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STRUCTURE AND FUNCTION RELATIONSHIPS IN THE PGC-1 FAMILY OF TRANSCRIPTIONAL COACTIVATORS

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Structure and function relationships in the PGC-1 family of transcriptional coactivators

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my loves,

My parents, Maman and Baba

For everything

ABSTRACT

PGC-1 coactivators are central regulators of several cellular processes, most notably the coupling between energy demands and supply. PGCs activate distinct biological processes in a tissue-specific manner. These include mitochondrial biogenesis, oxidative phosphorylation, oxygen transport, gluconeogenesis, angiogenesis, and muscle fiber-type specification. The family consists of three members: PGC-1α, PGC-1β, and PRC, among which the most studied is PGC-1α. Transcription of a single PGC-1α gene produces different isoforms (e.g. PGC-1α1 to α4) with different biological functions. All of the PGC-1α isoforms share some domain similarity but PGC-1α2, α3 and α4 lack the C-terminal domains present in PGC-1α1. The actions of PGC-1α1 are strongly linked to energy metabolism, whereas PGC-1α4 regulates skeletal muscle hypertrophy. The functions of PGC-1α2 and PGC-1α3 have remained unknown.

To study the mechanism of action of the new PGC-1α isoforms, we performed a protein complex purification and identified protein partners of all PGC-1α variants. We found that all PGC-1α isoforms can function as positive regulators of transcription by associating with members of the Mediator complex, histone acetyltransferase complexes, and splicing factors. Furthermore, we identified several transcription factors associated with each PGC-1α isoform, which allowed us to predict how different target genes are regulated. Interestingly, we also observed that PGC-1α isoforms can regulate splicing events and can affect the exon composition of their corresponding target transcripts.

Here, we report for the first time that PGC-1α1 can dimerize with other PGC-1α isoforms, suggesting that some of the functions of PGC-1α might be mediated by heterodimers.

Since PGC-1α1 is a key modulator of cellular metabolism in several tissues, it has gained considerable attention as potential target for the treatment of metabolic disorders. For that reason, we developed a screening platform to identify chemicals that can induce PGC-1α1 protein accumulation. From this screen, we identified several candidate small molecules as potential PGC-1α1 activators, which were validated in brown adipocytes. We identified 4 compounds that can increase PGC-1α1 protein accumulation, target gene expression, and mitochondrial respiration. These compounds could represent the beginning of a new class of therapeutics against obesity and related disorders.

LIST OF SCIENTIFIC PAPERS

I. **Izadi, M.**, Nakadai, T., Jerdychowski, M.P., Ferreira, D.M.S., Cervenka, R., Agudelo, L.Z., Correia, J.C., Martinez-Redondo, V., Ketscher, L., Roeder, R.G. and Ruas, J.L. Integrated analysis of PGC-1α isoform-specific nuclear interactome and transcriptome reveals novel partners and mechanisms of action. (2018). *Manuscript*.

II. Martínez-Redondo, V., Jannig, P.R., Correia, J.C., Ferreira, D.M.S., Cervenka, I., Lindvall, J.M. Sinha, I., **Izadi, M.**, Pettersson-Klein, A.T., Agudelo, L.Z., Gimenez-Cassina, A., Brum, P.C., Wright, K.D. and Ruas, J.L. Peroxisome proliferator-activated receptor gamma coactivator-1α isoforms selectively regulate multiple splicing events on target genes. (2016). *Journal of Biological Chemistry*. 291; 15169-15184.

III. Pettersson-Klein, A.T*, **Izadi, M***, Ferreira, D.M.S., Cervenka, I., Correia, J.C., Martínez-Redondo, V., Southern, M., Cameron, M., Kamenecka, T., Agudelo, L.Z., Porsmyr-Palmertz, M., Martens, U., Lundgren, B., Otrocka, M., Jenmalm-Jensen, A., Griffin, P.R. and Ruas, J.L. Small molecule PGC-1α1 protein stabilizers induce adipocyte Ucp1 expression and uncoupled mitochondrial respiration. (2018). *Molecular Metabolism*.9; 28-42.

