	differentiation		NM_002436.2	membrane protein, palmitoylated 1, 55kDa
LPIN1	212274_at	260 ±	23.7	430 ±	25.8	0.018	differentiation, adipocytes/muscle		D80010.1	lipin 1
	212276_at	228 ±	19.4	403 ±	38.7	0.027	differentiation, adipocytes/muscle		D80010.1	lipin 1

CDK5RAP1	218315_s_at	370 ±	22.0	486 ±	28.7	0.017	differentiation, neurones		NM_016408.1	CDK5 regulatory subunit associated protein 1
NDRG2	206453_s_at	193 ±	16.7	363 ±	20.6	0.009	differentiation, neurons		NM_016250.1	NDRG family member 2
	214279_s_at	87 ±	6.6	175 ±	9.0	0.005	differentiation, neurons		W74452	NDRG family member 2
REC14	221532_s_at	583 ±	33.0	827 ±	48.9	0.023	differentiation, osteoblasts?		AF309553.1	recombination protein REC14
PVALB	205336_at	55 ±	5.6	108 ±	5.7	0.013	development, muscle		NM_002854.1	parvalbumin
ALDH3A2	202054_s_at	178 ±	15.7	287 ±	17.8	0.026	development, nervous system		NM_000382.1	aldehyde dehydrogenase 3 family, member A2
ALOX5AP	204174_at	682 ±	75.5	1883 ±	179.5	0.012	immune response		NM_001629.1	arachidonate 5-lipoxygenase-activating protein
IGHG3	217369_at	149 ±	17.2	246 ±	17.2	0.045	immune response		AJ275383	immunoglobulin heavy constant gamma 3 (G3m marker)
P5-1	206082_at	157 ±	15.7	282 ±	14.0	0.009	immune response		NM_006674.1	MHC class I region ORF
PSG4	208191_x_at	88 ±	5.1	175 ±	7.0	0.002	immune response		NM_002780.1	pregnancy specific beta-1-glycoprotein 4
POR1	202109_at	387 ±	29.6	546 ±	27.5	0.027	signaling	cell motility/ cell adhesio	NM_012402.1	partner of RAC1 (arfaptin 2)
TSC1	209390_at	283 ±	21.6	433 ±	19.6	0.015	signaling	cell adhesion	AF013168.1	tuberous sclerosis 1
PTPN1	202716_at	247 ±	16.9	319 ±	18.0	0.005	signaling	differentiation	NM_002827.1	protein tyrosine phosphatase, non-receptor type 1
MPP2	207984_s_at	157 ±	11.4	270 ±	11.5	0.007	signaling	cytoskeleton interaction	NM_005374.1	membrane protein, palmitoylated 2 (MAGUK p55 subfamily member 2)
ADCY9	204497_at	109 ±	6.7	165 ±	7.5	0.013	signaling		AB011092.1	adenylate cyclase 9
ASM3A	213624_at	31 ±	2.1	54 ±	4.0	0.012	signaling		AA873600	acid sphingomyelinase-like phosphodiesterase
ITPK1	210740_s_at	232 ±	18.1	334 ±	16.3	0.009	signaling		AF279372.1	inositol 1,3,4-triphosphate 5/6 kinase
MAP2K1IP1	217971_at	335 ±	22.6	556 ±	37.9	0.010	signaling		NM_021970.1	mitogen-activated protein kinase kinase 1 interacting protein 1
MAP3K7	206854_s_at	222 ±	18.5	343 ±	25.6	0.019	signaling		NM_003188.1	mitogen-activated protein kinase kinase kinase 7
PIK3R4	212740_at	211 ±	25.2	355 ±	16.6	0.044	signaling		BF740111	phosphoinositide-3-kinase, regulatory subunit 4, p150
SNRK	209481_at	224 ±	23.7	594 ±	36.5	0.006	signaling		AF226044.1	SNF-1 related kinase
PPP1R3C	204284_at	259 ±	18.3	661 ±	58.7	0.010	signaling		N26005	protein phosphatase 1, regulatory (inhibitor) subunit 3C
OSBPL11	218304_s_at	171 ±	15.6	271 ±	15.4	0.015	signaling	lipid/steroid metabolism	NM_022776.1	oxysterol binding protein-like 11
OSBPL1A	208158_s_at	130 ±	10.4	261 ±	12.5	0.006	signaling	lipid/steroid metabolism,	NM_018030.1	oxysterol binding protein-like 1A
	209485_s_at	82 ±	7.5	227 ±	18.1	0.005	signaling	lipid/steroid metabolism,	W19983	oxysterol binding protein-like 1A
ALS2CR3	202125_s_at	196 ±	18.4	442 ±	35.0	0.010	signaling		NM_015049.1	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 3
SLC9A1	209453_at	469 ±	45.2	702 ±	39.1	0.023	signaling		M81768.1	solute carrier family 9 (sodium/hydrogen exchanger), isoform 1 (antiporter, Na·
BART1	202092_s_at	615 ±	36.4	856 ±	41.7	0.007	signaling		NM_012106.1	binder of Arl Two
SNX6	217789_at	605 ±	60.7	951 ±	67.6	0.043	signaling		NM_021249.1	sorting nexin 6
STARD7	200028_s_at	1915 ±	140.7	3050 ±	139.4	0.010	signaling		NM_020151.1	START domain containing 7
SACM1L	202797_at	293 ±	29.9	455 ±	19.6	0.041	signaling		NM_014016.1	SAC1 suppressor of actin mutations 1-like (yeast)
FRAT2	209864_at	75 ±	4.9	159 ±	13.2	0.011	signaling (WNT)	proliferation	AB045118.1	frequently rearranged in advanced T-cell lymphomas 2
GPR3	214613_at	123 ±	8.0	215 ±	13.5	0.008	signaling, G protein		AW024085	G protein-coupled receptor 3
GPS2	209350_s_at	344 ±	23.7	597 ±	48.3	0.021	signaling, G protein inhibitor		AL157493.1	G protein pathway suppressor 2
P450RAI-2	219825_at	29 ±	2.4	70 ±	11.3	0.044	signaling, inactivation of RA signal		NM_019885.1	cytochrome P450 retinoid metabolizing protein
KCNG1	214595_at	82 ±	10.6	189 ±	17.0	0.019	signaling, muscle contraction		AI332979	potassium voltage-gated channel, subfamily G, member 1
MTMR4	212277_at	405 ±	28.2	764 ±	38.1	0.005	signaling, myogenesis		AB014547.1	myotubularin related protein 4
	214268_s_at	697 ±	52.7	1292 ±	90.4	0.015	signaling, myogenesis		AL042220	myotubularin related protein 4
IMPA1	203011_at	277 ±	24.7	605 ±	32.4	0.002	signaling, PI		NM_005536.2	inositol(myo)-1(or 4)-monophosphatase 1
SMPD1	209420_s_at	283 ±	24.6	400 ±	20.2	0.043	signaling, sphingomyelin metabolism		M59916.1	sphingomyelin phosphodiesterase 1, acid lysosomal (acid sphingomyelinase)
APBA2BP	210720_s_at	215 ±	12.7	289 ±	13.3	0.004	signaling?	metabolism, amyloid	AB039947.1	amyloid beta (A4) precursor protein-binding, family A, member 2 binding protei
IBTK	210970_s_at	186 ±	13.5	323 ±	15.6	0.011	signaling?		AF235049.1	inhibitor of Bruton's tyrsoine kinase
SV2A	203069_at	273 ±	18.0	411 ±	16.8	0.005	synaptic vesicle transport		NM_014849.1	synaptic vesicle glycoprotein 2A
SYNJ1	212990_at	136 ±	12.6	212 ±	14.0	0.043	synaptic vesicle transport		AB020717.1	synaptojanin 1
SYNGR3	205691_at	46 ±	3.8	173 ±	27.5	0.018	synaptic vesicle transport		NM_004209.2	synaptogyrin 3
ACP2	202767_at	277 ±	23.7	417 ±	23.7	0.018	proteolysis/lysosome		NM_001610.1	acid phosphatase 2, lysosomal
CTSC	201487_at	211 ±	12.7	321 ±	14.3	0.005	proteolysis		NM_001814.1	cathepsin C
ZMPSTE24	202939_at	798 ±	50.5	1053 ±	50.7	0.027	proteolysis/processing of farnesylated proteins		NM_005857.1	zinc metalloproteinase (STE24 homolog, yeast)
TIMP3	201150_s_at	70 ±	5.5	158 ±	12.1	0.013	proteolysis/extracellular matrix degradation		NM_000362.2	tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflam

HSU79252	204593_s_at	89 ±	8.1	211 ±	11.2	0.009	unknown	apoptosis?	AA046752	hypothetical protein HSU79252
KIAA0089	212510_at	322 ±	26.8	661 ±	64.3	0.019	unknown	carbohydrate metabolisr	AA135522	KIAA0089 protein
---	210501_x_at	1593 ±	98.3	2481 ±	154.2	0.010	unknown	cell cycle?	AF119846.1	Homo sapiens, Similar to LATS (large tumor suppressor, Drosophila) homolog
SGT	201396_s_at	289 ±	19.7	404 ±	26.8	0.038	unknown	chaperone	NM_003021.2	small glutamine-rich tetratricopeptide repeat (TPR)-containing
PTK9L	202009_at	234 ±	15.8	425 ±	48.4	0.033	unknown	cytoskeleton	NM_007284.1	PTK9L protein tyrosine kinase 9-like (A6-related protein)
DKFZP586I222	36030_at	358 ±	25.7	523 ±	26.3	0.017	unknown	cytoskeleton, intermedia	AL080214	intermediate filament-like MGC:2625
C16orf5	218183_at	107 ±	7.9	160 ±	12.1	0.033	unknown	cytoskeleton, regulation?	NM_013399.1	chromosome 16 open reading frame 5
---	214603_at	763 ±	47.8	1270 ±	77.1	0.009	unknown	development? Embryona	U82671	melanoma antigen, family A, 2
SPATA2	204434_at	79 ±	10.3	164 ±	14.9	0.042	unknown	development?spermatog	NM_006038.1	spermatogenesis associated 2
AHCYL1	200849_s_at	615 ±	37.4	798 ±	49.8	0.036	unknown	differentiation	AA479488	S-adenosylhomocysteine hydrolase-like 1
	200850_s_at	845 ±	63.8	1181 ±	86.6	0.035	unknown	differentiation	NM_006621.1	S-adenosylhomocysteine hydrolase-like 1
NIFU	209075_s_at	386 ±	27.8	807 ±	54.9	0.009	unknown	energy homeostasis?	AY009128.1	nitrogen fixation cluster-like
KIAA0265	209255_at	243 ±	21.2	437 ±	40.7	0.022	unknown	enyzme, transferase, ox	AF277177.1	KIAA0265 protein
	209256_s_at	346 ±	18.5	567 ±	27.6	0.006	unknown	enyzme, transferase, ox	AF277177.1	KIAA0265 protein
FLJ10055	213836_s_at	63 ±	5.5	184 ±	14.3	0.005	unknown	enzyme, hydrolase	AW052084	hypothetical protein FLJ10055
FLJ10769	217940_s_at	755 ±	55.0	1063 ±	76.7	0.049	unknown	enzyme, kinase, carboh	NM_018210.1	hypothetical protein FLJ10769
FLJ10851	219277_s_at	104 ±	10.6	278 ±	18.7	0.005	unknown	enzyme, oxidoreductase	NM_018245.1	hypothetical protein FLJ10851
LOC51185	218142_s_at	198 ±	11.9	244 ±	15.3	0.050	unknown	enzyme, peptidase, prot	NM_016302.1	protein x 0001
MDS026	221772_s_at	155 ±	12.6	254 ±	22.4	0.041	unknown	enzyme, phosphatase	AB040974.1	uncharacterized hematopoietic stem/progenitor cells protein MDS026
LOC57107	219307_at	150 ±	15.1	265 ±	15.7	0.028	unknown	enzyme, synthase? Prot	NM_020381.1	candidate tumor suppressor protein
DKFZP586A05	207761_s_at	50 ±	4.5	87 ±	6.8	0.019	unknown	enzyme, transferase?	NM_014033.1	DKFZP586A0522 protein
KIAA0205	202651_at	48 ±	4.4	80 ±	4.4	0.028	unknown	enzyme,phopholipid bios	NM_014873.1	KIAA0205 gene product
GDBR1	202151_s_at	322 ±	36.4	1031 ±	54.4	0.006	unknown	enzyme?, protease?, ub	NM_016172.1	putative glialblastoma cell differentiation-related
HIG1	217845_x_at	2267 ±	127.5	2925 ±	144.1	0.004	unknown	hypoxia?	NM_014056.1	likely ortholog of mouse hypoxia induced gene 1
MRS2L	218536_at	95 ±	7.1	181 ±	14.7	0.017	unknown	magnesium homeostasis	AF052167.1	MRS2-like, magnesium homeostasis factor (S. cerevisiae)
	218538_s_at	61 ±	5.8	161 ±	11.6	0.007	unknown	magnesium homeostasis	NM_020662.1	MRS2-like, magnesium homeostasis factor (S. cerevisiae)
CARF	218929_at	148 ±	10.1	242 ±	10.7	0.011	unknown	nucleic acid binding?	NM_017632.1	collaborates/cooperates with ARF (alternate reading frame) protein
FLJ10486	218947_s_at	153 ±	8.5	248 ±	13.7	0.009	unknown	nucleic acid binding?	NM_018109.1	hypothetical protein FLJ10486
RNPC1	212430_at	128 ±	9.2	265 ±	12.8	0.003	unknown	nucleic acid binding?	AL109955	RNA-binding region (RNP1, RRM) containing 1
FLJ10377	218593_at	533 ±	30.7	770 ±	37.3	0.005	unknown	protein synthesis?	NM_018077.1	hypothetical protein FLJ10377
FLJ10989	218949_s_at	203 ±	13.5	333 ±	19.9	0.012	unknown	protein synthesis?	NM_018292.1	hypothetical protein FLJ10989
KIAA0664	212456_at	488 ±	35.3	1090 ±	57.8	0.004	unknown	protein synthesis?	AB014564.1	KIAA0664 protein
KIAA1185	212904_at	873 ±	60.9	1242 ±	79.8	0.032	unknown	protein synthesis?	AB033011.1	KIAA1185 protein
DDX35	218579_s_at	124 ±	9.3	278 ±	12.8	0.003	unknown	RNA helicase?	NM_021931.1	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 35
FLJ20542	217994_x_at	816 ±	53.9	1097 ±	58.7	0.015	unknown	RNA splicing?	NM_017871.1	hypothetical protein FLJ20542
GHITM	209248_at	1254 ±	99.1	2120 ±	111.4	0.011	unknown	signaling	AL136713.1	growth hormone inducible transmembrane protein
	209249_s_at	1989 ±	129.2	2802 ±	129.3	0.005	unknown	signaling	AF131820.1	growth hormone inducible transmembrane protein
HSPC135	221046_s_at	318 ±	32.6	512 ±	48.6	0.050	unknown	signaling, small GTPase	NM_014170.1	HSPC135 protein
KIAA0645	205223_at	124 ±	10.6	240 ±	12.1	0.009	unknown	signaling?	NM_014662.1	KIAA0645 gene product
ATP10D	213238_at	162 ±	12.3	225 ±	12.1	0.035	unknown	small molecule transpor	AI478147	ATPase, Class V, type 10D
KIAA0326	213196_at	281 ±	16.7	412 ±	20.8	0.005	unknown	transcription?, developm	AI924293	KIAA0326 protein
FLJ13725	45749_at	900 ±	48.6	1136 ±	57.8	0.009	unknown	transcriptional regulator	AA400206	hypothetical protein FLJ13725
KIAA0427	204303_s_at	84 ±	8.3	143 ±	8.4	0.029	unknown	transcriptional regulator	NM_014772.1	KIAA0427 gene product
MGC10334	220734_s_at	305 ±	28.6	642 ±	27.5	0.010	unknown	transcriptional regulator	NM_030575.1	hypothetical protein MGC10334
MGC2508	219088_s_at	118 ±	6.3	215 ±	20.6	0.021	unknown	transcriptional regulator	AA639585	hypothetical protein MGC2508
ZFP106	217781_s_at	220 ±	13.5	370 ±	19.1	0.008	unknown	transcriptional repressor	NM_022473.1	zinc finger protein 106