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II. Agudelo, L.Z., Ferreira, D.M.S., Cervenka, I., Bryzgalova, G., Jannig, P.R., Pettersson-Klein, A.T., Dadvar, S., Lakshmikanth, T., Sustarsic, E.G., Porsmyr-Palmertz, M., Correia, J.C., **Izadi, M.**, Martínez-Redondo, V., Ueland, P.M., Midttun, Ø., Gerhart-Hines, Z., Brodin, P., Pereira, T., Berggren, P.O and Ruas, J.L. Kynurenic acid and GPR35 regulate adipose tissue energy homeostasis and inflammation. (2018), *Cell metabolism*. 27; 378-392.

CONTENTS

LIST OF ABBREVIATIONS

ALS	Amyotrophic lateral sclerosis
AMPK	AMP-activated protein kinase
ATF-2	Activating transcription factor-2
BAF60	BRG1 associated factor 60a
BAT	Brown adipose tissue
CBP	CREB-binding protein
CREB	CAMP responsive element binding protein
Dio2	Deiodinase-2
DMD	Duchene muscular dystrophy
DMSO	Dimethyl sulfoxide
ERRα	Estrogen-related Receptor α
FOXO1	Forkhead Box O1
GCN5	General control of amino acid synthesis protein 5-like 2
G6Pase	Glucose 6-phosphatase
GSK3β	Glycogen synthase kinase 3β
GTFs	General transcription factors
HAT	Histone acetyltransferase
HCF	Host cell factor
HDAC	Histone deacetylase
HNF-4α	Hepatocyte nuclear factor-4α
IGF1	Insulin-like growth factor-1
MEF2C	Myocyte-specific enhancer factor 2C
Myf5	Myogenic factor 5
NMJ	Neuromuscular junction
Ndrg4	N-myc downstream-regulated gene 4
NRF-1	Nuclear respiratory factor-1
Osbpl1a	Oxysterol binding protein like 1a1
Pax 7	Paired box 7
PBX1	Pre-B-cell leukemia transcription factor
PEPCK	Phosphoenolpyruvate carboxy kinase

PIC	Pre-initiation complex
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator-1α
PGC-1β	Peroxisome proliferator-activated receptor γ coactivator-1β
PPARα	Peroxisome proliferator-activated receptor α
PPARγ	Peroxisome proliferator-activated receptor γ
PRC	PGC-1-related coactivator
PRMT1	Protein arginine methyltransferase
RNF34	Ring finger protein 34
ROS	Reactive oxygen species
RRM	RNA recognition motif
RS	Arg/Ser- rich domain
RXR	Retinoid X receptors
Sirt1	Sirtuin 1
SnRNP	Small nuclear ribonucleoproteins
SREBP	Sterol regulatory element binding protein
SWI/SNF	SWItch/Sucrose non-fermentable
TBP	TATA binding protein
TF	Transcription factor
TFAM	Mitochondrial transcription factor
TRAP	Triiodothyronine receptor auxiliary receptor protein
TSS	Transcription start site
UCP1	Uncoupling protein 1
VEGF	Vascular endothelial growth factor
XBP1	X-box binding protein 1
YY1	Ying yang 1

1 INTRODUCTION

1.1 TRANSCRIPTIONAL REGULATION IN EUKARYOTIC CELLS

Transcription is the process by which specific parts of the DNA sequence are copied into RNA, thus leading to gene expression. This process is tightly regulated in eukaryotic cells to ensure that gene expression is according to cell requirements and environmental signals. Transcriptional regulation is coordinated by a myriad of proteins such as transcription factors (TF), chromatin modifying and remodeling complexes and coregulators (coactivators or corepressors). In addition, transcription involves specific DNA sequences such as promoters and enhancers.

One of the essential requirements for transcription is the presence of an RNA polymerase (RNApol) enzyme. Mammalian eukaryotic cells have 3 classes of RNApol that are responsible for transcribing different types of genes. RNApol I transcribes large ribosomal RNA genes. Protein coding genes and some small RNAs are transcribed using RNApol II. RNApol III transcribes tRNA, 5S RNA and other small structural RNA [1-3]. Initially, gene expression depends on the assembly of a pre-initiation complex (PIC) containing one of the mentioned RNA polymerases on the core promoter region. Each RNApol has specific general initiation factors that contribute to accurate initiation of transcription from a core promoter region. PICs are assembled by several general transcription factors (GTFs) [4] to start transcription from the correct position on the promoter region [5-7].