FLJ11198	202394_s_at	302 ±	23.7	426 ±	25.2	0.039	unknown	transport, abc	NM_018358.1	hypothetical protein FLJ11198
SMC2L1	204240_s_at	277 ±	16.7	435 ±	24.5	0.007	unknown	transporter?abc	NM_006444.1	SMC2 structural maintenance of chromosomes 2-like 1 (yeast)
DSCR3	203635_at	291 ±	19.7	462 ±	18.8	0.004	unknown	transport, protein?	NM_006052.1	Down syndrome critical region gene 3
KIAA0453	212323_s_at	164 ±	14.7	322 ±	27.7	0.013	unknown	ubiquitination? Protein tr	AB007922.2	KIAA0453 protein
	212326_at	50 ±	4.7	89 ±	6.3	0.017	unknown	ubiquitination? Protein tr	AB007922.2	KIAA0453 protein
---	200878_at	116 ±	12.3	177 ±	12.8	0.041	unknown		AF052094.1	Homo sapiens clone 23698 mRNA sequence
---	200979_at	92 ±	7.4	182 ±	14.7	0.012	unknown		BF739979	Homo sapiens cDNA FLJ34891 fis, clone NT2NE2017562.
---	201410_at	509 ±	30.1	816 ±	40.5	0.006	unknown		AI983043	Homo sapiens cDNA FLJ30436 fis, clone BRACE2009037.
---	202681_at	469 ±	38.8	958 ±	53.3	0.008	unknown		AI346043	Homo sapiens cDNA FLJ31653 fis, clone NT2RI2004190.
---	209630_s_at	132 ±	10.4	202 ±	12.0	0.028	unknown		AL043967	Homo sapiens cDNA FLJ38058 fis, clone CTONG2014898.
---	212099_at	225 ±	60.1	1341 ±	112.5	0.002	unknown		AI263909	Human HepG2 3' region cDNA, clone hmd1f06.
---	213656_s_at	237 ±	20.1	402 ±	20.2	0.018	unknown		BF593594	Homo sapiens mRNA; cDNA DKFZp434I139 (from clone DKFZp434I139)
---	213758_at	90 ±	7.1	175 ±	8.9	0.005	unknown		AW337510	Homo sapiens cDNA: FLJ23483 fis, clone KAIA04052.
---	217501_at	87 ±	6.0	121 ±	6.1	0.013	unknown		AI339732	Homo sapiens, clone IMAGE:5268928, mRNA
---	221988_at	62 ±	6.3	123 ±	7.1	0.008	unknown		AA463853	ESTs, Moderately similar to cytokine receptor-like factor 2; cytokine receptor C
---	222275_at	67 ±	4.9	101 ±	5.6	0.013	unknown		AI039469	ESTs, Weakly similar to hypothetical protein FLJ20378 [Homo sapiens] [H.sap
---	222369_at	92 ±	5.1	147 ±	7.5	0.005	unknown		AW971254	ESTs, Weakly similar to hypothetical protein FLJ20378 [Homo sapiens] [H.sap
BLCAP	201032_at	257 ±	22.3	493 ±	33.8	0.015	unknown		NM_006698.1	bladder cancer associated protein
BRP44L	218024_at	265 ±	25.8	987 ±	53.0	0.002	unknown		NM_016098.1	brain protein 44-like
C11orf15	218065_s_at	462 ±	28.7	657 ±	30.9	0.006	unknown		NM_020644.1	chromosome 11 open reading frame 15
C13orf17	219116_s_at	129 ±	8.1	163 ±	8.9	0.033	unknown		NM_018185.1	chromosome 13 open reading frame 17
C14orf108	218139_s_at	239 ±	21.2	597 ±	24.2	0.003	unknown		NM_018229.1	chromosome 14 open reading frame 108
C14orf137	219369_s_at	56 ±	3.8	113 ±	11.4	0.019	unknown		NM_023112.1	chromosome 14 open reading frame 137
C20orf24	217835_x_at	1658 ±	98.6	2807 ±	106.0	0.002	unknown		NM_018840.1	chromosome 20 open reading frame 24
C20orf44	217935_s_at	195 ±	16.8	314 ±	14.4	0.014	unknown		NM_018244.1	chromosome 20 open reading frame 44
C20orf45	217851_s_at	30 ±	2.3	57 ±	3.9	0.010	unknown		NM_016045.1	chromosome 20 open reading frame 45
CASKIN2	61297_at	173 ±	11.6	248 ±	16.0	0.031	unknown		AL037338	cask-interacting protein 2
CGI-72	222133_s_at	120 ±	7.3	174 ±	8.8	0.005	unknown		AK022280.1	CGI-72 protein
CGI-87	203122_at	119 ±	8.4	187 ±	15.3	0.027	unknown		NM_016030.1	CGI-87 protein
CMT2	203094_at	371 ±	24.9	548 ±	33.8	0.019	unknown		NM_014628.1	gene predicted from cDNA with a complete coding sequence
DFNA5	203695_s_at	153 ±	11.6	249 ±	16.5	0.020	unknown		NM_004403.1	deafness, autosomal dominant 5
DJ465N24.2.1	209007_s_at	195 ±	12.1	300 ±	24.8	0.023	unknown		AF267856.1	hypothetical protein dJ465N24.2.1
DKFZP434K04	212228_s_at	428 ±	38.5	1039 ±	41.5	0.003	unknown		AC004382	hypothetical protein DKFZp434K046
DKFZp434K12	218149_s_at	131 ±	11.2	383 ±	51.6	0.013	unknown		NM_017606.1	hypothetical protein DKFZp434K1210
DKFZP564B16	202427_s_at	837 ±	58.7	1441 ±	66.5	0.005	unknown		NM_015415.1	DKFZP564B167 protein
DKFZP564I122	211774_s_at	106 ±	7.4	202 ±	15.5	0.013	unknown		BC006122.1	DKFZP564I122 protein
DKFZP586E19	213312_at	66 ±	8.4	127 ±	6.7	0.013	unknown		NM_020425.1	hypothetical protein DKFZp586E1923
DKFZP586P01	213199_at	219 ±	16.6	336 ±	15.9	0.008	unknown		AL080220.1	DKFZP586P0123 protein
	36552_at	147 ±	13.0	254 ±	13.7	0.015	unknown		AL080220	DKFZP586P0123 protein
ECM1	209365_s_at	425 ±	32.3	1383 ±	144.5	0.007	unknown		U65932.1	extracellular matrix protein 1
FLJ10055	203827_at	85 ±	9.5	275 ±	29.3	0.008	unknown		NM_017983.1	hypothetical protein FLJ10055
FLJ10241	45828_at	517 ±	44.2	945 ±	80.9	0.023	unknown		AI768100	hypothetical protein FLJ10241
FLJ10420	220731_s_at	443 ±	26.0	652 ±	45.1	0.019	unknown		NM_018090.1	hypothetical protein FLJ10420
FLJ10737	215792_s_at	568 ±	36.7	978 ±	46.2	0.003	unknown		AL109978.1	hypothetical protein FLJ10737
FLJ10846	219074_at	108 ±	11.4	214 ±	8.7	0.012	unknown		NM_018241.1	hypothetical protein FLJ10846
FLJ10996	219774_at	70 ±	4.4	110 ±	9.8	0.023	unknown		NM_019044.1	hypothetical protein FLJ10996

FLJ12666	217893_s_at	106 ±	7.0	160 ±	8.3	0.018	unknown		NM_024595.1	hypothetical protein FLJ12666
FLJ12875	218246_at	295 ±	24.1	581 ±	28.5	0.005	unknown		NM_024544.1	hypothetical protein FLJ12875
FLJ13057	218458_at	106 ±	8.3	227 ±	23.3	0.011	unknown		NM_022471.1	hypothetical protein FLJ13057 similar to germ cell-less
FLJ13448	219397_at	424 ±	21.5	650 ±	24.6	0.002	unknown		NM_025147.1	hypothetical protein FLJ13448
FLJ13657	219276_x_at	207 ±	23.9	516 ±	24.6	0.007	unknown		NM_024828.1	hypothetical protein FLJ13657
FLJ20288	208773_s_at	232 ±	15.1	315 ±	15.6	0.012	unknown		AL136943.1	FLJ20288 protein
FLJ20360	218331_s_at	145 ±	13.0	223 ±	13.7	0.042	unknown		NM_017782.1	hypothetical protein FLJ20360
FLJ20420	217972_at	778 ±	52.0	1357 ±	61.5	0.005	unknown		NM_017812.1	hypothetical protein FLJ20420
FLJ20507	219460_s_at	202 ±	13.2	394 ±	17.3	0.003	unknown		NM_017849.1	hypothetical protein FLJ20507
FLJ20758	217895_at	537 ±	28.0	689 ±	32.2	0.010	unknown		NM_017952.1	hypothetical protein FLJ20758
FLJ21901	219002_at	222 ±	27.7	1016 ±	62.4	0.004	unknown		NM_024622.1	hypothetical protein FLJ21901
FLJ22104	222209_s_at	48 ±	7.6	100 ±	6.7	0.039	unknown		AK000684.1	hypothetical protein FLJ22104
GL004	219137_s_at	267 ±	16.9	401 ±	17.2	0.007	unknown		NM_020194.1	GL004 protein
GNRPX	218290_at	447 ±	27.6	600 ±	34.5	0.015	unknown		NM_018049.1	likely ortholog of mouse guanine nucleotide releasing protein x
HSA011916	200035_at	483 ±	28.6	628 ±	40.7	0.035	unknown		NM_015343.1	likely ortholog of Xenopus dullard
HSPC002	219260_s_at	244 ±	19.8	407 ±	27.3	0.018	unknown		NM_015362.1	HSPC002 protein
HSPC125	219006_at	362 ±	30.6	930 ±	38.3	0.002	unknown		NM_014165.1	HSPC125 protein
HSPC132	218403_at	419 ±	25.0	626 ±	37.6	0.011	unknown		NM_016399.1	hypothetical protein HSPC132
HSU79252	204594_s_at	184 ±	11.7	412 ±	21.5	0.005	unknown		NM_013298.1	hypothetical protein HSU79252
HT008	218099_at	483 ±	30.4	637 ±	28.7	0.014	unknown		NM_018469.1	uncharacterized hypothalamus protein HT008
HTCD37	209586_s_at	390 ±	21.7	469 ±	23.2	0.033	unknown		AF123539.1	TcD37 homolog
KIAA0157	212835_at	96 ±	9.0	162 ±	10.0	0.024	unknown		D63877.1	KIAA0157 protein
KIAA0174	200851_s_at	822 ±	76.6	1364 ±	60.5	0.023	unknown		NM_014761.1	KIAA0174 gene product
KIAA0232	212441_at	307 ±	23.7	425 ±	28.3	0.018	unknown		D86985.2	KIAA0232 gene product
KIAA0258	203169_at	78 ±	5.6	134 ±	6.9	0.007	unknown		NM_014785.1	KIAA0258 gene product
KIAA0265	210111_s_at	442 ±	23.5	687 ±	39.3	0.010	unknown		AF277175.1	KIAA0265 protein
KIAA0350	212786_at	81 ±	7.9	144 ±	7.1	0.008	unknown		AA731693	KIAA0350 protein
KIAA0795	212882_at	316 ±	22.5	517 ±	35.4	0.024	unknown		AB018338.1	KIAA0795 protein
KIAA0962	212911_at	35 ±	2.1	55 ±	2.5	0.005	unknown		AB023179.1	KIAA0962 protein
KIAA1100	203286_at	183 ±	10.6	295 ±	12.3	0.002	unknown		NM_014901.1	KIAA1100 protein
LAP1B	212408_at	555 ±	52.4	803 ±	35.7	0.042	unknown		AK023204.1	DKFZP586G011 protein
LOC113251	212714_at	476 ±	30.4	710 ±	43.0	0.017	unknown		AL050205.1	c-Mpl binding protein
	214155_s_at	36 ±	2.4	55 ±	5.2	0.031	unknown		AI743740	c-Mpl binding protein
LOC51125	217819_at	787 ±	45.9	973 ±	58.0	0.026	unknown		NM_016099.1	HSPC041 protein
LOC51161	219114_at	163 ±	13.9	328 ±	25.2	0.012	unknown		NM_016210.1	g20 protein
LOC51204	221069_s_at	376 ±	26.4	695 ±	40.4	0.005	unknown		NM_016360.1	clone HQ0477 PRO0477p
LOC51295	218225_at	176 ±	15.5	366 ±	30.0	0.013	unknown		NM_016581.1	ECSIT
LOC55831	217882_at	577 ±	37.5	841 ±	59.5	0.029	unknown		NM_018447.1	30 kDa protein
LOC57019	208424_s_at	239 ±	16.7	442 ±	29.6	0.010	unknown		NM_020313.1	hypothetical protein LOC57019
LOC57019	208968_s_at	828 ±	71.4	1418 ±	74.9	0.015	unknown		BC002568.1	hypothetical protein LOC57019
LOC91966	212961_x_at	689 ±	38.6	1004 ±	61.9	0.020	unknown		L43578.1	hypothetical protein LOC91966
	213315_x_at	728 ±	41.2	1116 ±	48.5	0.006	unknown		L43577	hypothetical protein LOC91966
	214112_s_at	403 ±	24.3	631 ±	43.3	0.021	unknown		AA543076	hypothetical protein LOC91966
LRPPRC	211615_s_at	521 ±	50.2	856 ±	46.5	0.033	unknown		M92439.1	leucine-rich PPR-motif containing
	211971_s_at	501 ±	35.3	901 ±	48.4	0.008	unknown		AF052133.1	leucine-rich PPR-motif containing
M9	212716_s_at	1157 ±	83.7	1953 ±	133.3	0.018	unknown		AW083133	muscle specific gene

MDS029	218597_s_at	527 ±	37.5	933 ±	80.0	0.022	unknown			NM_018464.1	uncharacterized hematopoietic stem/progenitor cells protein MDS029
MDS030	218992_at	218 ±	17.9	377 ±	25.3	0.017	unknown			NM_018465.1	uncharacterized hematopoietic stem/progenitor cells protein MDS030
MGC11308	212861_at	236 ±	15.8	375 ±	24.6	0.023	unknown			BF690150	hypothetical protein MGC11308
MGC13033	209688_s_at	151 ±	11.6	262 ±	11.2	0.011	unknown			BC005078.1	hypothetical protein MGC13033
MGC14376	214696_at	183 ±	12.0	270 ±	18.2	0.033	unknown			AF070569.1	hypothetical protein MGC14376
MGC21654	214061_at	117 ±	11.6	203 ±	17.7	0.038	unknown			AI017564	unknown MGC21654 product
MGC2198	209329_x_at	941 ±	61.1	1427 ±	75.6	0.004	unknown			BC000587.1	hypothetical protein MGC2198
MGC4276	209273_s_at	365 ±	28.0	552 ±	38.9	0.023	unknown			BG387555	hypothetical protein MGC4276 similar to CG8198
MGC4276	209274_s_at	444 ±	32.1	708 ±	43.5	0.008	unknown			BC002675.1	hypothetical protein MGC4276 similar to CG8198
NSPC1	210023_s_at	533 ±	40.5	814 ±	54.8	0.019	unknown			BC004952.1	likely ortholog of mouse nervous system polycomb 1
PARL	218271_s_at	612 ±	38.1	840 ±	43.1	0.014	unknown			NM_018622.1	presenilins associated rhomboid-like protein
SS18L1	213140_s_at	148 ±	8.5	224 ±	19.9	0.036	unknown			AB014593.1	synovial sarcoma translocation gene on chromosome 18-like 1
TERA	220147_s_at	1024 ±	73.0	1456 ±	74.5	0.033	unknown			NM_021238.1	TERA protein
TSBP	202060_at	159 ±	12.6	311 ±	17.3	0.005	unknown			NM_014633.1	likely ortholog of mouse TPR-containing, SH2-binding phosphoprotein
ZNF294	215596_s_at	278 ±	16.1	430 ±	18.7	0.004	unknown			AL163248	zinc finger protein 294
ZNF363	212749_s_at	315 ±	29.6	530 ±	26.5	0.029	unknown			AL050144.1	zinc finger protein 363
	214281_s_at	315 ±	17.8	483 ±	30.8	0.021	unknown			AA524525	zinc finger protein 363

Table 2

Genes that were significantly induced by PGC-1 and repressed by glucocorticoids

Product	Probe set ID	control vehicle	± SEM	control cort	± SEM	PGC-1α vehicle	± SEM	PGC-1α cort	± SEM	classification	Description	RefSeq
NRIP1	202599_s_at	189 ±	24.6	260 ±	21.9	679 ±	18.6	280 ±	30.0	transcriptional coreguator	nuclear receptor interacting protein 1	NM_003489
PRPF4B	202126_at	280 ±	25.3	225 ±	14.3	408 ±	16.0	250 ±	22.7	mRNA processing	PRP4 pre-mRNA processing factor 4 homolog B (yeast)	AA156948
DNAJA3	205963_s_at	844 ±	46.3	721 ±	53.9	1134 ±	48.9	880 ±	53.2	protein folding	DnaJ (Hsp40) homolog, subfamily A, member 3	NM_005147
PCK1	208383_s_at	16 ±	1.0	17 ±	0.9	73 ±	2.4	19 ±	1.0	metabolism	phosphoenolpyruvate carboxykinase 1 (soluble)	NM_002591
CYP24A1	206504_at	15 ±	0.9	14 ±	0.8	35 ±	2.1	18 ±	1.0	mitochondrial, calcium homeostasis	cytochrome P450, family 24, subfamily A, polypeptide 1	NM_000782
ADCY9	204497_at	109 ±	6.7	85 ±	6.9	165 ±	7.5	110 ±	8.1	signaling	adenylate cyclase 9	AB011092
PPP1R3C	204284_at	259 ±	18.3	279 ±	19.8	661 ±	58.7	257 ±	17.6	signaling	protein phosphatase 1, regulatory (inhibitor) subunit 3C	N26005
FLJ13448	219397_at	424 ±	21.5	395 ±	21.9	650 ±	24.6	478 ±	27.9	signaling	hypothetical protein FLJ13448	NM_025147
BMP2	205290_s_at	138 ±	7.6	73 ±	7.6	1192 ±	112.9	119 ±	12.8	differentiation	bone morphogenetic protein 2	NM_001200
	205289_at	66 ±	3.2	45 ±	3.1	674 ±	54.9	55 ±	4.8	differentiation	bone morphogenetic protein 2	AA583044
SYNJ1	212990_at	136 ±	12.6	125 ±	9.2	212 ±	14.0	137 ±	11.9	neurotransmitter transport	synaptojanin 1	AB020717
KIAA0795	212882_at	316 ±	22.5	265 ±	20.7	517 ±	35.4	352 ±	22.6	unknown	KIAA0795 protein	AB018338
ZNF363	212749_s_at	315 ±	29.6	251 ±	18.9	530 ±	26.5	330 ±	30.1	unknown	zinc finger protein 363	AL050144
TERA	220147_s_at	1024 ±	73.0	699 ±	60.2	1456 ±	74.5	947 ±	80.8	unknown	TERA protein	NM_021238
SS18L1	213140_s_at	148 ±	8.5	128 ±	7.7	224 ±	19.9	138 ±	8.7	unknown	synovial sarcoma translocation gene on chromosome 18-li	AB014593
G0S2	213524_s_at	48 ±	5.3	50 ±	5.8	1555 ±	98.2	217 ±	26.4	cell cycle/cell growth	putative lymphocyte G0/G1 switch gene	NM_015714
MRPS30	218398_at	330 ±	26.2	260 ±	21.6	553 ±	34.2	390 ±	23.3	mitochondrial protein synthesis, proap	mitochondrial ribosomal protein S30	NM_016640
HMGE	212434_at	361 ±	24.6	301 ±	24.2	598 ±	29.3	438 ±	27.0	mitochondrial, protein folding	GrpE-like protein cochaperone	AL542571
B4GALT5	221485_at	435 ±	24.7	330 ±	23.8	855 ±	25.6	512 ±	36.3	posttranslational protein modification	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, poly	NM_004776
HSPB7	218934_s_at	296 ±	25.0	215 ±	24.9	1055 ±	44.9	757 ±	37.5	protein folding	heat shock 27kDa protein family, member 7 (cardiovascula	NM_014424
TIMP3	201150_s_at	70 ±	5.5	42 ±	3.3	158 ±	12.1	70 ±	4.9	proteolysis/extracellular matrix degrad	tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystr	NM_000362
VEGF	211527_x_at	197 ±	16.1	130 ±	15.0	286 ±	15.4	213 ±	12.8	signaling	vascular endothelial growth factor	M27281
	210512_s_at	365 ±	28.5	199 ±	22.7	662 ±	24.1	432 ±	25.7	signaling	vascular endothelial growth factor	AF022375
NCOA4	210774_s_at	1296 ±	79.1	1239 ±	85.3	2399 ±	97.3	1902 ±	94.1	transcriptional coactivator	nuclear receptor coactivator 4	AL162047