A second level of transcriptional regulation is mediated by a large number of proteins called TFs that recognize and bind to specific DNA sequences located in the regulatory regions. These TFs express and activate in response to special environmental cues and lead to inhibition or increase in the expression of their specific target genes [8]. The third group of transcriptional regulators exerts their role in a DNA-binding independent manner. These proteins are called coactivators or corepressors depending on if they activate or repress transcription, respectively. These proteins affect gene expression through remodeling chromatin structures/modifying histones or they affect pre-initiation complex formation and function [7, 9].

1.1.1 Transcription by RNA polymerase II

RNApol II is a protein complex with ability to perform RNA synthesis and also proofreading of the nascent transcript [1]. The first step in transcription by RNApol II is

binding to sequence specific promoter region by the action of the Transcription Factor IID (TFIID) complex, component of the PIC. TFIID contains the TATA-binding protein (TBP) and 13 TBP-associated factors, which bind to TATA element in the promoter. This leads to a bend in DNA that acts as a platform for PIC assembly [10, 11]. Later binding of TFIIB stabilizes the interaction of TFIID to the DNA on the TATA element and this complex is further stabilized by TFIIA binding [12]. Moreover, TFIIB joining to the complex leads to the addition of RNApol II in association with TFIIF to the PIC and it helps in positioning the active site of RNApol II at the transcription start site (TSS) [13]. Binding of TFIIE and consequently TFIIH will complete assembly of the PIC [14], which modulate promoter melting (opening DNA duplex on the promoter region) with ATP-dependent helicase activity of TFIIH [15-17] that leads to formation of an open complex between DNA and RNApol II. Finally, in the presence of all nucleotide triphosphates (NTPs), RNApol II is able to clear the promoter and enters the transcription elongation phase [18] (Figure 1).

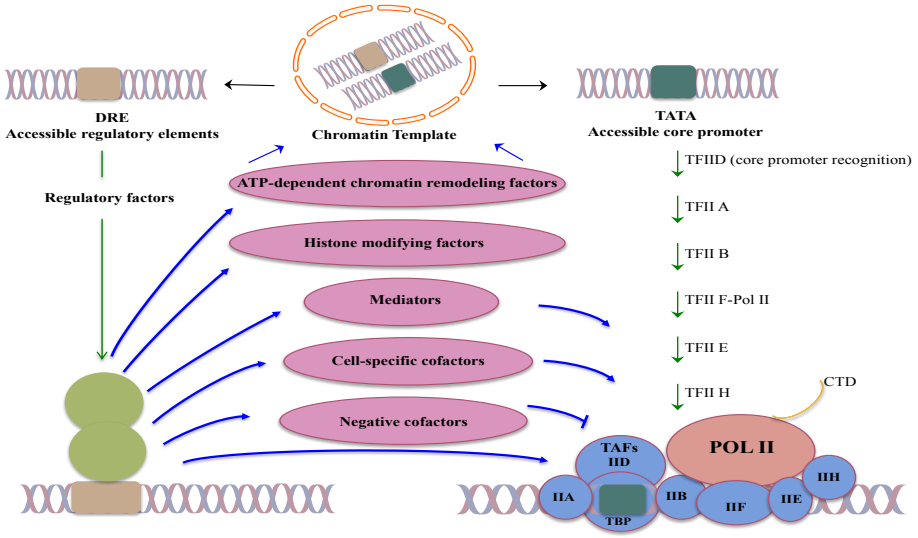

Figure 1. Schematic model for the regulation of PIC assembly and regulation of transcription by RNApol II. Assembly of a PIC including RNApol II and general transcription factors on the TATA element of the core promoter. PIC assembly and function is regulated sequentially by binding of regulatory factors to distal regulatory regions on the DNA**,** binding of chromatin remodeling complexes and histone modifying factors to modify chromatin structure and finally, binding of other regulatory factors that facilitate function of the general transcription machinery. (Adapted from Robert G. Roeder, 2005, *FEBS letters*)

1.1.2 Transcriptional coregulators

Transcriptional coregulators are proteins that can directly cooperate with the transcription machinery and increase (coactivators) or decrease (corepressor) the levels of gene

expression. This group of proteins might have enzymatic activity to change chromatin structure or they might act as a scaffold and provide a regulatory platform for binding of chromatin modifiers and transcriptional machinery [19].

Histone modifying and chromatin remodeling complexes

Eukaryotic genomic DNA is organized in a compact chromatin structure that is not readily accessible to the general transcription machinery. Several protein complexes contribute to regulating DNA accessibility for gene expression. These protein complexes exert their function by remodeling chromatin or through chromosomal histone modifications. All of these modifications alter the accessibility of DNA for binding of activators or repressors and further gene regulation. Histone modifying enzymes are a group of enzymes that modulate gene expression by mediating covalent acetylation, methylation, or ubiquitylation of lysine residues, methylation of arginine residues, and phosphorylation of serine and threonine residues at N-terminal regions of histones [20]. Among proteins with functional histone acetyltransferase activity (HAT) are CREB-binding protein CBP [21], the E1A binding protein p300 [22], and the p300/CBP-associated factor p/CAF [23]. Protein arginine methyltransferase PRMT1 [24] and histone deacetylase proteins (HDAC) such as GCN5 [25] are other examples of histone modifying enzymes that regulate gene expression through interaction with different transcription factors. ATP-dependent chromatin remodeling complexes such as SWI/SNF complex are the second group that can increase or decrease the accessibility of DNA for gene regulation [9]. These proteins mobilize nucleosomes to facilitate access of the transcription machinery and regulators to DNA [26]. SWI/SNF complex has been shown to be important for ligand-dependent activation of transcription by nuclear receptors [27].

Mediator Complex

Another important coactivator complex necessary for the expression of protein-coding genes is the mediator complex. The human mediator complex was initially identified as a thyroid hormone receptor-associated protein (TRAP) [28]. Since then, several structural and functional studies have elucidated how the mediator functions in the control of transcriptional activation and repression. The mediator complex facilitates transcription by making a bridge between gene-specific transcription factors bound to specific regulatory elements on DNA and RNApol II in the PIC complex [29, 30]. The mediator also

participates in releasing RNApol II from the promoter and transition from initiation to elongation process by stimulating phosphorylation of the C-terminal domain (CTD) of RNApol II [31]. Disturbances in mediator function have been associated with several diseases including cancer, metabolic and neurological disorders and cardiovascular diseases [32].

1.2 SPLICING

Several processes convert nascent transcript into mature RNA including 5'cap, RNA splicing and polyadenylation at 3' end of the mRNA molecule. These three processing reactions can occur coupled with transcription. RNA splicing was discovered in 1977 [33, 34] and is one of the main steps to generate a mature mRNA in a eukaryotic system. This process involves the removal of intron sequences and joining exon sequences together. Splicing events have been characterized in different systems and happen through the excision of intron sequences at the conserved splicing sites found on the 5'(GU dinucleotide) and 3' (AG dinucleotide) end of introns [35]. In eukaryotes, the pre-mRNA splicing site recognition and the splicing reaction are catalyzed by the spliceosome complex. This complex is composed of small nuclear ribonucleoproteins (snRNPs) U1, U2, U4, U5, U6 and auxiliary factors including U2AF65 and U2AF35. All these proteins (with the help of RNA-dependent ATPases and helicases) lead to two steps of esterification and removal of intron sequence (cutting and sewing on the splice sites) [36].