Table 3

Genes that were induced by PGC-1α and GR together

Product	Probe set ID	control vehicle	± SEM	control cort	± SEM	PGC-1α vehicle	± SEM	PGC-1α cort	± SEM	classification 1	classification 2	accession number	Description
PHLDA1	217997_at	45 ±	3.9	53 ±	2.7	48 ±	3.5	90 ±	6.5	apoptosis	cell cylce/ cell growth	AA576961	pleckstrin homology-like domain, family A, member 1
BCL10	205263_at	368 ±	24.0	456 ±	26.9	456 ±	29.6	704 ±	38.0	apoptosis		AF082283.1	B-cell CLL/lymphoma 10
CTGF	209101_at	86 ±	11.8	219 ±	18.1	109 ±	10.8	464 ±	19.2	cell adhesion		M92934.1	connective tissue growth factor
TPBG	203476_at	503 ±	75.5	816 ±	37.2	332 ±	43.4	1148 ±	57.7	cell adhesion/ cell motility		NM_006670.1	trophoblast glycoprotein
CDKN1A	202284_s_at	330 ±	36.3	1065 ±	50.2	334 ±	45.5	1936 ±	86.4	cell cylce/ cell growth	DNA damage repair	NM_000389.1	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
AREG	205239_at	16 ±	0.9	20 ±	1.1	17 ±	1.1	44 ±	1.4	cell cylce/ cell growth	signaling	NM_001657.1	amphiregulin (schwannoma-derived growth factor)
DDX16	203694_s_at	612 ±	45.2	634 ±	51.7	752 ±	57.3	1119 ±	59.7	cell cylce/ cell growth		NM_003587.2	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 16
GAS2L1	31874_at	142 ±	19.9	254 ±	21.4	407 ±	24.3	842 ±	26.4	cytoskeleton	signaling, cell growth	Y07846	growth arrest-specific 2 like 1
CRK	202224_at	376 ±	29.0	448 ±	23.2	483 ±	26.5	735 ±	40.8	cytoskeleton/ cell motility	cell cylce/ cell growth	NM_016823.1	v-crk sarcoma virus CT10 oncogene homolog (avian)
	202225_at	258 ±	18.7	322 ±	32.8	345 ±	24.7	492 ±	22.5	cytoskeleton/ cell motility	cell cylce/ cell growth	NM_016823.1	v-crk sarcoma virus CT10 oncogene homolog (avian)
	202226_s_at	579 ±	35.2	696 ±	37.2	722 ±	44.8	1031 ±	67.0	cytoskeleton/ cell motility	cell cylce/ cell growth	NM_016823.1	v-crk sarcoma virus CT10 oncogene homolog (avian)
GAS2L1	208258_s_at	70 ±	5.9	89 ±	6.6	121 ±	12.4	191 ±	8.9	cytoskeleton/ cell motility	signaling, cell growth	NM_006478.1	growth arrest-specific 2 like 1
	209729_at	168 ±	14.8	246 ±	20.2	405 ±	37.3	716 ±	44.5	cytoskeleton/ cell motility	signaling, cell growth	BC001782.1	growth arrest-specific 2 like 1
BBS1	218471_s_at	88 ±	6.9	148 ±	7.8	116 ±	12.9	390 ±	21.6	development		NM_024649.1	Bardet-Biedl syndrome 1
USP2	207213_s_at	59 ±	4.3	73 ±	4.1	59 ±	4.9	134 ±	5.7	deubiquitination	apoptosis, activator	NM_004205.1	ubiquitin specific protease 2
IL22RA1	220056_at	54 ±	3.8	96 ±	4.1	53 ±	6.0	232 ±	16.2	immune response		NM_021258.1	interleukin 22 receptor, alpha 1
SLC19A2	209681_at	80 ±	11.3	261 ±	11.9	122 ±	10.4	399 ±	25.0	metabolism, AS transport		AF153330.1	solute carrier family 19 (thiamine transporter), member 2
CA2	209301_at	39 ±	2.9	66 ±	6.1	58 ±	3.2	95 ±	4.1	metabolism, carbon		M36532.1	carbonic anhydrase II
HSPA6	213418_at	109 ±	10.4	124 ±	7.1	109 ±	8.9	240 ±	11.5	protein folding	stress response	NM_002155.1	heat shock 70kDa protein 6 (HSP70B')
PARG1	203910_at	94 ±	10.8	215 ±	8.7	65 ±	10.4	430 ±	13.3	signaling	cytoskeleton, GAP	NM_004815.1	PTPL1-associated RhoGAP 1
NY-REN-45	217894_at	24 ±	2.0	41 ±	2.4	28 ±	1.9	66 ±	3.0	signaling		NM_016121.1	NY-REN-45 antigen
TEM6	217853_at	297 ±	23.9	301 ±	18.3	221 ±	23.2	433 ±	28.3	signaling		NM_022748.1	tumor endothelial marker 6
C6orf4	215411_s_at	618 ±	28.2	486 ±	29.2	438 ±	44.8	723 ±	34.7	signaling	immune response	AL008730	chromosome 6 open reading frame 4
TSPAN-3	200973_s_at	650 ±	37.3	748 ±	40.5	636 ±	52.0	960 ±	45.2	signaling	cell growth, cell motility	NM_005724.1	tetraspan 3
LIFR	205876_at	22 ±	1.6	28 ±	1.9	28 ±	1.6	45 ±	3.2	signaling	differentiation, apoptosis	NM_002310.2	leukemia inhibitory factor receptor
PDE4DIP	212390_at	140 ±	8.5	180 ±	9.4	140 ±	9.7	323 ±	32.6	signaling		AB007923.1	phosphodiesterase 4D interacting protein (myomegalin)
APXL	204967_at	104 ±	7.3	116 ±	6.0	102 ±	8.5	225 ±	19.0	signaling		NM_001649.1	apical protein-like (Xenopus laevis)
PITPN	201191_at	206 ±	12.2	274 ±	12.5	214 ±	13.5	375 ±	17.8	signaling	lipid metabolism	H15647	phosphotidylinositol transfer protein
HSD11B2	204130_at	30 ±	2.5	34 ±	2.4	31 ±	2.0	55 ±	4.1	signaling, glycocorticoid	metabolism, cholesterol	NM_000196.1	hydroxysteroid (11-beta) dehydrogenase 2
ACVR1B	213198_at	230 ±	15.7	236 ±	13.1	246 ±	15.2	362 ±	31.0	signaling	growth, differentiation	AL117643.1	activin A receptor, type IB
MAP3K7	206854_s_at	222 ±	18.5	334 ±	22.0	343 ±	25.6	512 ±	22.9	signaling		NM_003188.1	mitogen-activated protein kinase kinase kinase 7
KIAA0790	213236_at	179 ±	16.4	409 ±	36.5	137 ±	17.6	825 ±	61.5	signaling	cell cycle, transcription	AK025495.1	KIAA0790 protein
	41644_at	356 ±	29.9	685 ±	47.6	300 ±	32.8	1274 ±	139.4	signaling	cell cycle, transcription	AB018333	KIAA0790 protein

VDR	204254_s_at	140 ±	13.3	151 ±	9.7	95 ±	11.1	263 ±	15.9	transcription factor		NM_000376.1	vitamin D (1,25- dihydroxyvitamin D3) receptor
ZNF3	212684_at	152 ±	10.9	129 ±	7.7	159 ±	8.7	326 ±	18.9	transcription factor	differentiation, proliferation?	AI752257	zinc finger protein 3 (A8-51)
TGIF	203313_s_at	87 ±	8.2	129 ±	8.9	51 ±	6.9	222 ±	7.9	transcription factor	development, brain	NM_003244.1	TGFB-induced factor (TALE family homeobox)
GTF2F1	202355_s_at	253 ±	24.5	289 ±	27.5	328 ±	23.8	465 ±	21.9	transcriptional co-factor		BC000120.1	general transcription factor IIF, polypeptide 1, 74kDa
---	203356_at	166 ±	14.0	250 ±	15.7	171 ±	11.3	372 ±	16.0	unknown		BE349584	Homo sapiens cDNA FLJ36423 fis, clone THYMU2011308.
---	208774_at	499 ±	49.6	913 ±	60.5	566 ±	40.4	1364 ±	86.8	unknown		AV700224	Homo sapiens cDNA FLJ39631 fis, clone SMINT2002210.
CGI-36	220417_s_at	428 ±	43.6	664 ±	37.9	528 ±	49.7	954 ±	47.7	unknown		NM_015963.1	CGI-36 protein
FLJ14054	219054_at	72 ±	8.2	177 ±	10.4	102 ±	10.2	294 ±	8.4	unknown		NM_024563.1	hypothetical protein FLJ14054
FLJ20366	218692_at	200 ±	19.6	369 ±	21.0	210 ±	18.7	701 ±	22.9	unknown		NM_017786.1	hypothetical protein FLJ20366
KIAA0232	212441_at	307 ±	23.7	484 ±	26.5	425 ±	28.3	644 ±	36.3	unknown		D86985.2	KIAA0232 gene product
KIAA0763	203906_at	178 ±	12.9	249 ±	15.5	242 ±	17.6	367 ±	18.9	unknown	protein trafficking	AI652645	KIAA0763 gene product
GTL3	217957_at	843 ±	58.6	1060 ±	50.7	870 ±	75.9	1562 ±	66.2	unknown	transcriptional regulator	NM_013242.1	likely ortholog of mouse gene trap locus 3
---	210230_at	163 ±	11.5	186 ±	16.5	156 ±	13.8	375 ±	52.1	unknown		BC003629.1	Homo sapiens cDNA: FLJ23438 fis, clone HRC13275.
---	212979_s_at	117 ±	14.1	183 ±	11.5	133 ±	9.2	249 ±	10.8	unknown		BF791738	Homo sapiens PRO2751 mRNA, complete cds
---	65585_at	118 ±	6.7	114 ±	6.2	122 ±	7.3	171 ±	10.4	unknown		AA527515	Homo sapiens cDNA FLJ40819 fis, clone TRACH2010771.
FLJ10408	220535_at	64 ±	5.0	90 ±	4.4	69 ±	7.2	302 ±	42.9	unknown		NM_018088.1	hypothetical protein FLJ10408
FLJ10587	218514_at	434 ±	26.3	486 ±	37.2	500 ±	30.1	852 ±	35.4	unknown		NM_018149.1	hypothetical protein FLJ10587
FLJ12571	219758_at	93 ±	6.2	107 ±	5.3	100 ±	7.0	171 ±	7.7	unknown		NM_024926.1	hypothetical protein FLJ12571
KIAA0329	204307_at	87 ±	5.5	100 ±	6.5	86 ±	5.4	163 ±	8.1	unknown		AB002295.1	KIAA0329 gene product
	204308_s_at	95 ±	7.5	149 ±	7.6	99 ±	6.7	270 ±	22.8	unknown		NM_014844.1	KIAA0329 gene product
TNRC15	212260_at	272 ±	19.3	291 ±	25.2	314 ±	20.1	504 ±	22.1	unknown		AL045800	trinucleotide repeat containing 15
	212261_at	521 ±	27.8	527 ±	26.9	543 ±	30.3	832 ±	40.5	unknown		AL045800	trinucleotide repeat containing 15

Table 4

Genes that were induced by GR in the absence and presence of PGC-1α

Product	Probe set ID	control vehicle	± SEM	control cort	± SEM	PGC-1α vehicle	± SEM	PGC-1α cort	± SEM	classification 1	classification 2	accession number	Description
PIG3	210609_s_at	230 ±	18.4	447 ±	41.1	214 ±	20.3	446 ±	28.9	apoptosis		BC000474.1	quinone oxidoreductase homolog
CRADD	209833_at	171 ±	11.5	301 ±	14.4	147 ±	11.1	250 ±	16.4	apoptosis, activator		U79115.1	CASP2 and RIPK1 domain containing adaptor with death domain
BIRC3	210538_s_at	17 ±	1.5	32 ±	1.7	17 ±	1.4	42 ±	2.5	apoptosis, inhibitor		U37546.1	baculoviral IAP repeat-containing 3
CTGF	209101_at	86 ±	11.8	219 ±	18.1	109 ±	10.8	464 ±	19.2	cell adhesion		M92934.1	connective tissue growth factor
BAIAP2	205293_x_at	129 ±	12.1	260 ±	16.5	103 ±	11.1	388 ±	22.9	cell adhesion, cytokinesis	cytoskeleton, signaling	AB017120.1	BAI1-associated protein 2
	205294_at	176 ±	15.7	318 ±	19.7	130 ±	13.1	436 ±	40.5	cell adhesion, cytokinesis	cytoskeleton, signaling	NM_017450.1	BAI1-associated protein 2
FLRT3	219250_s_at	37 ±	2.1	183 ±	26.9	26 ±	2.4	67 ±	8.0	cell adhesion/ extracellular matrix	cell adhesion	NM_013281.1	fibronectin leucine rich transmembrane protein 3
COL4A2	211964_at	43 ±	5.5	159 ±	15.9	59 ±	6.4	227 ±	35.2	cell adhesion/ extracellular matrix		AK025912.1	collagen, type IV, alpha 2
	211966_at	47 ±	3.5	100 ±	10.4	48 ±	4.4	125 ±	11.0	cell adhesion/ extracellular matrix		AK025912.1	collagen, type IV, alpha 2
CDKN1A	202284_s_at	330 ±	36.3	1065 ±	50.2	334 ±	45.5	1936 ±	86.4	cell cycle	DNA damage repair	NM_000389.1	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CDC7L1	204510_at	409 ±	36.3	566 ±	25.5	322 ±	29.0	707 ±	39.1	cell cycle/cell growth	replication	NM_003503.2	CDC7 cell division cycle 7-like 1 (S. cerevisiae)
ING1	210350_x_at	92 ±	6.4	218 ±	21.9	100 ±	7.0	168 ±	14.1	cell cycle/cell growth inhibitor	apoptosis	AF044076.1	inhibitor of growth family, member 1
	209808_x_at	225 ±	14.9	530 ±	47.9	218 ±	15.7	393 ±	28.1	cell cycle/cell growth inhibitor	apoptosis	AW193656	inhibitor of growth family, member 1
PA26	218346_s_at	147 ±	14.2	350 ±	17.0	237 ±	16.8	344 ±	18.4	cell cycle/cell growth inhibitor		NM_014454.1	p53 regulated PA26 nuclear protein
OKL38	219475_at	144 ±	11.2	336 ±	39.0	152 ±	11.9	290 ±	20.7	cell cycle/cell growth inhibitor		NM_013370.1	pregnancy-induced growth inhibitor
PDZ-GEF1	203097_s_at	294 ±	34.4	652 ±	21.5	304 ±	29.2	623 ±	40.8	cytoskeletal organisation		NM_014247.1	PDZ domain containing guanine nucleotide exchange factor(GEF)1
GAS2L1	31874_at	142 ±	19.9	254 ±	21.4	407 ±	24.3	842 ±	26.4	cytoskeleton	signaling, cell growth	Y07846	growth arrest-specific 2 like 1
F3	204363_at	207 ±	52.5	1570 ±	79.5	316 ±	74.5	1702 ±	144.8	immune response	coagulation	NM_001993.2	coagulation factor III (thromboplastin, tissue factor)
PTX3	206157_at	84 ±	18.5	547 ±	28.6	74 ±	24.0	633 ±	42.9	immune response		NM_002852.1	pentaxin-related gene, rapidly induced by IL-1 beta
CD163	215049_x_at	48 ±	4.0	77 ±	3.8	52 ±	3.7	85 ±	4.7	immune response, anti-	oxidative stress	Z22969.1	CD163 antigen
BBS1	218471_s_at	88 ±	6.9	148 ±	7.8	116 ±	12.9	390 ±	21.6	development		NM_024649.1	Bardet-Biedl syndrome 1
SLC19A2	209681_at	80 ±	11.3	261 ±	11.9	122 ±	10.4	399 ±	25.0	metabolism, AS transport		AF153330.1	solute carrier family 19 (thiamine transporter), member 2
XK	206698_at	21 ±	1.4	59 ±	3.5	19 ±	1.6	53 ±	5.7	metabolism, AS transport		NM_021083.1	Kell blood group precursor (McLeod phenotype)
PDK4	205960_at	26 ±	3.2	81 ±	5.5	26 ±	3.1	141 ±	17.1	metabolism, carbohydrate		NM_002612.1	pyruvate dehydrogenase kinase, isoenzyme 4
CA2	209301_at	39 ±	2.9	66 ±	6.1	58 ±	3.2	95 ±	4.1	metabolism, carbon		M36532.1	carbonic anhydrase II
H105E3	209279_s_at	238 ±	25.5	638 ±	70.2	288 ±	30.3	870 ±	90.7	metabolism, cholesterol biosynthesis		BC000245.1	NAD(P) dependent steroid dehydrogenase-like; H105e3
	215093_at	78 ±	8.5	229 ±	22.5	104 ±	11.3	268 ±	29.2	metabolism, cholesterol biosynthesis		U82671	NAD(P) dependent steroid dehydrogenase-like; H105e3
FACL1	207275_s_at	385 ±	24.7	775 ±	47.9	356 ±	28.2	619 ±	35.8	metabolism, lipid catabolism		NM_001995.1	fatty-acid-Coenzyme A ligase, long-chain 1
ATP1A1	220948_s_at	1335 ±	99.2	1929 ±	122.5	1619 ±	113.9	2633 ±	191.8	metabolism, nutrient transport		NM_000701.1	ATPase, Na+/K+ transporting, alpha 1 polypeptide
GLUL	200648_s_at	122 ±	10.4	211 ±	22.5	105 ±	11.6	252 ±	18.3	neurotransmitter synthesis	AS metabolism, oxidative stres	NM_002065.1	glutamate-ammonia ligase (glutamine synthase)
	215001_s_at	205 ±	30.0	615 ±	39.2	178 ±	27.5	607 ±	76.0	neurotransmitter synthesis	AS metabolism, oxidative stres	AL161952.1	glutamate-ammonia ligase (glutamine synthase)
CHST3	209834_at	188 ±	12.9	385 ±	32.6	180 ±	16.8	403 ±	16.9	posttranslational protein modification		AB017915.1	carbohydrate (chondroitin 6) sulfotransferase 3
	32094_at	150 ±	11.7	294 ±	22.2	153 ±	14.1	301 ±	12.9	posttranslational protein modification		AB017915	carbohydrate (chondroitin 6) sulfotransferase 3
FKBP5	204560_at	99 ±	8.9	328 ±	47.3	83 ±	8.8	268 ±	39.5	posttranslational protein modification	transcriptional regulator	NM_004117.1	FK506 binding protein 5
AP3S1	202442_at	1216 ±	77.6	1649 ±	72.7	1002 ±	90.0	1795 ±	93.4	protein trafficking/vesicle transport	signaling	NM_001284.1	adaptor-related protein complex 3, sigma 1 subunit
GOLPH4	213568_at	20 ±	1.6	35 ±	1.9	29 ±	2.4	47 ±	4.2	protein trafficking/vesicle transport		AI811298	golgi phosphoprotein 4
RAB20	219622_at	124 ±	9.9	225 ±	13.8	141 ±	11.3	238 ±	13.2	protein trafficking/vesicle transport		NM_017817.1	RAB20, member RAS oncogene family