Alternative splicing is the process that generates multiple mRNAs from a single gene and was discovered together with splicing [33, 34]. Alternative splicing increases the capacity of the eukaryotic genome to generate more proteins (protein isoforms with different peptide sequences, chemical and biological activities) from a single gene [37]. The presence of weak or strong splicing sites can result in different modes of alternative splicing (use of alternative 5' splice site or alternative 3' splice site and inclusion of alternative cassette exon). Alternative splicing can also happen through alternative polyadenylation sites or transcription initiation at different promoters. Most of the studies have focused on cis-acting elements on the mRNA and trans-acting regulatory proteins that bind to these sequences to increase or silence the usage of adjacent splicing sites but it also has been shown that more than half of human genes have alternative promoters [38]. All of the mentioned mechanisms of alternative splicing contribute to protein diversity. Differences in peptide sequences can alter post-translational modifications, protein stability, localization, ligand binding, allosteric regulation and enzymatic activity and finally lead to different

cellular and developmental regulation. Misregulation of alternative splicing is linked to diseases such as cancer and developmental disorders [39].

1.3 THE PGC-1 FAMILY OF TRANSCRIPTIONAL COACTIVATORS

Proteins of the (PGC-1) family of coactivators are main components in the regulation of energy metabolism in many tissues, often in response to higher energy demands. The first member of the PGC-1 family, PGC-1α, was identified in the late 1990s in brown adipose tissue as a key regulator of genes involved in cold-induced thermogenesis [40]. Since then, from PGC-1α DNA sequence and protein homology searches, PGC-1β and PRC (PGC-1-related coactivator) joined the PGC-1 family [41, 42]. These proteins have high homology in structural features and share some biological activities, such as the ability to regulate mitochondrial biogenesis in different tissues. Since PGCs do not have a DNA-binding domain they exert their biological functions through interaction with transcription factors and other coactivator complexes. Most PGC-1s exert their biological function through a few shared protein domains among them (Figure 2).

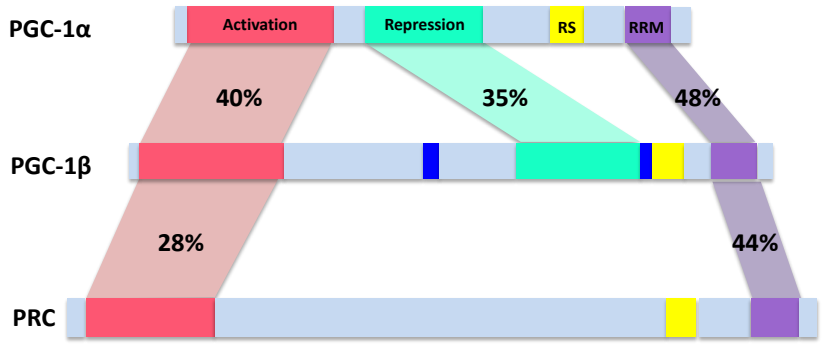

Figure 2. Schematic representation of the PGC-1 family members homology. Homology in PGC-1α, PGC-1β, and PRC structures. They all share Activation, Arg/Ser- rich domain (RS), and RNA recognition motif (RRM). The Repression domain is only present in PGC-1α and PGC-1β. (Adapted from Lin J, 2005, *Cell Metabolism*).

Among this family of coactivators, PGC-1α has been especially studied in different research areas such as energy metabolism, cardiovascular disease, neurodegenerative diseases, skeletal muscle physiology, and even mood disorders. Many studies have determined the functions of PGC-1 family in different physiological and disease conditions, thus elucidating some of the underlying mechanisms by which these transcriptional regulators modulate cellular adaptation to different challenges (Figure 3).