BLM	205733_at	179 ±	12.1	267 ±	16.9	157 ±	12.3	340 ±	17.5	recombination		NM_000057.1	Bloom syndrome
EP400	212375_at	268 ±	21.6	533 ±	26.9	226 ±	22.1	474 ±	28.2	replication, DNA repair		BE880591	E1A binding protein p400
	212376_s_at	355 ±	22.7	619 ±	38.5	293 ±	27.6	588 ±	33.6	replication, DNA repair		BE880591	E1A binding protein p400
NOL3	221566_s_at	84 ±	4.7	110 ±	7.7	84 ±	6.6	173 ±	19.0	RNA processing	apoptosis	AF043244.1	nucleolar protein 3 (apoptosis repressor with CARD domain)
MPHOSPH10	212885_at	410 ±	31.0	689 ±	27.5	385 ±	31.7	585 ±	40.7	RNA processing		AL545921	M-phase phosphoprotein 10 (U3 small nucleolar ribonucleoprotein)
RNAC	218544_s_at	262 ±	24.8	389 ±	22.3	226 ±	19.0	432 ±	35.3	RNA processing	ribosome biogenesis	NM_005772.1	RNA cyclase homolog
MAP3K7	206854_s_at	222 ±	18.5	334 ±	22.0	343 ±	25.6	512 ±	22.9	signaling		NM_003188.1	mitogen-activated protein kinase kinase kinase 7
PARG1	203910_at	94 ±	10.8	215 ±	8.7	65 ±	10.4	430 ±	13.3	signaling	cytoskeleton, GAP	NM_004815.1	PTPL1-associated RhoGAP 1
NY-REN-45	217894_at	24 ±	2.0	41 ±	2.4	28 ±	1.9	66 ±	3.0	signaling		NM_016121.1	NY-REN-45 antigen
AIM1	212543_at	619 ±	62.8	1573 ±	69.8	488 ±	76.9	1668 ±	92.3	signaling	cytoskeleton	U83115.1	absent in melanoma 1
ARHE	212724_at	508 ±	29.5	694 ±	40.9	372 ±	31.2	608 ±	62.1	signaling	cytoskeleton	BG054844	ras homolog gene family, member E
PITPN	201190_s_at	323 ±	25.5	489 ±	34.7	357 ±	28.2	593 ±	31.9	signaling		H15647	phosphotidylinositol transfer protein
FST	204948_s_at	184 ±	16.4	474 ±	19.2	171 ±	19.1	292 ±	17.2	signaling	development	NM_013409.1	follistatin
RGS19	204336_s_at	260 ±	19.3	454 ±	34.4	248 ±	18.4	494 ±	46.9	signaling		NM_005873.1	regulator of G-protein signalling 19
RGS2	202388_at	156 ±	22.5	694 ±	49.7	135 ±	26.1	894 ±	68.9	signaling		NM_002923.1	regulator of G-protein signalling 2, 24kDa
PANX1	204715_at	145 ±	12.8	256 ±	11.2	95 ±	13.0	285 ±	12.1	signaling		NM_015368.1	pannexin 1
BDNF	206382_s_at	103 ±	9.4	174 ±	9.3	66 ±	6.0	116 ±	7.4	signaling	survival	NM_001709.1	brain-derived neurotrophic factor
TNFRSF21	218856_at	358 ±	26.7	552 ±	24.8	222 ±	25.3	463 ±	27.5	signaling	immune response, apoptosis	NM_016629.1	tumor necrosis factor receptor superfamily, member 21
RASA3	206220_s_at	80 ±	5.8	126 ±	5.7	81 ±	6.3	119 ±	8.2	signaling		NM_007368.1	RAS p21 protein activator 3
PELI2	219132_at	42 ±	3.0	62 ±	2.4	37 ±	4.0	58 ±	3.7	signaling		NM_021255.1	pellino homolog 2 (Drosophila)
FZD1	204451_at	101 ±	8.0	233 ±	29.7	76 ±	8.3	149 ±	13.3	signaling		NM_003505.1	frizzled homolog 1 (Drosophila)
FZD7	203705_s_at	32 ±	2.4	52 ±	5.5	31 ±	2.4	64 ±	7.7	signaling		AI333651	frizzled homolog 7 (Drosophila)
	203706_s_at	82 ±	6.0	173 ±	9.8	61 ±	7.3	261 ±	7.9	signaling		NM_003507.1	frizzled homolog 7 (Drosophila)
KIAA0790	213236_at	179 ±	16.4	409 ±	36.5	137 ±	17.6	825 ±	61.5	signaling	cell cycle, transcription	AK025495.1	KIAA0790 protein
	41644_at	356 ±	29.9	685 ±	47.6	300 ±	32.8	1274 ±	139.4	signaling	cell cycle, transcription	AB018333	KIAA0790 protein
BCMP1	209656_s_at	270 ±	38.5	610 ±	43.7	250 ±	25.9	524 ±	49.9	signaling		AL136550.1	brain cell membrane protein 1
AKAP2	202759_s_at	548 ±	32.8	766 ±	52.9	491 ±	38.6	740 ±	42.9	signling		BE879367	A kinase (PRKA) anchor protein 2
AKAP7	205771_s_at	76 ±	10.9	235 ±	13.4	70 ±	9.4	216 ±	11.0	signling		AL137063	A kinase (PRKA) anchor protein 7
GADD45A	203725_at	815 ±	55.9	1385 ±	69.9	581 ±	63.8	1255 ±	96.8	stress response	DNA repair, cell cycle arrest	NM_001924.2	growth arrest and DNA-damage-inducible, alpha
SNN	218032_at	89 ±	19.6	733 ±	30.0	80 ±	26.3	846 ±	46.5	stress response		AF070673.1	stannin
SNN	218033_s_at	58 ±	7.2	149 ±	5.6	58 ±	6.6	183 ±	8.0	stress response		NM_003498.1	stannin
FOXO3A	210655_s_at	282 ±	27.0	411 ±	19.3	189 ±	20.6	449 ±	23.3	transcription factor	apoptosis	AF041336.1	forkhead box O3A
	204131_s_at	461 ±	36.6	837 ±	61.4	295 ±	33.7	783 ±	38.7	transcription factor	apoptosis	N25732	forkhead box O3A
	204132_s_at	146 ±	16.7	240 ±	9.4	93 ±	10.9	227 ±	13.3	transcription factor	apoptosis	NM_001455.1	forkhead box O3A
CEBPD	203973_s_at	268 ±	49.5	1514 ±	50.3	501 ±	57.6	1403 ±	76.4	transcription factor	immune response	NM_005195.1	CCAAT/enhancer binding protein (C/EBP), delta
ELK1	203617_x_at	698 ±	53.0	1299 ±	77.8	681 ±	71.1	1349 ±	91.1	transcription factor		NM_005229.2	ELK1, member of ETS oncogene family
	210376_x_at	313 ±	32.4	606 ±	25.9	332 ±	27.6	740 ±	29.2	transcription factor		M25269.1	ELK1, member of ETS oncogene family
KLF7	204334_at	199 ±	20.3	309 ±	12.0	168 ±	13.3	282 ±	18.8	transcription factor		AA488672	Kruppel-like factor 7 (ubiquitous)
MBNL2	203640_at	331 ±	30.4	632 ±	30.9	415 ±	39.7	742 ±	64.9	transcription factor		BE328496	muscleblind-like 2 (Drosophila)
PRKCBP1	209049_s_at	148 ±	8.9	206 ±	14.6	118 ±	10.2	226 ±	13.9	transcription factor, possible		BC001004.1	protein kinase C binding protein 1

ZNF145	205883_at	32 ±	4.1	156 ±	17.9	38 ±	4.2	149 ±	8.4	transcription factor, possible	apoptosis, growth, differentiati	NM_006006.1	zinc finger protein 145 (Kruppel-like, expressed in promyelocytic leukemia)
ZNF216	210275_s_at	1558 ±	103.3	2803 ±	164.4	1401 ±	113.8	3320 ±	147.1	transcription factor, possible		AF062347.1	zinc finger protein 216
	217741_s_at	722 ±	46.6	1379 ±	153.6	706 ±	65.6	1849 ±	93.2	transcription factor, possible		AW471220	zinc finger protein 216
HMGB3	203744_at	599 ±	41.7	953 ±	51.0	606 ±	60.2	1070 ±	102.6	transcription factor, possible		NM_005342.1	high-mobility group box 3
ZNF189	207513_s_at	176 ±	32.8	1031 ±	43.8	301 ±	37.1	1224 ±	105.9	transcription factor, possible		NM_003452.1	zinc finger protein 189
HIC2	212966_at	44 ±	4.8	81 ±	5.9	56 ±	7.2	115 ±	11.7	transcription factor, possible	development	AB028943.1	hypermethylated in cancer 2
BCL6	203140_at	94 ±	7.0	175 ±	10.2	85 ±	8.2	202 ±	9.3	transcription factor, repressor		NM_001706.1	B-cell CLL/lymphoma 6 (zinc finger protein 51)
MAFB	218559_s_at	378 ±	26.7	633 ±	29.9	277 ±	38.2	728 ±	29.7	transcription factor, repressor		NM_005461.1	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)
DSIPI	207001_x_at	101 ±	14.9	242 ±	15.7	102 ±	8.5	227 ±	22.5	transcriptional coactivator	immune response	NM_004089.1	delta sleep inducing peptide, immunoreactor
	208763_s_at	217 ±	43.7	1594 ±	112.2	256 ±	51.4	1690 ±	105.8	transcriptional coactivator	immune response	AL110191.1	delta sleep inducing peptide, immunoreactor
SP110	208012_x_at	279 ±	26.6	435 ±	24.8	295 ±	22.0	501 ±	22.8	transcriptional coactivator		NM_004509.1	SP110 nuclear body protein
CTNNBIP1	203081_at	217 ±	18.0	361 ±	31.4	212 ±	15.3	420 ±	22.7	transcriptional co-regulator	signaling, development, differe	NM_020248.1	catenin, beta interacting protein 1
PER1	202861_at	170 ±	15.4	462 ±	42.1	170 ±	15.9	716 ±	106.0	transcriptional coregulator	circadian clock	NM_002616.1	period homolog 1 (Drosophila)
	36829_at	321 ±	22.6	606 ±	27.1	312 ±	26.6	751 ±	75.3	transcriptional coregulator	circadian clock	AF022991	period homolog 1 (Drosophila)
NEDD4	213012_at	378 ±	28.2	849 ±	51.4	268 ±	30.9	857 ±	50.6	transcriptional coactivator	ubiquitination/proteasome degi	D42055.1	neural precursor cell expressed, developmentally down-regulated 4
---	203356_at	166 ±	14.0	250 ±	15.7	171 ±	11.3	372 ±	16.0	unknown		BE349584	Homo sapiens cDNA FLJ36423 fis, clone THYMU2011308.
---	208774_at	499 ±	49.6	913 ±	60.5	566 ±	40.4	1364 ±	86.8	unknown		AV700224	Homo sapiens cDNA FLJ39631 fis, clone SMINT2002210.
CGI-36	220417_s_at	428 ±	43.6	664 ±	37.9	528 ±	49.7	954 ±	47.7	unknown		NM_015963.1	CGI-36 protein
FLJ14054	219054_at	72 ±	8.2	177 ±	10.4	102 ±	10.2	294 ±	8.4	unknown		NM_024563.1	hypothetical protein FLJ14054
FLJ20366	218692_at	200 ±	19.6	369 ±	21.0	210 ±	18.7	701 ±	22.9	unknown		NM_017786.1	hypothetical protein FLJ20366
KIAA0232	212441_at	307 ±	23.7	484 ±	26.5	425 ±	28.3	644 ±	36.3	unknown		D86985.2	KIAA0232 gene product
LOC51668	214163_at	23 ±	2.2	42 ±	2.5	22 ±	1.9	58 ±	6.1	unknown	cell cycle?	AV700696	HSPCO34 protein
FLJ21079	219582_at	174 ±	16.2	563 ±	36.1	141 ±	14.8	418 ±	32.7	unknown	cell growth?	NM_024576.1	hypothetical protein FLJ21079
HRIHFB2122	216210_x_at	245 ±	16.6	429 ±	30.9	272 ±	20.1	517 ±	28.8	unknown	cytoskeleton organisation	AA046650	Tara-like protein
	202795_x_at	223 ±	14.8	353 ±	22.6	236 ±	16.5	421 ±	19.8	unknown	cytoskeleton organisation	NM_007032.1	Tara-like protein
	210276_s_at	104 ±	9.1	220 ±	20.7	118 ±	11.1	293 ±	14.8	unknown	cytoskeleton organisation	AF281030.1	Tara-like protein
EPB41L4B	220161_s_at	56 ±	5.4	168 ±	7.5	58 ±	7.4	190 ±	21.7	unknown	cytoskeleton, cell shape	NM_019114.1	erythrocyte membrane protein band 4.1 like 4B
DJ971N18.2	201581_at	446 ±	38.1	656 ±	31.8	544 ±	32.9	797 ±	58.0	unknown	enzyme, isomerase, protein m	AL544094	hypothetical protein DJ971N18.2
SLA	203761_at	107 ±	10.5	204 ±	15.5	98 ±	12.0	268 ±	26.8	unknown	enzyme, kinase, signaling	NM_006748.1	Src-like-adaptor
KIAA0111	201303_at	2911 ±	174.9	3963 ±	219.6	3311 ±	188.6	4275 ±	212.2	unknown	mRNA processing	NM_014740.1	KIAA0111 gene product
PSCDBP	209606_at	19 ±	2.2	54 ±	3.8	18 ±	1.9	53 ±	2.6	unknown	transcription factor, possible	L06633.1	pleckstrin homology, Sec7 and coiled/coil domains, binding protein
FLJ11078	219354_at	157 ±	14.0	239 ±	17.0	177 ±	10.9	260 ±	12.2	unknown	transcriptional regulator, inhibit	NM_018316.1	hypothetical protein FLJ11078
RNF144	204040_at	109 ±	8.4	179 ±	8.9	109 ±	14.0	185 ±	15.0	unknown	ubiquitination/proteasome degi	NM_014746.1	ring finger protein 144
---	217185_s_at	311 ±	44.9	589 ±	49.5	312 ±	33.1	630 ±	59.0	unknown		Z95118	---
---	217202_s_at	127 ±	15.0	243 ±	26.2	94 ±	10.8	249 ±	32.9	unknown		U08626	---
---	221902_at	130 ±	9.8	259 ±	19.0	119 ±	14.9	280 ±	18.4	unknown		AL567940	ESTs, Weakly similar to A43932 mucin 2 precursor, intestinal - human (frag
	64942_at	306 ±	24.6	485 ±	20.0	298 ±	23.4	533 ±	37.7	unknown		AI937160	ESTs, Weakly similar to A43932 mucin 2 precursor, intestinal - human (frag
---	64418_at	111 ±	7.2	162 ±	11.3	119 ±	8.4	197 ±	13.8	unknown		AI472320	Homo sapiens cDNA FLJ34482 fis, clone HLUNG2004067.
---	221841_s_at	55 ±	10.5	216 ±	12.1	77 ±	17.1	280 ±	19.3	unknown		BF514079	Homo sapiens cDNA FLJ38575 fis, clone HCHON2007046.
C14orf132	218820_at	510 ±	49.4	869 ±	32.3	377 ±	45.9	806 ±	44.4	unknown		NM_020215.1	chromosome 14 open reading frame 132

DKFZP434J214	212665_at	488 ±	134.2	4824 ±	270.1	725 ±	219.2	4725 ±	309.5	unknown		AL556438	DKFZP434J214 protein
DKFZP564G202	212202_s_at	175 ±	11.0	242 ±	14.2	177 ±	17.8	287 ±	19.3	unknown		AF132733.1	DKFZP564G2022 protein
	212204_at	456 ±	28.5	606 ±	31.8	459 ±	32.1	697 ±	30.3	unknown		AF132733.1	DKFZP564G2022 protein
DKFZP586F242	212345_s_at	412 ±	37.2	656 ±	37.1	333 ±	31.3	732 ±	38.7	unknown		BE675139	hypothetical protein DKFZp586F2423
FLJ20287	218104_at	442 ±	40.7	707 ±	32.0	442 ±	36.5	855 ±	34.7	unknown		NM_017746.1	hypothetical protein FLJ20287
FLJ20748	222116_s_at	415 ±	46.2	769 ±	57.2	350 ±	39.1	717 ±	63.2	unknown		AL157485.1	hypothetical protein FLJ20748
FLJ21313	218706_s_at	77 ±	7.1	217 ±	7.9	102 ±	7.3	181 ±	16.4	unknown		AW575493	hypothetical protein FLJ21313
HYA22	201904_s_at	254 ±	28.1	525 ±	25.3	223 ±	23.3	596 ±	27.5	unknown		BF031714	HYA22 protein
	201906_s_at	259 ±	25.9	511 ±	30.2	222 ±	24.9	622 ±	30.0	unknown		NM_005808.1	HYA22 protein
KIAA0469	203068_at	798 ±	52.3	1405 ±	65.4	737 ±	67.2	1237 ±	70.7	unknown		NM_014851.1	KIAA0469 gene product
KIAA0870	212975_at	161 ±	10.5	290 ±	20.3	131 ±	11.5	273 ±	17.2	unknown		AB020677.2	KIAA0870 protein
LBH	221011_s_at	229 ±	21.1	529 ±	43.3	201 ±	23.1	578 ±	48.1	unknown		NM_030915.1	likely ortholog of mouse limb-bud and heart gene
LHFPL2	212658_at	269 ±	14.1	433 ±	21.8	196 ±	16.3	324 ±	23.1	unknown		N66633	lipoma HMGIC fusion partner-like 2
LOC283537	214719_at	132 ±	17.4	356 ±	31.8	207 ±	24.9	434 ±	45.6	unknown		AK026720.1	hypothetical protein LOC283537
LOC57406	221552_at	72 ±	5.1	120 ±	7.3	75 ±	5.0	119 ±	9.5	unknown		BC001698.1	lipase protein
MDS006	220606_s_at	80 ±	7.0	160 ±	7.8	80 ±	6.7	172 ±	12.1	unknown		NM_020233.1	x 006 protein
MGC3222	217795_s_at	589 ±	41.8	1096 ±	67.6	686 ±	46.0	1546 ±	127.4	unknown		W74580	hypothetical protein MGC3222
MGC4701	210054_at	62 ±	4.8	131 ±	8.8	60 ±	5.9	137 ±	5.9	unknown		BC003648.1	hypothetical protein MGC4701
PRO2577	218172_s_at	189 ±	20.2	337 ±	31.3	199 ±	14.2	380 ±	25.3	unknown		NM_018630.1	hypothetical protein PRO2577
PRO2730	213549_at	73 ±	8.7	151 ±	11.8	85 ±	7.6	197 ±	12.1	unknown		AI890972	hypothetical protein PRO2730
RERE	200940_s_at	323 ±	20.8	487 ±	23.6	300 ±	27.8	605 ±	32.6	unknown		AB036737.1	arginine-glutamic acid dipeptide (RE) repeats
ZNF259	200054_at	352 ±	32.5	597 ±	50.9	325 ±	30.8	661 ±	48.0	unknown		NM_003904.1	zinc finger protein 259

Appendix II

The PGC-1 – related protein PERC is a selective coactivator of estrogen receptor α.

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Appendix II

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 16, Issue of April 19, pp. 13918–13925, 2002

Printed in U.S.A.

The PGC-1-related Protein PERC Is a Selective Coactivator of Estrogen Receptor α*

Received for publication, February 4, 2002
Published, JBC Papers in Press, February 19, 2002, DOI 10.1074/jbc.M201134200

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Peroxisome proliferator-activated receptor γ coactivator-1 (PGC-1) is a tissue-specific coactivator that enhances the activity of many nuclear receptors and coordinates transcriptional programs important for energy metabolism. We describe here a novel PGC-1-related coactivator that is expressed in a similar tissue-specific manner as PGC-1, with the highest levels in heart and skeletal muscle. In contrast to PGC-1, the new coactivator shows high receptor specificity. It enhances potently the activity of estrogen receptor (ER) α, while having only small effects on other receptors. Because of its nuclear receptor selectivity, we have termed the new protein PERC (PGC-1 related Estrogen Receptor Coactivator). We show here that the coactivation function of PERC relies on a bipartite transcriptional activation domain and two LXXLL motifs that interact with the AF2 domain of ERα in an estrogen-dependent manner. PERC and PGC-1 are likely to have different functions in ER signaling. Whereas PERC acts selectively on ERα and not on the second estrogen receptor ERβ, PGC-1 coactivates strongly both ERs. Moreover, PERC and PGC-1 show distinct preferences for enhancing ERα in different promoter contexts. Finally, PERC enhances the ERα-mediated response to the partial agonist tamoxifen, while PGC-1 modestly represses it. The two coactivators are likely to mediate distinct, tissue-specific responses to estrogens.

Nuclear receptors are ligand-regulated transcription factors with a broad range of functions in development, physiology, and behavior. They include steroid hormone receptors for glucocorticoids, mineralocorticoids, progestins, estrogens, and androgens, as well as receptors for thyroid hormone, retinoids, vitamin D, and intermediary metabolites (1). They use a conserved DNA binding domain (DBD)[1] to interact with specific sites in the genome, termed hormone response elements (HREs). DNA-bound receptors can activate the expression of genes in the vicinity of HREs, via two transcriptional activation functions, denoted AF1 and AF2. AF1 lies in the N-terminal part of the receptors and varies significantly from one receptor to another. AF2 is located at the conserved ligand binding domain (LBD) and relies on an agonist ligand-induced protein conformation (2–5). Depending on cellular and promoter context, AF1 and AF2 act independently or synergistically to regulate gene expression.

A large number of proteins that interact with the AF2 domain and enhance the activity of nuclear receptors have been identified (reviewed in Refs. 6–8). They include the three members of the p160 steroid receptor coactivator (SRC) family (SRC-1/NcoA-1, TIF2/GRIP1/NcoA-2, AIB1/pCIP/ACTR/RAC3/SRC-3), the cointegrators CBP and p300, components of the Mediator complex, individual coactivators such as PGC-1, NRIF3, ASC-2/RAP250, PELP1, and CAPER, and the family of CITED proteins (6–12). Most of these coactivators harbor one or multiple LXXLL motifs (L being leucine and *X* any amino acid) within short amphipathic helices (13, 14). These LXXLL motifs, also called NR boxes, interact with a hydrophobic pocket of the ligand-activated LBD of the receptors, thereby recruiting the coactivators to target DNA sites (15–17). The diverse coactivators are then thought to regulate transcription via enzymatic modification of chromatin or other transcription proteins, and/or physical recruitment of components of the transcriptional machinery (reviewed in Refs. 6–8). The multitude of nuclear receptor coactivators suggests that at least some of them carry distinct and specific functions. They may do so by interacting with specific subsets of receptors, acting in selective cell types, directing receptor function to subsets of target genes or conferring regulation by other signals.

Of the so far identified AF2 coactivators, most interact with many, if not all, nuclear receptors. Although particular LXXLL motifs of SRC-1, TIF2, and SRC-3 display preferences for specific receptors, the three p160 coactivators can enhance the activity of most nuclear receptors (18, 19). CBP and p300 are general coactivators, not only of nuclear receptors but also of many nonreceptor transcription factors (7). AF2 coactivators that display receptor specificity include NRIF3, PELP1, CAPER, and CITED1. NRIF3 enhances selectively the activity of the thyroid hormone receptor (TR) and retinoid X receptor (RXR), without affecting the glucocorticoid (GR), estrogen (ER) or vitamin D receptors (9). The other three receptor-selective coactivators potentiate preferentially the activity of the two ERs, ERα and ERβ (10–12). None of the ER-specific AF2 coactivators described so far distinguish between ERα and ERβ,

* This work was supported by the Swiss National Science Foundation, the University of Basel, Novartis Stiftung (to S. N. S.), and the Max Cloëtta Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF468496 and AF468497.

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[1] The abbreviations used are: DBD, DNA binding domain; HREs, hormone response elements; LBD, ligand binding domain; SRC, steroid receptor coactivator; TR, thyroid hormone receptor; RXR, retinoid X receptor; GR, glucocorticoid receptor; ER, estrogen receptor; PPAR, peroxisome proliferator-activated receptor; PGC-1, PPARγ coactivator 1; HNF, hepatocyte nuclear factor; NRF, nuclear respiratory factor; PRC, PGC-1-related coactivator; PERC, PGC-1-related estrogen receptor coactivator; ERE, estrogen response element; aa, amino acid(s); PR, progesterone receptor; MR, mineralocorticoid receptor; AR, androgen receptor; AD, activation domain; RRM, RNA recognition motif; β-gal, β-galactosidase; HA, hemagglutinin.

receptors that bind similar ligands and carry distinct biological functions (20, 21).

Few coactivators show tissue-specific expression. One of them is PGC-1, which is expressed at high levels in tissues such as heart, skeletal muscle, kidney, and brown fat (22–24). PGC-1 expression is induced also in a tissue-specific manner, in response to particular physiological states such as exposure to cold or fasting (22, 25, 26). Induction of PGC-1 in response to signals indicating metabolic needs of an organism can then lead to the activation of pathways important for energy homeostasis, such as adaptive thermogenesis, mitochondrial biogenesis, fatty acid oxidation, and gluconeogenesis (22, 25–29). PGC-1 interacts with and enhances the activity of many nuclear receptors, like the peroxisome proliferator-activated receptors (PPAR) α and γ, TR, GR, ERα, hepatocyte nuclear factor 4 (HNF4), as well as nonreceptor transcription factors like the nuclear respiratory factor 1 (NRF1) (22, 24, 26–28, 30). A characteristic feature of PGC-1, not shared by other nuclear receptor coactivators, is its C-terminal domain. It harbors sequence motifs typical of RNA processing regulators and has been implicated in the regulation of pre-mRNA splicing (31).

The existence of sequence-related coactivators, such as the three p160 SRC proteins, or CBP and p300, may reflect the evolutionary adaptation of duplicated genes to similar but distinct biological functions. Recently, a PGC-1 related coactivator (PRC) that is ubiquitously expressed and enhances the activity of NRF1 was described (32). Here, we report the cloning and characterization of a third member of the family. PERC (PGC-1 related estrogen receptor coactivator) is expressed in a tissue-specific manner and displays a striking preference for functional interactions with ERα among the nuclear receptors.

EXPERIMENTAL PROCEDURES

Cloning of PERC—Total RNA was isolated from HeLa cells with the Trizol reagent (Invitrogen). Full-length cDNA was synthesized either by standard procedures using oligo(dT) primers or with the GeneRacer kit (Invitrogen). Oligo(dT)-primed cDNA was used to amplify sequences from exon 2 to the end of the predicted PERC open reading frame. The 5′ part and first exon of the cDNA, which were absent from the published genome sequence, were amplified in a nested polymerase chain reaction (PCR), using internal exon 3-specific PERC primers, 5′ GeneRacer Primers, and cDNA synthesized with the GeneRacer kit. Multiple clones were analyzed and sequenced. Two types of PERC cDNAs were found at a ratio of 1:1 (of 12 clones). They differed by a 117-bp sequence, which corresponds to exon 4 of PERC. Restriction sites were introduced by PCR at the 5′ and 3′ ends of the PERC coding sequences, and full-length PERC (including the 117-bp exon 4) and PERC-s (lacking exon 4) clones were constructed by standard subcloning procedures. The PERC sequences have been submitted to the GenBank™ data base under accession numbers AF468496 and AF468497. The full-length PERC is the human homolog of the recently described mouse PGC-1β (33).

Expression Analysis—Total RNA was isolated from tissues of 6–8-week-old mice using the Trizol reagent and checked for its integrity by agarose gel electrophoresis and ethidium bromide staining. RNA (400 ng) was converted to cDNA in a 20-μl reaction at 45 °C for 45 min using MultiScribe reverse transcriptase (Applied Biosystems) and random hexamer primers according to the manufacturer's instructions. Real-time PCR with the LightCycler system (Roche Diagnostics) was used for the amplification and quantification of PERC, PGC-1, and β-actin cDNA. LightCycler reactions were performed in a final volume of 15 μl, using 3 μl of cDNA, 10 pmol of specific primers, and the LC FastStart SYBRGreen kit (Roche Diagnostics) as recommended by the manufacturer (denaturation at 95 °C for 15 s, annealing at 60 °C for 5 s, extension at 72 °C for 10 s; 40 cycles, with the PCR product being monitored at 72 °C at the end of each cycle). A melting curve from 65 to 95 °C (0.05 °C/s) at the end of the reaction was used to check the purity and nature of the product. In all cases, a single PCR product was detected. Primers were chosen with the help of the OLIGO 4 program and were from different exons, so as to avoid amplification of possible DNA contamination of the RNA preparation. The sequences of the primers and the sizes of the PCR products were as follows: 5′-CAA GCT CTG ACG CTC TGA AGG-3′ (exon 4) and 5′-TTG GGG AGC AGG CTT TCA C-3′ (exon 5) for PERC (product 201 bp), 5′-GGA GCC GTG ACC ACT GAC A-3′ (exon 4) and 5′-TGG TTT GCT GCA TGG TTC TG-3′ (exon 5) for PGC-1 (product 176 bp), 5′-GGT CAT CAC TAT TGG CAA CGA G-3′ (exon 3) and 5′-GTC AGC AAT GCC TGG GTA CA-3′ (exon 4) for β-actin (product 196 bp). Control reactions performed on plasmid DNA confirmed that the PGC-1 primers could not amplify PERC sequences and vice versa. For quantification, standard amounts for each template (from 400,000 to 128 plasmid copies, in 1:5 dilutions) were analyzed in parallel to the samples. The cycle numbers needed for a log-linear phase product to reach the crossing point, which was set above the background noise, were plotted against the logarithm of the input plasmid copy number and fitted to a standard curve. The cDNA copy numbers for each gene were calculated from the standard curve, and the copy numbers of PERC and PGC-1 were normalized to the number of β-actin copies in the sample. Results shown are from duplicate reactions, using the same cDNA preparation. Similar results were obtained from independent preparations of cDNAs from two female and two male mice.

Plasmid Constructs—PERC deletion and point mutants were generated by standard PCR methods and verified by sequencing. All PERC variants were subcloned into pcDNA3/HA, pcDNA3/GAL4DBD (containing Gal4 DBD as a *Hin*dIII/*Nde*I fragment from pGBKT7 (CLONTECH)), and pGADT7 (CLONTECH). More information on the plasmids is available on request. Expression plasmids p6RGR, p6RMR, pSVARo, pSG5/ERα, pcDNA3/HA-hPGC-1, and pSG5/SRC-1e, as well as the luciferase reporter plasmids pTAT3-Luc, pERE-tk-Luc (one copy of the vitellogenin A2 ERE fragment (−334 to −289 nucleotides, relative to transcription initiation) (vERE)), and pGK1 have been described (24). The following expression and luciferase reporter plasmids were generously provided: pSG5/hPR (34), pSG5/hERβ (E. Treuter), pSV-SPORT1/mPPARγ2 and p3xPPRE-tk-Luc (M. Meyer), pSG5/hTRβ and pSG5/mRXRα (H. Gronemeyer), pMMTV-LTR-Luc (35), pminPbLUC-neo (F. Hamy), pC3-Luc (5). For the expression of the Gal4DBD/hERα-LBD fusion in yeast, the hERα-LBD (308C) was amplified by PCR from pSG5/ERα and subcloned into pGBKT7 to yield pGBKT7/hERα(308C). To generate hERα AF2 mutant L539/540A, the LBD was amplified by PCR from pRST7/hERα-LL (30) and subcloned either into pGBKT7 to yield pGBKT/hERα(308C)-LL or into pSG5/ERα to yield pSG5/hERα-LL. The luciferase reporter plasmids pΔ(vERE)x1-Luc and pΔ(vERE)x2-Luc were constructed by cloning the vERE-containing *Hin*dIII fragment from pERE-tk-Luc into the *Hin*dIII site upstream of the minimal alcohol dehydrogenase promoter of pΔLuc (35). pΔ(cERE)x1-Luc and pΔ(cERE)x2-Luc have a monomer or dimer of the sequence 5′-GAG CTC GAG AGG TCA CAG TGA CCT GTC-3′ (consensus (cERE) half-sites are underlined) at the *Sal*I site of pΔ-Luc. pΔ(DR4)x2-Luc has the sequence 5′-CTT AGG TCA CTT CAG GTC AGC CTC GAG GGA GGT CAC TTC AGG TCA GTC-3′ (DR4 half-sites are underlined) at the *Hin*dIII/*Sal*I sites of pΔ-Luc.

Cell Culture and Transfections—COS7 and U2OS cells were cultured in Dulbecco's modified Eagle's medium supplemented with 9% fetal bovine serum. Charcoal-stripped fetal bovine serum was used when assaying hormone responses. Media lacking phenol red were used in experiments with AR or ERs. Cells were seeded into six-well plates 24 h prior to transfection by the calcium phosphate precipitation method. All transfections included 0.2 μg of p6RlacZ for normalization of transfection efficiency. Standard amounts of expression and reporter plasmids per transfection in coactivation assays were: 1 μg of nuclear receptor expression plasmid, 1 μg of luciferase reporter, 0.5 μg of pcDNA3/HA-PERC (and its variants) or pcDNA3/HA-hPGC-1. For coactivation of AR in COS7 and coactivation of ERα in U2OS, 1 μg of pcDNA3/HA-PERC, pcDNA3/HA-hPGC-1, and pSG5/hSRC-1e was used. When assaying the transcriptional activity of the Gal4DBD-PERC fusion proteins in COS7, 0.5 μg of pcDNA3/GAL4DBD-PERC (or its variants) and 1 μg of the Gal4-responsive pGK1 luciferase reporter were transfected. After overnight exposure to the DNA-calcium phosphate precipitate, cells were washed and incubated for an additional 24 h in fresh medium containing either hormone or vehicle (0.1% ethanol or Me_2SO). Assays for luciferase and β-gal activities were performed as described previously (24). Luciferase values normalized to β-gal activity are referred to as luciferase units. Data shown represent the mean ± S.D. of four to six values from at least two independent experiments performed in duplicates.

Yeast Two-hybrid Interaction Assay—A diploid yeast strain with integrated Gal4-responsive β-gal reporters (CG1945xY187, CLONTECH) was transformed by the lithium acetate transformation method with pGBKT7/hERα(308C) or pGBKT7/hERα(308C)-LL (Gal4 DBD fused to the hERα LBD) and pGADT7/PERC constructs (Gal4 AD fused to PERC wild type or mutants). Transformants were grown to stationary phase, diluted 1:20 in selective media containing either ethanol

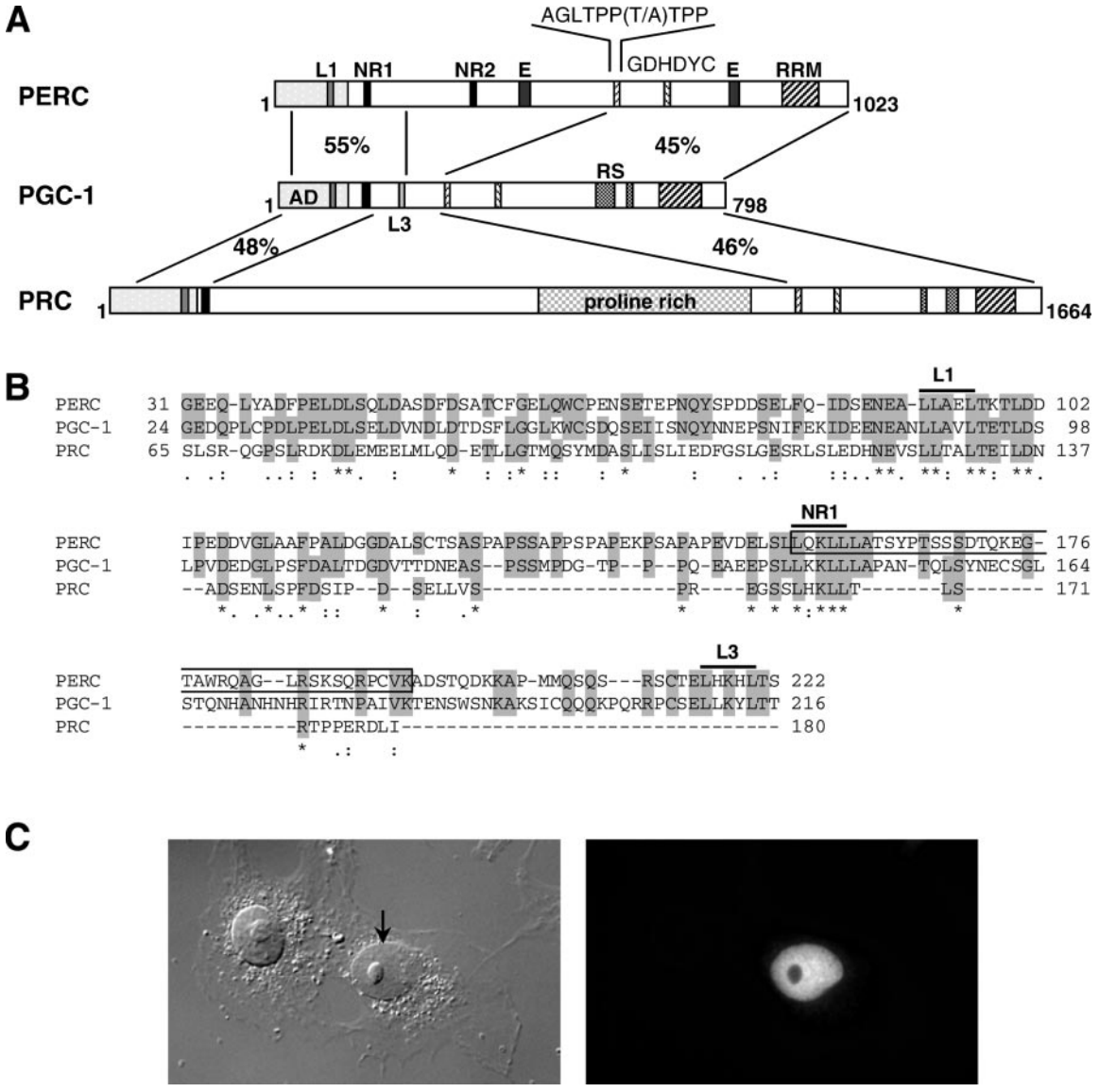

FIG. 1. **PERC is a new member of the PGC-1 protein family.** *A*, schematic representation of the PERC protein, its sequence features, and comparison with PGC-1 and PRC. The *shaded part* of the N terminus indicates the predominantly acidic region. Amphipathic α-helical leucine-rich motifs are marked as L1, L3, NR1, and NR2; of these, NR1 and NR2 conform to the L*XX*LL sequence. Also indicated are two regions rich in glutamic acids (*E*) (aa 430–450 and 807–824), two sequence motifs (AGLTPP(T/A)TPP and GDHDYC) that are highly conserved among the three proteins, and the putative RRM. The percent similarities of the conserved regions among PGC-1 and PERC, or among PGC-1 and PRC, are shown in between the protein diagrams. Serine/arginine-rich regions (*RS*) are present in PGC-1 and PRC but not PERC. Finally, PRC is characterized by a unique, long proline-rich region. *B*, multiple sequence alignment (Clustal W) of the conserved N-terminal region. The alternatively spliced exon 4 of PERC is *boxed*. Identical residues in at least two of the proteins are *shaded*; residues marked by *asterisk*, *colon*, and *period* are identical, conserved, or semi-conserved, respectively, in all three proteins. *C*, PERC localizes to the nucleus. *Right*, HA-tagged PERC protein in transiently transfected COS7 cells was detected by immunofluorescence, using a monoclonal mouse anti-HA antibody and a goat anti-mouse rhodamine-conjugated antibody. *Left*, differential interference contrast image acquisition of the same field. The *arrow* indicates the nucleus of a HA-PERC expressing cell.

vehicle (0.1%) or 10 μM 17β-estradiol (E2), grown for an additional 16 h at 30 °C in 96-well plates, and assayed for β-gal activity as described previously (35).