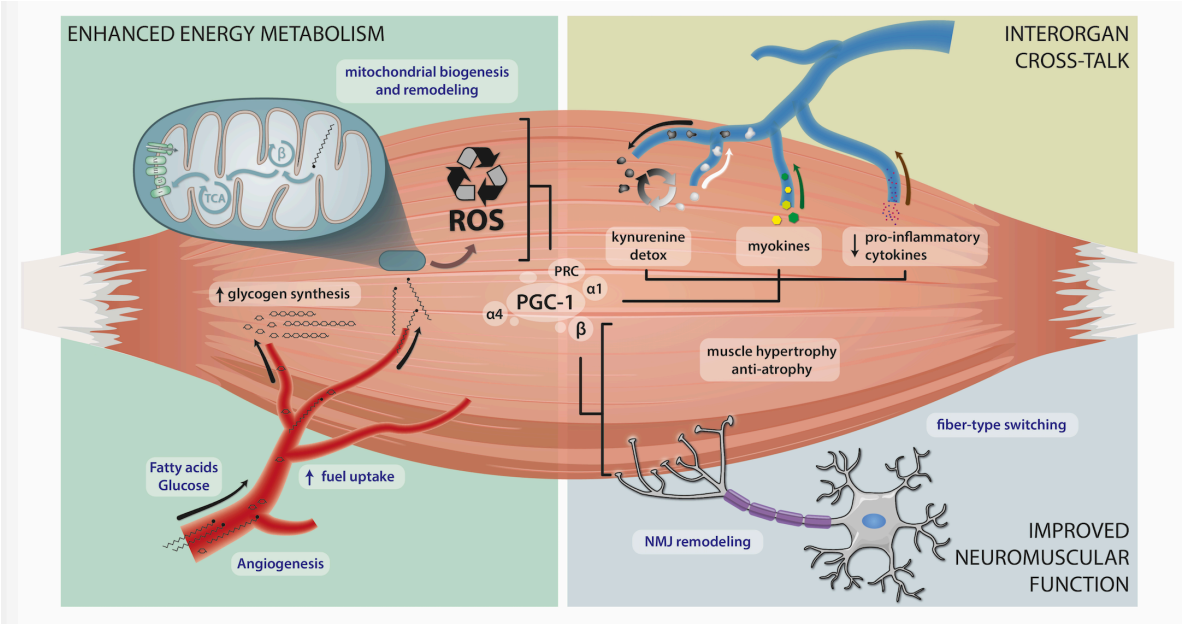

Figure 3. Skeletal muscle PGC-1s orchestrate systemic adaptations to physiological cues. Different PGC-1 proteins activate specific gene programs to regulate cellular adaptation to diverse challenges. They regulate fuel supply, uptake and utilization. (Adapted from Correia et al, 2015, *Trends in endocrinology and metabolism*)

Since PGC-1α gene can be transcribed into multiple splice variants (please see 1.3.3), we refer to the canonical transcript as PGC-1α1. PGC-1α1 is a highly regulated coactivator whose activity and expression can be regulated by diverse stimuli in a tissue-specific manner. These include cold exposure in brown adipose tissue (BAT), fasting in liver, and exercise in skeletal muscle (among others). However, while in skeletal muscle PGC-1α1 is involved in fiber type switching toward more oxidative phenotype [43, 44], in brown and beige adipose tissue it induces adaptive thermogenesis and energy release in the form of heat [40]. Therefore, increased PGC-1α1 levels in either tissue could potentially have positive effects on whole body metabolism. The key role of PGC-1α1 in fuel handling makes this protein an interesting target in the treatment of type 2 diabetes, obesity, or any disorders associated with skeletal muscle wasting [45-48]. In fact, transgenic animals with skeletal muscle-specific PGC-1α1 overexpression are protected from muscular atrophy, diet-induced obesity, age-related sarcopenia, and insulin resistance [49]. Adipose-specific PGC-1α genetic deletion disturbs the mitochondrial and thermogenic gene networks, which makes those mice more prone to high-fat diet-induced insulin resistance [50]. Reduced PGC-1α1 levels have been reported in skeletal muscle and adipose tissue of diabetic patients, which could contribute to the pathogenesis of the disease [51]. Finally, PGC-1α1 has also been implicated in the detoxification of reactive oxygen species (ROS) [48] and protection against neurodegenerative disorders like Huntington's and Alzheimer's diseases

and also amyotrophic lateral sclerosis (ALS) [52, 53].

The tissue expression profile of PGC-1β is similar to PGC-1α but its expression is not regulated by the same signals as PGC-1α. For instance, PGC-1α induces hepatic gluconeogenesis during fasting, while PGC-1β induced upon fasting modulates hepatic lipogenesis [54].

The function of PRC has been studied *in vitro* and the role of PRC seems to be limited to the regulation of mitochondrial biogenesis in amplifying cells [55]. The main problem of studying the role of PRC *in vivo* is that disruption of the PRC-encoding gene, results in embryonic lethality. Other functions recently described for the PGC-1 family include lipid synthesis, lipoprotein secretion, angiogenesis, hematopoiesis and immune response [56].