Immunofluorescence—COS7 cells were transfected with the HA-PERC expression vector pcDNA3/HA-PERC using FuGENE (Roche Molecular Biochemicals). PERC was detected in fixed cells by fluorescence microscopy, using a mouse monoclonal antibody against the HA epitope (HA.11, Babco) and a rhodamine-conjugated goat anti-mouse antibody (Jackson Laboratories) as described previously (24).

RESULTS

Identification and Sequence Analysis of a PGC-1-related cDNA—Sequencing of the human genome revealed a locus on chromosome 5 with significant sequence similarity to PGC-1 and distinct from the PGC-1-related coactivator PRC (32). Using primers designed against the predicted coding sequences, we amplified and cloned cDNAs representing this PGC-1 homolog (see "Experimental Procedures"). Sequence analysis of the identified cDNAs indicated the existence of two isoforms, likely resulting from alternative splicing. The longer cDNA encodes a protein of 1023 amino acids (aa), which we named PERC. The short isoform, referred to as PERC-s, is identical to PERC except that it lacks aa 156 to 194, sequences that correspond to exon 4 of the gene. Fig. 1*A* shows a diagram of the predicted open reading frame of PERC, indicating interesting sequence features and homologies to the related proteins PGC-1 and PRC. The greatest similarity between the three proteins is in the C-terminal half of PERC (45–46% over 450 aa). This region includes a RNA recognition motif (RRM), which has been implicated in the regulation of RNA processing (31), and two

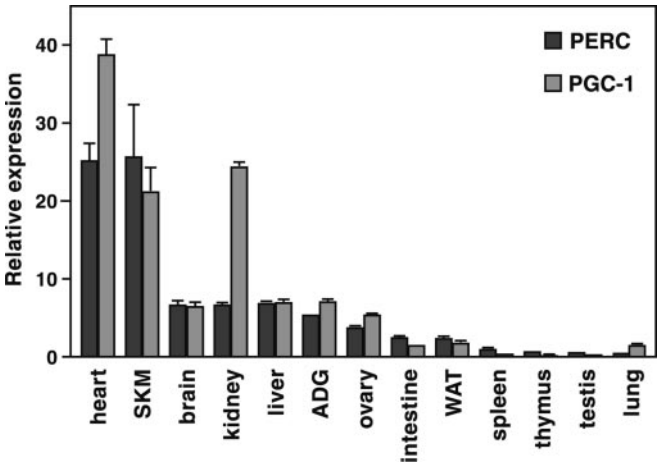

FIG. 2. **PERC mRNA is expressed in a tissue-specific manner.** Levels of mouse PERC and PGC-1 mRNAs in different mouse tissues were determined by real-time quantitative RT-PCR (see "Experimental Procedures"), normalized to β-actin mRNA levels, and expressed as copies of PERC or PGC-1 per 1000 β-actin copies in each sample. Data shown are from a 6–8-week-old male, except for the ovary RNA, which is from a 6–8-week-old female. Comparable results were obtained with cDNAs prepared from tissues of one more male and female. *SKM*, skeletal muscle; *ADG*, adrenal gland; *WAT*, white adipose tissue.

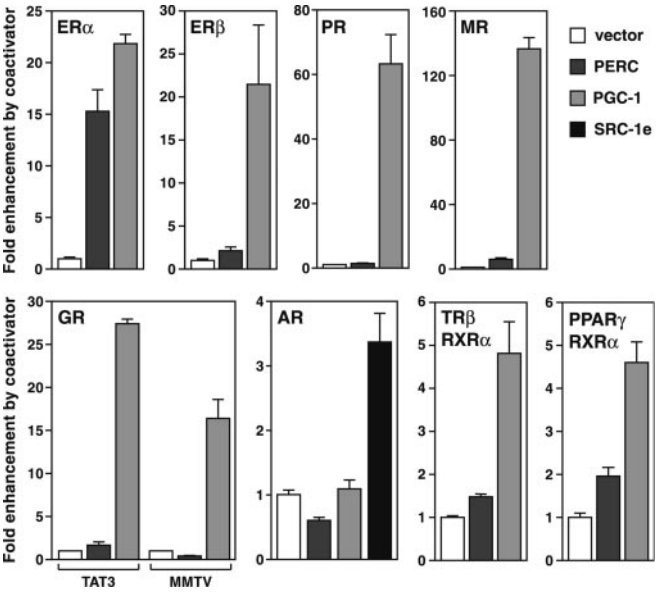

FIG. 3. **PERC selectively enhances the activity of ERα.** COS7 cells were cotransfected with expression plasmids for the indicated nuclear receptors, the corresponding luciferase reporter constructs (pERE-tk-Luc for ERα and ERβ; pTAT3-Luc for PR, MR, and GR; pMMTV-LTR-Luc for GR; pminPbLUCneo for AR; pΔ(DR4)x2-Luc for TRβ/RXRα; 3xPPRE-Luc for PPARγ/RXRα), and either pcDNA3 control vector (*white bars*) or expression vectors for PERC (*dark gray bars*), PGC-1 (*light gray bars*), or SRC-1e (*black bars*). Cells were treated with 50 nM 17β-estradiol (*ERα* and *ERβ*), progesterone (*PR*), aldosterone (*MR*), or corticosterone (*GR*), 100 nM dihydrotestosterone (*AR*), T3 (*TRβ*/*RXRα*), or 1 μM rosiglitazone and 1 μM 9-*cis*-retinoic acid (*PPARγ*/*RXRα*) for 24 h and assayed for luciferase activity. Data are expressed as fold enhancement of nuclear receptor activity by coactivator in the presence of hormone, *i.e.* activity in the presence of hormone and absence of coactivator was set equal to 1 for all receptors.

conserved short motifs of as yet unknown function (Fig. 1*A*). In contrast to PGC-1 and PRC, which have short serine/arginine-rich stretches (RS motif) N-terminal to the RRM, PERC has no RS domain. Instead, PERC has two glutamic acid-rich stretches (aa 430–450 and aa 807–824). A similar stretch of glutamic acids has been described in the nuclear receptor coactivator PELP1 (10). The second conserved region between the three proteins is the N-terminal region (Fig. 1, *A* and *B*). The first 130 aa of PERC are predominantly acidic residues, interspersed with leucines (25% aspartic and glutamic acids, 14% leucines, and just one basic residue). Alignment of this region with PGC-1 and PRC highlights the presence of a conserved leucine-rich motif (aa 92–96 of PERC), termed L1 here. In addition, PERC has two L*XX*LL motifs, indicated as NR1 and NR2 in Fig. 1. NR1 shows sequence conservation to the L*XX*LL motifs of PGC-1 and PRC, while NR2 is unique to PERC. The similarity between PERC and PGC-1 extends beyond NR1 and includes the region of a third Leu-rich motif of PGC-1; a Leu-motif is, however, not discernible in this region of PERC (Fig. 1*B*). Finally, consistent with the presence of nuclear localization signal sequences, PERC is a nuclear protein (Fig. 1*C*).

PERC Is Expressed in a Tissue-specific Manner—To determine PERC mRNA levels in different tissues in a quantitative and sensitive manner, we employed real-time RT-PCR with RNA from mouse tissues. Primers were chosen so as to detect specifically the long, exon 4+ PERC transcript. As seen in Fig. 2, PERC was detected at highest levels (>20 copies of PERC/1000 copies of β-actin) in heart and skeletal muscle. Intermediate levels (5–10 copies of PERC/1000 copies of β-actin) were seen in brain, kidney, liver, and adrenal gland. Lower PERC levels were detectable in ovary, intestine, and white adipose tissue. Expression in spleen, thymus, testis, and lung was below 1 copy/1000 copies of actin. The tissue distribution of PERC appears very similar to that of PGC-1 (22–24). Quantitation of PGC-1 mRNA in the same tissue samples demonstrated that the two genes are indeed expressed with similar profiles and at similar levels in most tissues. A notable exception is the kidney, where PGC-1 levels were significantly higher.

The PGC-1 Homolog Selectively Enhances the Activity of ERα—The similarity to PGC-1 and the presence of two L*XX*LL motifs suggested that PERC could function as a coactivator of nuclear receptors. To test this, we evaluated the effect of PERC overexpression on the ligand-dependent trancriptional activity of different nuclear receptors. We introduced full-length nuclear receptors, with or without PERC, in COS7 cells and assessed their ability to induce the expression of appropriate luciferase reporters in the presence of hormone. To our surprise, PERC had either no or just marginal effects on ERβ, progesterone receptor (PR), mineralocorticoid receptor (MR), GR, androgen receptor (AR), TRβ/RXRα, or PPARγ/RXRα, especially when compared with the activity of PGC-1 under the same conditions (Fig. 3). The one nuclear receptor where PERC functioned as a potent coactivator was ERα. The selective activation of ERα was not due to a special feature of the estrogen-responsive luciferase construct (single copy of vERE upstream of the thymidine kinase promoter), because ERβ function at the same estrogen-responsive reporter was minimally affected by PERC. Moreover, PERC had at most a 2-fold effect on GR activity irrespective of whether this was measured with a reporter having three tyrosine aminotransferase GREs or part of the MMTV LTR. Neither PERC nor PGC-1 had any effect on AR, which was however responsive to the effects of SRC-1, a coactivator of the p160 family. We concluded that PERC shows a remarkable selectivity for ERα, while its homolog PGC-1 can activate potently most nuclear receptors.

PERC Interacts with ERα in a LXXLL-, AF2-, and ligand-dependent Manner—To determine whether PERC and ERα interacted physically, and if so, to find out the requirements for such an interaction, we employed the yeast two-hybrid system. As shown in Fig. 4, the LBD of ERα interacted with full-length PERC in a ligand-dependent manner. Mutations in helix 12 of the ERα LBD (L539A/L540A) abolished the interaction, indi-

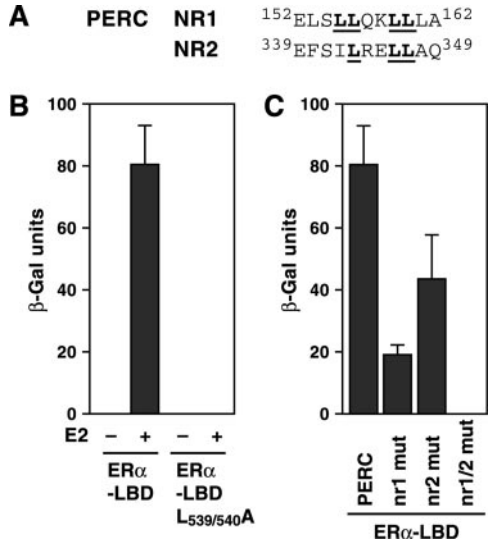

FIG. 4. **PERC interacts physically with the LBD of ERα in a ligand, AF-2, and LXXLL-motif dependent manner.** *A*, amino acid sequences of PERC motifs NR1 and NR2. Leucines indicated in *bold* were substituted with alanines in PERC nr1 and nr2 mutants. *B*, yeast expressing Gal4DBD-ERα-LBD (wild type or AF2 mutant L539/540A) and either Gal4AD alone (not shown) or Gal4AD-PERC were grown in the absence or presence of 10 μM 17β-estradiol (*E2*) and assayed for β-gal activity. No activity was detected in yeast expressing Gal4DBD-ERα-LBD and Gal4AD. *C*, yeast expressing Gal4DBD-ERα-LBD and the indicated Gal4AD-PERC variants were grown in the presence of 10 μM 17β-estradiol and assayed for β-gal activity.

cating that it depends on the structural integrity of the AF2 domain (4, 36). To test the involvement of the two L*XX*LL motifs of PERC in the interaction with ERα, we substituted the leucines in each motif by alanines (Fig. 4*A*). Mutations in either NR1 or NR2 alone reduced the interaction, while the double nr1/nr2 mutation abolished it (Fig. 4*C*). In conclusion, PERC interacts via two motifs, NR1 and NR2, with a ligand-dependent conformation of the ERα AF2 domain.

We next determined whether the requirements of the interaction detected by the two-hybrid assay were also important for the ability of PERC to enhance the activity of full-length ERα. Coexpression of PERC with the receptor in COS7 cells enhanced the activity of ERα in the presence of the agonist estradiol, but had no effect in the absence of hormone or the presence of the antagonist tamoxifen (Fig. 5*A*). Enhancement required an intact AF2 function, because the AF2 mutation L539A/L540A abolished responsiveness to PERC (Fig. 5*A*). Finally, mutations in either motif NR1 or NR2 reduced PERC activity, and the double nr1/nr2 mutation abolished coactivation (Fig. 5*B*). These findings demonstrated that PERC function in ERα signaling depends on an agonist ligand and intact complementing interaction surfaces: AF2 of ERα and NR1/NR2 of PERC. Interestingly, NR1 is missing in the natural isoform PERC-s, which lacks the 39 aa encoded by exon 4. Coexpression of this short isoform showed indeed that PERC-s had a reduced ability, similar to that of the PERC nr1 mutant, to enhance the hormone-dependent activity of ERα. Consequently, mechanisms that regulate the alternative splicing of exon 4 of PERC could modulate cellular responses to estrogens.

A Potent Bipartite Transcriptional Activation Domain (AD) in the N Terminus of PERC Is Required for Coactivation—The N-terminal region and in particular motif L1 of PERC is well conserved among the three members of the PGC-1 family (Fig. 1*B*). In PGC-1 and PRC, this region harbors a potent trancriptional AD (24, 28, 32). To test whether PERC also carries such an AD, we asked if full-length PERC tethered to DNA activates transcription. A fusion of PERC to the DBD of Gal4, which recruits PERC to a Gal4-responsive luciferase reporter, indeed activated transcription strongly (Fig. 6*A*). Deletion of the first 91 aa of PERC abolished activation, indicating that the N-terminal part is essential for the activation function (Fig. 6*A*). The first 91 aa (N91) fused to the Gal4 DBD were sufficient to activate transcription. However, full transcriptional activity of PERC required additional sequences up to aa 128. Gal4 DBD fused to aa 1–128 (N128) was the strongest PERC activator, enhancing transcription by more than 20,000-fold in COS7 cells (Fig. 6*A*). Within the 91–128 region, the conserved motif L1 contributed to the activation function. Point mutations that substituted the leucines of L1 with alanines reduced PERC transcriptional activity, in the context of both full-length PERC and the N128 construct (Fig. 6*A*). Our findings suggest a bipartite N-terminal AD. The first part is encoded by aa 1–91 and is essential, while the second part relies on motif L1 and contributes to full activity. This bipartite AD function is crucial for the ability of PERC to enhance the activity of ERα (Fig. 6*B*). Deletion of the first 91 aa or mutations in motif L1 abolished or reduced, respectively, PERC coactivation (Fig. 6*B*), suggesting that both components of the AD are required for full function of PERC in ER signaling.

PERC and PGC-1 Confer Distinct Functional Properties to Ligand-activated ERα—To address whether PERC and PGC-1 fulfill similar functions when acting with ERα, we compared the effects of the two coactivators on estrogen signaling in different contexts. First, we evaluated PERC and PGC-1 function on ERα-activated transcription at different promoter contexts (Fig. 7*A*). A single consensus ERE upstream of the minimal alcohol dehydrogenase promoter was preferentially responsive to PGC-1 activity. PERC caused a small, reproducible 2–3-fold enhancement, compared with a 10-fold increase by PGC-1. ERα acting from two copies of the consensus ERE or a longer vitellogenin A2 ERE fragment (−334 to −289 nucleotides, relative to transcription initiation) upstream of the same minimal promoter was equally responsive to the two coactivators. On the other hand, two copies of the vitellogenin ERE fragment, or a 1.8-kb fragment of the estrogen-responsive complement 3 (C3) promoter, were enhanced stronger by PERC than by PGC-1 (Fig. 7*A*). These observations suggest that PERC and PGC-1 may selectively activate distinct ERα targets genes.

ERα signaling depends on the nature of the activating ligand, as well as the cellular and promoter context (5, 37, 38). In particular, there are classes of ER ligands that act in a tissue-selective manner. For example, tamoxifen is an antagonist in the mammary gland but an agonist in the bone, uterus, and cardiovascular system (reviewed in Ref. 39). One of the underlying molecular mechanisms for the agonist action of tamoxifen is thought to involve the cooperation of tamoxifen-bound ERα with tissue-specific cofactors. To determine how PERC and PGC-1 affect the response to tamoxifen, we employed the C3 promoter, which has been characterized for its responsiveness to this agonist (38, 40). In the osteosarcoma cells U2OS, tamoxifen activated the C3 promoter strongly, although not as well as estradiol (Fig. 7*B*). PERC expression further enhanced the tamoxifen response by 2-fold. In contrast, PGC-1 modestly repressed the tamoxifen-induced response (Fig. 7*B*). These findings suggest that the relative activities of PERC and PGC-1 may contribute to the tissue-specific action of partial agonists like tamoxifen.

DISCUSSION

We report here the cloning and characterization of PERC, a new member of the PGC-1 family of proteins and a coactivator of ERα. In contrast to PGC-1, which activates many nuclear receptors, PERC shows a unique receptor selectivity. It po-

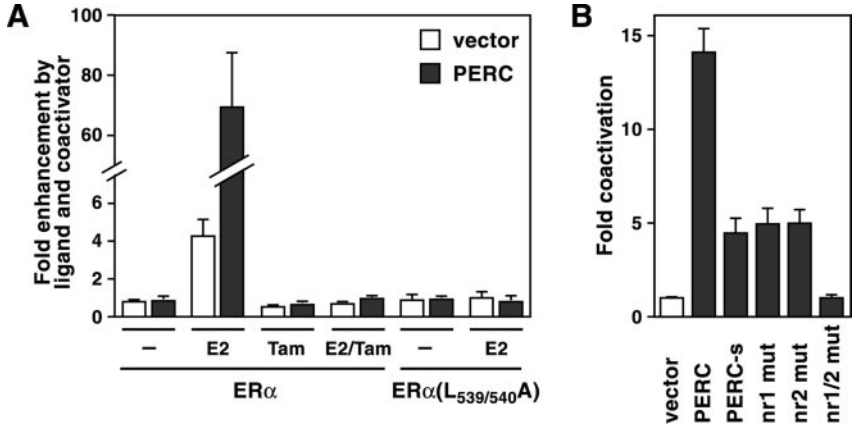

FIG. 5. **Coactivation of ERα by PERC depends on an agonist ligand and the integrity of AF2 of ERα and NR1/NR2 of PERC.** *A*, COS7 cells transfected with expression plasmids for ERα (wild type or AF2 mutant), the ER-responsive luciferase reporter pERE-tk-Luc, and either pcDNA3 control vector (*white bars*) or PERC expression vector (*dark gray bars*) were treated for 24 h with ethanol vehicle (−), 50 nM 17β-estradiol (*E2*), 5 μM tamoxifen (*Tam*) or both ligands (*E2*/*Tam*) and assayed for luciferase activity. Data are expressed as fold enhancement by PERC, with activity in the absence of PERC and ligand set equal to 1. *B*, COS7 cells transfected with an expression plasmid for ERα, the ER-responsive luciferase reporter pERE-tk-Luc, and either pcDNA3 vector control (*white bars*) or expression vectors for PERC and its indicated variants (*dark gray bars*) were treated for 24 h with 50 nM 17β-estradiol and assayed for luciferase activity. PERC-s lacks exon 4 (aa 156–194). Data are expressed as fold enhancement of ERα activity by PERC in the presence of hormone.

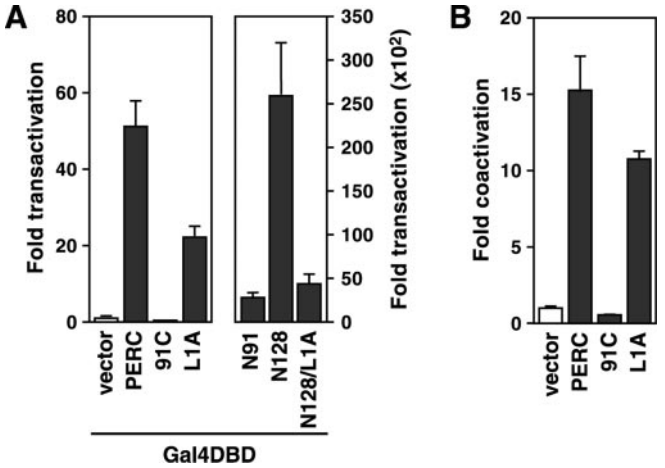

FIG. 6. **A bipartite transcriptional activation domain in PERC is required for coactivation of ERα.** *A*, transcriptional activity of PERC. COS7 cells transfected with the luciferase reporter pGK1 and either Gal4DBD control vector or the indicated Gal4DBD-PERC variants were assayed for luciferase activity. Data are expressed relative to the activity in cells expressing the Gal4DBD alone (vector), which was set equal to 1. Note that the *y* axis scales are different in the two panels. *B*, coactivation function of PERC. COS7 cells transfected with an ERα expression plasmid, the ER-responsive luciferase reporter pERE-tk-Luc, and either pcDNA3 vector control (*white bars*) or expression vectors for PERC and its indicated variants (*dark gray bars*) were treated for 24 h with 50 nM 17β-estradiol and assayed for luciferase activity. Data are expressed as fold enhancement of ERα activity by PERC in the presence of hormone. *91C*, aa 91–1023 of PERC; *N91*, aa 1–91 of PERC; *N128* and *N128*/*L1A*, aa 1–128 of PERC wild type and PERC L1A mutant, respectively.

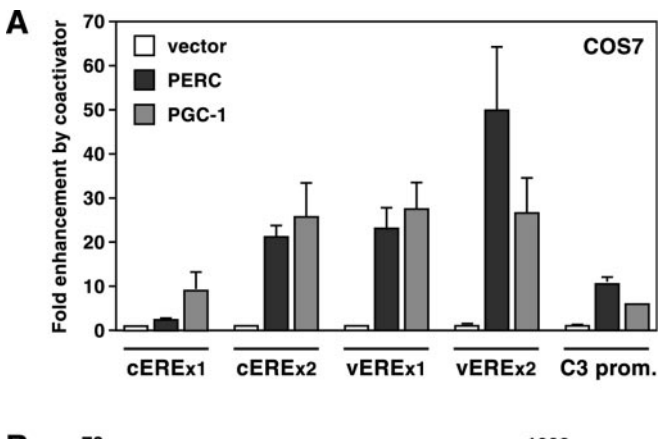

FIG. 7. **PERC and PGC-1 confer differential promoter- and ligand-specific activation of ERα.** *A*, coactivation of ERα by PERC or PGC-1 in different promoter contexts. COS7 cells transfected with an ERα expression plasmid, the different ER-responsive luciferase reporters (*cERE×1*, *cERE×2*, *vERE×1*, *vERE×2*, and *C3* promoter) and either pcDNA3 control vector (*white bars*) or expression vectors for PERC (*dark gray bars*) and PGC-1 (*light gray bars*) were treated for 24 h with 50 nM 17β-estradiol and assayed for luciferase activity. Data are expressed as fold enhancement of ERα activity by each coactivator in the presence of hormone. *B*, the activity of tamoxifen-bound ERα in U2OS osteosarcoma cells is enhanced by PERC but not by PGC-1. U2OS cells transfected with an ERα expression plasmid, the reporter pC3-Luc, and either pcDNA3 control vector (*white bars*) or expression vectors for PERC (*dark gray bars*) and PGC-1 (*light gray bars*) were treated for 24 h with ethanol vehicle (−), 50 nM 17β-estradiol (*E2*), or 5 μM tamoxifen (*Tam*) and assayed for luciferase activity.

tently enhances the ligand-dependent activity of ERα, while having only minimal effects on the activity of the related receptor ERβ or other nuclear receptors tested here. Furthermore, PERC and PGC-1 confer distinct properties to ERα signaling. Thus, the relative activities of the two coactivators may contribute to the specific profiles of estrogen responses in different tissues.

PERC, PGC-1, and the recently described PRC (32) define a new, small family of coactivators. The conserved features of the family reside primarily in the N- and C-terminal domains, which carry the effector functions of these coactivators: activation of transcription and regulation of pre-mRNA processing

(24, 28, 31, 41). Thus, the three coactivators are likely to employ similar mechanisms to mediate their biological functions. Whether PERC, which lacks the RS domain of PGC-1, is able to regulate RNA processing has to be addressed in future experiments. PERC, PGC-1, and PRC also share sequence similarities outside the effector domains: the L*XX*LL motifs that enable interactions with nuclear receptors and additional small conserved motifs that may represent interaction surfaces for other transcription factors or regulators (Fig. 1). At the same time, the significant sequence divergence, particularly in the unique central domains of the proteins, suggests that the three members of the family have acquired unique functions and roles.

The mechanism by which the N-terminal AD of PERC regulates transcription is not clear yet. The corresponding region of PGC-1 can interact with SRC-1 and CBP, suggesting that it acts as a scaffold for the recruitment of other coactivators (41). Our studies here indicate a bipartite AD that contacts more than one target. The reduced transcriptional activity of the L1A mutant points to the conserved motif L1 as one of the interaction surfaces. An additional contact must reside in the first 90 aa, which are essential and sufficient for transcriptional activation. Neither SRC-1 nor CBP overexpression enhanced PERC transcriptional activity, implicating targets other than these two coregulators. Since PGC-1 and PERC are strong activators of transcription in yeast, which do not have SRC-1 or CBP, it seems likely that the N-terminal ADs can contact evolutionary conserved components of the transcriptional machinery (24).[2] Delineation of the exact interaction surfaces of PGC-1, PRC, and PERC, as well as identification of the proteins they contact, will shed light on the mechanisms by which these ADs act.

An important feature of the PGC-1 family is the presence of L*XX*LL motifs, which mediate interactions with the LBDs of nuclear receptors. PERC has two canonical L*XX*LL motifs: NR1, which is conserved in PGC-1 and PRC, and NR2, which is unique to PERC (28, 30, 32, 42). Both NR boxes contribute to the physical interaction with ERα and to efficient coactivation of this receptor. Notably, the presence of NR1 depends on the inclusion of the small exon 4. The detection of two PERC isoforms, with and without this exon, and the decreased ability of the short PERC-s to activate ERα, suggest that regulation of this alternative splicing event could be used to modulate ERα signaling. Interestingly, the mouse homolog of PERC, which was recently described as PGC-1β, harbors an additional L*XX*LL motif that is upstream of NR1 (aa 140–144) and not conserved in the human protein (33). We do not know yet whether this third motif functions as a nuclear receptor interaction domain, and if so, whether it enables functional interactions with ERα or other receptors. Although no data have been presented yet on the ability of the mouse protein to coactivate the different receptors we have tested here, it is possible that the mouse and human homologs may have diverged in their nuclear receptor specificity.

Our experiments demonstrate clearly that PERC is a coactivator of ERα. The fact that this coactivation function depends on a physical interaction between the L*XX*LL motifs of PERC and the AF2 domain of ERα raises the question of why PERC has only minor effects on many other nuclear receptors that harbor similar AF2 domains. The reason for this receptor selectivity is not clear, particularly since PERC can interact physically with other ligand-activated receptors, such as GR.[2] One possible explanation is that the affinity of the GR-PERC interaction is lower than that of GR with other endogenous AF2 coactivators. If so, PERC may not get recruited efficiently at GR target sites. An alternative explanation is that the physical interaction mediated by the PERC NR boxes and the receptor AF2 binding pocket is a necessary, but not sufficient, step for coactivation. Coactivators have been proposed to undergo conformational changes subsequent to docking to transcription factors. These changes may enable their enzymatic activities or the recruitment of additional regulators (41, 43). Similarly, the conformation of nuclear receptors may change upon interaction with coactivators. Thus, specificity in the functional interaction between PERC and ERα could be due to conformational changes subsequent to binding that may activate either PERC, by unmasking its AD, or ERα, by enabling its AF1 activity. Consistent with an activation step for PERC, we have observed that deletion of C-terminal and central domains of PERC result in a much more potent transcriptional regulator (Fig. 6). It seems likely that the PERC AD is masked in the context of the full-length protein, similar to what has been shown for PGC-1 (41).

Besides their differences in nuclear receptor specificity, PERC and PGC-1 display distinct preferences for the promoter context in which they enhance ERα activity. The two types of EREs we have tested, a vERE and a synthetic cERE, contain the same consensus core but differ in the flanking sequences. Such differences have been shown before to influence ERα-ERE interactions (44). Moreover, the vitellogenin fragment includes additional 5′ sequences, where a second, nonconsensus ERE can be discerned (−312 to −298 nucleotides, relative to transcription initiation). Finally, due to the difference in the length of the flanking sequences, the dimerized elements vERE×2 and cERE×2 present ERα binding sites with different spacing. Thus, multiple properties, such as flanking sequences, the presence of additional nonconsensus sites, and the spacing between EREs, may account for the distinct utilization of PERC and PGC-1 at the different promoters. Notably, PERC seems to prefer promoters with multiple sites, such as the dimerized EREs, or the C3 promoter that has at least three EREs (40). Different response elements may induce distinct nuclear receptor conformations and thereby influence either the recruitment of the coactivators or the activity of the recruited coactivators (45, 46).

An additional context that reveals differences in PERC and PGC-1 function is the ability of the two coactivators to promote the agonistic effect of the partial agonist tamoxifen. In a cell and promoter context where tamoxifen is an agonist, PERC enhances this agonist activity, while PGC-1 represses it. In this respect, PERC acts like the p160 coactivators, which can enhance the agonist activity of tamoxifen (47–49). Presumably, PERC can interact, directly or indirectly, with the tamoxifen-induced conformation of ERα. PGC-1 cannot do so, at least in the context of the C3 promoter in U2OS cells. Because of its antagonist activity in the mammary gland, tamoxifen is used to treat estrogen-dependent breast tumors. Many of these tumors develop resistance to tamoxifen and some start recognizing it as an agonist (reviewed in Ref. 39). Our findings suggest that the nature, as well as the relative levels of different AF2 coactivators, may determine the cellular response to tamoxifen. Evaluation of PERC and PGC-1 levels in breast tumors will be important to test whether these two coactivators contribute to the responsiveness, or lack of, to endocrine therapy.

PERC mRNA distribution is very similar to that of PGC-1. PGC-1 function in heart, muscle, and liver may mediate physiological state signals to tissue-specific transcriptional activation of proteins that regulate energy and glucose homeostasis. For example, in response to exposure to cold, PGC-1 induces the expression of uncoupling proteins and stimulates energy

[2] D. Kressler and A. Kralli, unpublished observations.

expenditure in brown fat and muscle, while in response to fasting, it stimulates gluconeogenesis in liver (Refs. 26 and 29 and reviewed in Ref. 50). The similar expression profile of PERC may be indicative of a second pathway that relates energy needs to specific metabolic responses, possibly under different regulatory input and with a different outcome. This could increase specificity and flexibility of the transcriptional responses. Estrogens can have profound effects on systems other than the reproductive one. In both males and females, estrogens have protective effects on the cardiovascular and skeletal system, regulate adipose function, and affect glucose and lipid metabolism (51–54). Mice that lack a functional ERα have increased adipose mass, develop mild glucose intolerance and insulin resistance, and show decreased energy expenditure (54, 55). Similarly, humans with deficiencies in estrogen signaling show a propensity for insulin resistance and altered lipid metabolism (52). It will be interesting to test whether these estrogen effects require PGC-1, PERC, or a combination of the two coactivators.

The mechanisms by which estrogens act in a tissue- and promoter-specific manner are complex (20, 21). Mice with genetic ablations of the p160 coactivators SRC-1 or SRC-3/AIB1 show only mild defects in estrogen signaling (56–58). Thus, it seems likely that multiple coactivators can cooperate with ERs to mediate appropriate tissue-specific and physiological state-dependent responses. The molecular unraveling of estrogen activity will require an understanding of all ERα and ERβ interactors as possible contributors to estrogen signaling. Here, we have described a tissue-specific coactivator, PERC, which shows a remarkable selectivity for ERα over other nuclear receptors. Future studies will define the reason for selectivity, as well as the biological role of PERC.

Acknowledgments—We thank J. Gustafsson, H. Gronemeyer, D. P. McDonnell, M. Meyer, M. Parker, D. Picard, and E. Treuter for generous gifts of plasmids; N. Yanze, V. Schmid, and T. Grange for assistance with quantitative real-time PCR; L. Dolfini for technical assistance; and U. Müller for helpful discussions.

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Appendix III

Material and methods

PGC-1α antibody

The first 293 aa of PGC-1α were expressed as GST-fusion protein in E. coli (BL21) with a 2-2.5 hour induction by IPTG (0.1 mM). Lysis was performed with 50 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl containing 0.1 mM PMSF, protease inhibitors and 0.2 mg/ml lysozyme by incubating on ice for 30 minutes. Subsequent, 0.1 % DOC was added and the lysate was incubated for 15 minutes at 37 °C. DNA was digested with 10 µg/ml DNase I for 30 minutes at room temperature and the reaction was stopped with EDTA. In the following, the lysate was cleared by centrifugation for 20 minutes at 4 000 g and the supernatant was loaded on a GST column. The following elution was performed with 10 mM reduced glutathione in 50 mM Tris, pH 8.0 by incubating 10 minutes at room temperature. The eluate was injected into rabbits 5 times (Eurogentec Inc, Belgium) to produce antiserum. The final bleeding was purified by ammonium sulfate precipitation as followed: After clearance for 30 minutes at 3 000g, 0.5 x volume of saturated ammonium sulfate was added slowly while stirring and mixture was incubated over night at 4°C. Large protein aggregates or proteins precipitating with lower concentration of ammonium sulfate were removed by centrifugation for 30 minutes at 3 000g. In the second step with 1 x volume ammonium sulfate for at least 4 hours the antibodies were precipitated, then they were collected by centrifugation, solved in PBS-/- and dialyzed versus PBS-/- over night.

Cell lines, Infections, and Transfections

SAOS2-GR(+)(Rogatsky, 1997) cells were cultured in Dulbecco's modified Eagle's medium (sigma D6429, with 4500mg/l glucose, l-glutamine and sodium pyruvate, substitutes pyridoxine hydrochloride for pyridoxal hydrochloride) supplemented with charcoal-stripped 9% fetal calf serum (FCS) and 200ng/ml G418 at 37 °C with 7.5 % CO_2 and split 1:3 every 4^{th} to 5^{th} day. For the analysis of the gene expression profiles, cells were kept in normal fetal calf serum. One day before infection, cells were plated

with 7-9x10^5 cells per well in a 10 cm dish with DMEM and stripped FCS and were kept from then on in medium with stripped FCS. For the other experiments cells were always kept in medium with charcoal-stripped fetal calf serum. The analysis of the mRNA levels, protein extracts, genomic DNA and living cells the FACS analysis was done with 1.8x10^5 cells in 6-well plates or 2-3x10^5 cells per well in 6 cm plates and around 8x10^4 cells were split in 12 well plates for immunofluorescence. Adenoviruses were thawn on ice and sonicated 3 times for around 10 sec and were pipetted (only with filter tips, dilutions were made to have always at least 10 µl volume per well) directly in the cell culture plates with fresh medium (5 ml for 10 cm, 1 ml for 6 well, 0.5 ml for 12 well, 1.5 ml for 6 cm plate). Incubation occurred for around 2 hours and then cells were washed and fresh medium was added. Infections were done at a multiplicity of infection (moi) of 20 (mRNA analysis, protein analysis of the RNAi), 40 (FACS analysis, DNA analysis and control protein analysis) or 100 (gene chip experiments). The adenoviruses expressing siRNA (pSUPER or ERR2-3) were always infected 3 days before the infection with GFP or PGC-1α at an MOI of 100. For transfections in presence of the siRNA for ERRα, around 8x10^5 cells were split in 10 cm plates and infected with the adenoviral vectors expressing siRNA (pSUPER or ERR2-3). After 3 days, cells were split in 6-well plates with 1.6x10^5 cells per well. The next day, cells were incubated with fresh medium while the $CaPO_4$ precipitate was prepared: DNA was mixed with 3 µl 2.5M $CaCl_2$ and Tris up to 25 µl per well of a 6-well plate and 25µl 2x HBS was added dropwise while vortexing. Precipitates were allowed to form for 10 minutes before adding to the cells. After incubating over night for around 14-16 hours, cells were washed once with 1x PBS -/-. Cell lysates were prepared 40 to 48 h after transfection and assayed for luciferase activity.

Transfections included 0.2 µg of p6RlacZ for normalization of transfection efficiency, 100 ng pcDNA3.HA-hPGC-1 and 250 ng of the luciferase reporter pERRα.prom.short-Luc. Different amounts of pcDNA3/ERRα from 250 ng to 61 ng were obtained by 1:4 dilution steps, were cotransfected. Samples were filled up with pcDNA3 to have the same amount of DNA.

Luciferase reporter assay

Cells were washed with PBS-/- (2 ml) twice, lysed by incubation with 100 µl reporter lysis buffer (Promega) on ice. Lysates were scraped off using a rubber policeman and transferred to an eppendorf tube. Next, the cell trash was removed by centrifugation for 3 minutes at 20 000 g, 4 °C. 20 µl of the lysate were used to measure luciferase activity using the Microlite TLX1 luminometer (Dynatech Laboraties, Inc.) and 20 µl were assayed for b-Gal activity using CPRG (Roche) as substrate and the Thermomax microplate reader (Molecular Devices, Inc.).

Buffers and chemicals

Buffers: The 2x HBS buffer for transfections contained 280 mM NaCl, 1.5 mM Na_2HPO_4 and 50 mM HEPES, pH 7.05. TBST consisted of 150 mM NaCl, 10 mM Tris pH 7.5, 0l05 % Tween-20.

Chemicals: The 250x protease inhibitor mix included aprotinin, leupeptin and pepstatin each at 250µg/ml in DMSO. PMSF was dissolved in isopropanol as a 250 mM (250x) stock. Corticosterone (Fluka, 27840) was diluted with 100 % EtOH down to $1x10^{-4}$ and added to the cells in a 1:2000 dilution (50 nM).

Westernblot analysis

Cells were incubated in lysis buffer (100 mM Tris pH 8, 0.5% NP40, 150 mM NaCl, 1 mM EDTA) supplemented with fresh 1mM PMSF and protease inhibitor mix for 10 min, 4˚C. Proteins separated by SDS-PAGE (7,5 % gels) were blotted onto nitrocellulose membranes using the semi-dry electrophoresis transfer system. Membranes were blocked for around 30 minutes to one hour at room temperature or over night (4 °C) by incubation with 9 % milk in TBST. The primary antibodies were diluted in TBST with 4.5 % milk for the PGC-1α antibody (Schreiber et al., 2003) in concentrations from 1: 6000 to 1: 8000 and with 9 % milk for the ERRα antibody (Johnston et al., 1997) in a concentration of 1:3000 to 1:3300 (kept at 4 °C with 0.018 % NaN_3 and recycled for several weeks). Incubation occured over night at 4 °C or at least for 1 hour at room temperature. Secondary antibodies were purchased from Biorad. Bound antibodies were detected by enhanced chemiluminescence SuperSignal (Pierce).

RNA analysis

Total RNA was isolated using the TRIzol® reagent (Invitrogen Life Technologies). Cells were washed once with PBS-/- and TRIzol® reagent was added to the cells (1ml per well in a 6-well plate, 2 ml for a 10 cm dish). After incubating the cells at room temperature for 3-5 minutes, cells were harvested by pipetting up and down. RNA was extracted with 0.2ml chloroform per 1ml Trizol, incubated for 2-3 minutes at room temperature and centrifuged for 15 minutes at 4°C, not more than 12 000 g. Next, the aqueous phase was transferred into a fresh tube and 0.5ml isopropanol was added. After 10 minutes incubation at room temperature, samples were centrifuged for 10 minutes at 4˚C at not more than 12 000 g. Supernatants were removed and washed with 1 ml 75% ethanol (DEPC treated water) and centrifuged for 5 minutes at 4˚C for not more than 7 500 g. After aspirating the ethanol, the pellets were dried for maximum 5 minutes and resuspended with 10-15 µl H_2O (DEPC treated). Samples were incubated for around 20 minutes on ice and then frozen at -70˚C. RNA was checked for its integrity by agarose gel electrophoresis and ethidium bromide staining.

DNA isolation

Infected cells were trypsinized after 48 or 60 hours of infection. One half was analyzed by westernblot to control for PGC-1α wt and mutant expression, from the other half, genomic and mitochondrial DNA was isolated. Cells were lysed with 10mM Tris, pH 8, 100 mM EDTA, 0.5 % SDS and RNA was digested with 100 µg/ml RNase A for 30 min at 37˚C, followed by incubation with 100 µg/ml proteinase K at 50 ˚C over night. After three extractions with 25:24:1 phenol/chloroform/isoamyl alcohol, aqueous phases were precipitated with one volume of isopropanol and incubated for around 3-4 hours at 4˚C with gentle inversion. Precipitates were collected by centrifugation at 9.000 x g for 20 min, dried and solved in TE buffer pH 8 over night at 4˚C. To completely solve the DNA and make it more accessible for quantitative PCR it was sheared with a syringe and subsequently incubated at 60 ˚C for around 20 min. The specificity of the signals was controlled by RNAse treatment, which did not have an effect, and DNAse treatment, which led to a destruction of the signal.

Quantitative RT-PCR analysis of mRNA and DNA

Per sample, 800 ng RNA was converted to 20 µl cDNA (TaqMan, Applied Biosystems) with random hexamer primers (25°C, 10 minutes, 46 °C, 45 minutes, 95 °C, 5 minutes) and specific transcripts were quantified by real-time PCR using the Light Cycler system (Roche Diagnostics). Samples were measured in a 15 µl volume with 1.5µl of a 5 µM primer stock solution for each primer, 1.2 µl $MgCl_2$ (25 mM) and 1.2 µl of the Mix including enzyme and SYBR green provided with the LightCycler FastStart DNA Master SYBR green I kit (Roche Diagnostics). At the end, 2 µl of the cDNA were added and samples were centrifuged for 1 minute at 770g. After preincubation at 95°C for 10 minutes, products were amplified (95°C for 15 seconds, 60 °C for 5 seconds and 72 °C for 10 seconds) for 40 cycles. A melting curve from 65 to 95°C (0.05°C/sec) at the end of the reaction was used to check the purity and nature of the product. In all cases, a single PCR product was detected. The sequences of the primers, which were designed in two different exons to avoid DNA contamination and the sizes of the PCR products are listed in a supplementary table following the material and method section. For quantification, either plasmid dilutions (5 dilutions 1:5) or 5 serial (1:3) cDNA dilutions from not infected cells were used as standard. All values were normalized to the mRNA levels of 36B4.

DNA was diluted down to 1 ng/µl. Cytochrome oxidase II was measured as mitochondrial encoded gene and normalized to β-actin as nuclear encoded gene. For the standards, DNA of not infected cells was serially diluted 4 times from 10 ng in 1:3 dilution steps. Copy numbers for quantification were set arbitrary. Quantification of both mRNA and DNA levels was done with the arithmetic fit points method, whereas the noise band and crossing line were set mostly around 0.4.

FACS analysis

After 48 hours of infection, SAOS2-GR(+) cells were incubated for 30 min at 37˚C with 500 nM MitoFluor Red 594 for flow cytometry (Molecular Probes Inc.), diluted in medium. To accumulate the dye in actively respiring mitochondria, cells were incubated for another 30 min with fresh medium at 37˚C. Cells were trypsinized, washed twice with PBS-/- and resuspended in 300-500 µl PBS-/- containing 1 mM EDTA to avoid cell clumps. Mitochodrial density was analyzed in living cells by flow cytometry (FACSCalibur, Beckton Dickinson) and data were analyzed using the

software WinMDI 2.8 developed by Joseph Trotter (http://www.scripps.edu). Each measurement represents 20 000 events. Similar GFP expression for PGC-1α wild type and mutant was detected in the FL1 channel, mitochondrial density was measured in the FL4 channel. In parallel, extracts were analyzed by western blot analysis to control for similar expression levels of PGC-1α wild type and mutant and the induction of ERRα protein levels.

Immunofluorescence

Cells were grown in 12well plates on coverslips with around $8x10^4$ cells per well. When mitochondrial density was analyzed, cells were incubated for 30 min at 37˚C with 500 nM CM-H_2XRos (Molecular Probes, Inc.) diluted in medium. To accumulate the dyes in actively respiring mitochondria, cells were incubated for another 30 min with fresh medium at 37˚C. Mitochodrial density was analyzed in living cells or after fixation with 3 % PFA in PBS+/+ by light microscopy (Zeiss, Axioplan 2). For staining with the PGC-1α antibody, cells were fixed with 3 % PFA, washed with PBS+/+, permeabilized with 0.1 % triton X-100 for 5 minutes and again washed with PBS+/+. After blocking for 30 minutes with 1 % BSA in PBS+/+, cells were incubated with the primary PGC-1α antibody (1:500) for 1-2 hours and subsequently washed twice with 0.5 % BSA and twice with PBS+/+. Staining with a secondary anti-rabbit antibody coupled to Cy3 was done for 30 minutes. After the subsequent washing steps as before, nuclei were stained with 50 ng/ml DAPI for 1-2 minutes, cells were washed and mounted with the vectashield mounting reagent (Vector Laboratories Inc., Burlingame, CA).

Target preparation and hybridization of the HG-U133A GeneChips

Microarray analysis was performed using HG-U133A GeneChips™ (Affymetrix, Santa Clara, USA). 10 µg of total RNA (isolated from SAOS2-GR(+) cells) was reverse transcribed using the SuperScript Choice system for cDNA synthesis (Life Technologies) according to the protocol recommended by Affymetrix (GeneChip Expression Analysis: Technical Manual (2001) p. 2.1.14-2.1.16). The oligonucleotide used for priming was 5'-ggccagtgaattgtaatacgactcactatagggaggcgg-$(t)_{24}$-3' (Genset Oligo, France) as recommended by Affymetrix. Double-stranded cDNA was cleaned

by phenol:chloroform extraction and the aqueous phase removed by centrifugation through Phase-lock Gel (Eppendorf). *In vitro* transcription was performed on 1 μg of cDNA using the Enzo BioArray High Yield RNA transcript labelling kit (Enzo Diagnostics, USA) following the manufacturer's protocol. The cRNA was cleaned using RNAeasy clean-up columns (Qiagen). To improve the recovery from the columns the elution water was spun into the matrix at 27 *g* and then left for one minute prior to the standard 8000 *g* centrifugation recommended by Qiagen. The cRNA was fragmented by heating in 1x fragmentation buffer (40 mM Tris-acetate pH 8.1, 100 mM KOAc, 30 mM MgOAc) as recommended by Affymetrix. 10 μg of fragmented cRNA were hybridised to a HG-U133A GeneChip (Affymetrix) using their standard procedure (45°C, 16 hours). Washing and staining was performed in a Fluidics Station 400 (Affymetrix) using the protocol EukGE-WS2v4 and scanned in an Affymetrix GeneChip scanner.

Microarray analysis

Chip analysis was performed using the Affymetrix Microarray Suite v5 and GeneSpring 5.1 (Silicon Genetics). Changes in gene expression were assessed by looking for concordant changes between replicates using a signed Wilcoxon rank test (as recommended by Affymetrix). The "change" p-value threshold was < 0.003. Any gene whose detection p-value was > 0.05 in all experimental conditions was discarded from the analysis as being unreliable data. The differentially regulated genes overlapping in all three experiments were identified using the GeneSpring software. Classification into functional groups was done with help of the annotations of the Affymetrix NetAffx Analysis Center, SOURCE, the National Center for Biotechnology Information PubMed and LocusLink and with the OXPHOS and human mitoDB_6_2002 lists curated at the Whitehead Institute Center for Genome Research (Mootha et al., 2003).

List of light cycler primers and primer information

ordered alphabetical according to gene names

symbol	gene name	organism	primer no.	5'-3' sequence	forward f reverse r symbol	information	plasmid name
36B4	ribosomal subunit	human	428	cgt tgc cag ccc aga aca ct	f	housekeeping gene	pGEM4/h36B4
	P0		429	tga cca gcc caa agg aga ag	r		
act	actin	mouse	397	ggt cat cac tat tgg caa cga g	f	housekeeping gene	pCR2.1/mactin
			398	gtc agc aat gcc tgg gta ca	r		
ATPase	ATPase Na+/K+ transporting alpha	human	649	caa acg cat ggc aag gaa a	f	induced by GR and further enhar	no
			650	tgc aat tct gga cag agc aag	r	by PGC-1 in SAOS2	
ATPs	ATP synthase beta	human	584	gca agg cag gga gac cag a	f	target of PGC1 in SAOS2	no
			585	ccc aaa gtc tca gga cca aca	r		
			586	tgg tcc tga gac ttt ggg ca	f		
			574	gct ttg gcg aca ttg ttg att	r		
BAII	BAII associated protein	human	690	agc tgg agc aga agg tgg ag	f	induced by GR and no effect	no
			691	gct gat ggc gtc gat gta ctg	r	by PGC-1 in SAOS2	
BBS1	Bardet Biedl syndrome	human	641	Cac acc ttt tct gcc tgcc t	f	induced by GR and further enhar	no
			652	Ttt cgg tca tca cca gtg gtc	r	by PGC-1 in SAOS2	
COX4i1	cytochrome c	human	563	caa gcg agc aat ttc cac ct	f	target of PGC1 in SAOS2	pBS/COX4i1 no4
	oxidase 4 iso 1		564	Ggt cac gcc gat cca tat aag	r		
CPT1	carnithine palmitoyltransferase 1	human	731	Ggc atc atc act ggc gtg t	f	target of PGC1 in SAOS2	no
			735	Tgc tgt ctc tca tgt gct gga	r		
cytc	cytochrome c	human	443	cca gtg cca cac cgt tga a	f	target of PGC1 in SAOS2	pBS/cytc
			444	tcc cca gat gat gcc ttt gtt	r		
Endo G	Endonuclease G	human	719	cgc agc tac caa aac gtc tat g	f	target of PGC1 in SAOS2	no
			720	Ctc gat ggg cac cag gaa g	r		
ERRa	ERR alpha	mouse	575	gga gga cgg cag aag tac aaa	f	target of PGC1 in SAOS2	pSG5/mERRa
			576	gcg aca cca gag cgt tca c	r		
			577	cca gga aga cag ccc cag t	f		
			578	cac acc cag cac cag cac t	r		
ERRa	ERR alpha	human	505	Aag aca gca gcc cca gtg aa	f	target of PGC1 in SAOS2	pcDNA3/Gal4.hERRa
			506	Aca ccc agc acc agc acc t	r		
GAPDH	GAPDH	human	125	gaa ggt gaa ggt cgg agt c	f	housekeeping gene	pCR2.1/hGAPDH
			126	gaa gat ggt gat ggg att tc	r		
IL22R	Interleukin receptor 22	human	645	Cgt gaa att cca gtc cag caa	f	induced by GR and further enhar	no
			646	Cgg tga ccc tgg cat agt aga	r	by PGC-1 in SAOS2	
IDH3A	Isocitrate dehydrogenase isoenzyme 3	human	738	Att gat cgg agg tct cgg tgt	f	target of PGC1 in SAOS2	no
			739	Cag gag ggc tgt ggg att c	r		
Lip	Lipase	human	686	caa acc ctc cat cct cat gct	f	induced by GR and no effect of	no
			687	cgc aga cca agt gca ggt tc	r	PGC-1 in SAOS2	
MCAD	acyl-Coenzyme A dehydrogenase	human	561	tgt gga ggt ctt gga ctt gga	f	target of PGC1 in SAOS2	pBS/MCAD no5
	C-4 to C-12 straight chain (ACADM)		565	tcc tca gtc att ctc ccc aaa	r		
			433	ttc ttc ttt tgc acg tgg cc	r		

mtTFA	mitochondrial transcription factor	human	568	ccg agg tgtg ttt tca tct gtc	f	target of PGC1 in SAOS2	no
			569	caa cgc tgg gca att ctt cta	r		
			570	gat gct tat agg gcg gag tgg	f		
			571	gct gaa cga ggt ctt ttt ggt	r		
NRF-1	nuclear respiratory factor 1	human	434	Cag cag gtc cat gtg gct act	f	no target of PGC-1	pBS.NRF-1
			438	gcc gtt tcc gtt tct ttc c	r		
NRF-2	nuclear respiratory factor 2	human	580	caa ggc aac aga tga aac gg	f	no target of PGC-1	no
			581	gac ttg ctg acc ccc tga act	r		
p21	cyclin-dependent kinase inhibitor p21	human	423	Cgg gat gag ttg gga gga g	f	induced by GR and further enhar	pCMV5/p21
			424	Cgg cgt ttg gag tgg tag aa	r		
PERC		mouse	359	Caa gct ctg acg ctc tga agg	f	5mM MgCl	pCR2.1/mPERC
			356	gtt tca cct cca gcc tca gag	r		
		human	412	cca ggc agg cct cag atc ta	f	5mM MgCl	pBS/HA(91-426)hPER
			413	gcc gag gtg agg tgc tta tg	r		
PGC-1	PPARg coactivator 1	mouse	408	gga gcc gtg acc act gac a	f		pBS/mPGC-1(63-690)
			409	tgg ttt gct gca tgg ttc tg	r		
		human	416	gca ccg aaa ttc tcc ctt gta	f		pcDNA3/hPGC-1
			418	ttt gcttgg ccc tct cag ac	r		
PDK4	pyruvate dehydrogenase isoenzyme 4	human	643	Aat gcg ggc aac agt tga a	f	GR target	no
			644	Caa gcc gta acc aaa acc ag	r		
RIP140	RIP140	human	716	Tga tgc ctc tat ttt ccc caa	f	target of PGC1 in SAOS2	no
			718	Atc ccc tcc acc caa ttt tt	r		
sc219m2	solute carrier family 19 member 2 (thia	human	658	tga tgt gct act cct ctc gcc	f	induced by GR and further enhar	no
			659	cat ttc tcc cca agt tga cca	r	by PGC-1 in SAOS2	
stannin	stannin	human	682	atc gtc atc ctc att gcc atc	f	induced by GR and no effect of	no
			683	ctt tct ctc cac gca cgg tc	r	PGC-1 in SAOS2	
TIM22	translocase of inner mitochondrial mer	human	740	Cca agt cca gcc aag agt gag	f	target of PGC1 in SAOS2	no
			741	Cag cgg taa aca ccc caa at	r		
Tra	thyroid receptor alpha	human	573	cat ctt tga act ggg caa gt	f	annealing temp 48degr, 5 sec,	no
			579	ctg agg ctt tag act tcc tga tc	r	50 cycles, only fresh cDNA	
zinc	zinc finger protein 145	human	698	aag ctg cac agt ggg atg aag	f	induced by GR and no effect of	no
			699	atg ggt ctg cct gtg tgt ctc	r	PGC-1 in SAOS2	

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Appendix IV

CURRICULUM VITAE

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1984 - 1993 Gymnasium in Remseck, Germany, Abitur

1993 - 1999 Undergraduate studies in Biology at the University of Stuttgart-Hohenheim. Main subjects: Zoophysiology, Biochemistry, Plant physiology, Membrane physiology

1998 – 1999 Diploma work in the institute of Physiology, University of Stuttgart-Hohenheim: "Caveolae and caveolins in olfactory sensory cells: a biochemical characterization"

2000 – 2004 Ph D thesis in Biochemistry in the Biozentrum, University of Basel, Switzerland: " The transcriptional coactivator PGC-1α as a modulator of ERRα and GR signaling: function in mitochondrial biogenesis."

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LIST OF PUBLICATIONS:

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Appendix V

ACKNOWLEDGEMENTS

I am deeply grateful to my supervisor, Natasha Kralli, for giving me the opportunity to carry out my PhD thesis in her lab. I have learned a lot from her, and more importantly, she teached me to be a good scientist. I am especially thankful for the last year, where she did a really good job in supervising me although being on different continents. I appreciate her patience, her really good food at the Christmas parties, and her support in my rougher times.

Thanks a lot also to all the members of the Kralli lab, especially to Darko, Didi, Roger and Kathrin, with further special thanks to Didi for the few months alone in a big lab. I think we all were a good team, thanks a lot for the support and the nice time we had, although we did not really have the same taste of music.

I would also like to thank all the people on the 5th floor, the one who left and the one who are still there, in particular my dear friend Nicole, who always helped me, what ever I needed, and my dear friends Beatrice and Reika. I will miss our cooking lessons. Furthermore Paul and Stephen for their lab space, Verena and Werner for all their organisatory help, the FACS and light cycler 'team' in the 4th and 6th floor, Martin Spiess for his support, and in particular Ed Oakeley for all his help with the Affymetrix analysis.

Special thanks goes to my parents, and also to my brothers, thanks for all the support and encouragement in the harder times. My special gratitude goes to Lars, 'das Beste was mir je passieren konnte!'

Thanks also to my friend Biggi, my 'cyber'-friends Meike and Karin, for their tolerance and patience.