1.3.1 Structure and modes of action

PGC-1 coactivators exert their role in transcriptional regulation via interaction with a variety of transcription factors (such as nuclear receptors) and other coactivators. As many other coactivators, this family of proteins does not have a DNA-binding domain. Thus, they work as a docking platforms for other proteins with, for example, histone acetyltransferase activity or involved in the assembly of the basal transcription machinery [56]. In this manner, they facilitate pre-initiation complex formation to efficiently regulate gene expression. The protein domain organization of PGC-1 coactivators is remarkably similar, which describes the partial overlap in their physiological functions [57]. All three members share structural domains in their N- and C-terminal regions. Their N-terminal region includes a highly conserved activation domain that is an interaction surface for the recruitment of histone acetyltransferase proteins, such as the SRC-1 (Steroid Receptor Coactivator-1)/p300 complex (Figure 4) [58]. The activation domain includes several leucine-rich LXXLL motifs, also called NR (Nuclear Receptor) boxes, which mediate association of PGC-1s with the ligand-binding domain of a variety of nuclear hormone receptors [42, 59]. Following the activation domain, PGC-1α has a repression domain that interacts with P160 and Sirt1 (Sirtuin 1). These factors regulate PGC-1α's biological activity.

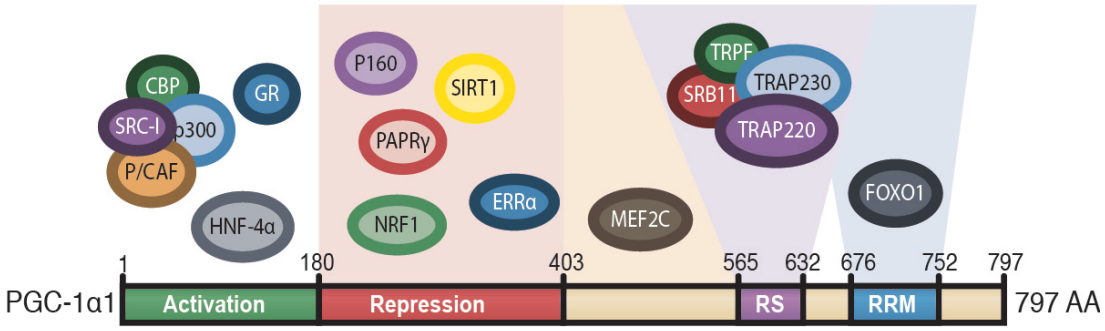

Figure 4. Protein complexes associated with PGC-1α1. PGC-1α1 binds to different protein partners through several domains in its structure. PGC-1α1 binds to the histone acetyltransferase (HAT) and TRAP/DRIP/Mediator complexes through the amino and carboxyl termini, respectively. SIRT1 (Sirtuin 1) and P160 bind to the repression domain, which also contains three p38 MAP kinase phosphorylation sites (P). LXXLL and LLXXL motifs are nuclear receptor binding sites. Splicing factors bind to RNA binding domain in C-terminus. GR (Glucocorticoid Receptor), HNF-4α (Hepatocyte Nuclear Factor-4α), PPARγ (Peroxisome Proliferator-Activated Receptor γ), ERR-α (Estrogen-related Receptor α), NRF-1 (Nuclear Respiratory Factor-1), MEF2C (Myocyte-specific Enhancer Factor 2C), FOXO1 (Forkhead Box O1).

The greatest structural similarity between the three PGC-1 coactivators is at the carboxy-terminal region [60, 61]. All three C-termini contain an RNA recognition motif (RRM) similar to the one found in SR proteins and heterogeneous nuclear ribonucleoproteins, which are known to mediate RNA binding. They also share an arginine-serine rich (RS) domain [40], before the RRM domain, which is typical of proteins involved in mRNA splicing [62]. The presence of RS and RRM domains in PGC-1 proteins suggests that they can be involved in RNA processing. Indeed, PGC-1α1 mutants that lack the RS and RRM domains cannot bind some splicing factors and therefore cannot regulate splicing processes in an *in vitro* system. This evidence suggests that PGC-1α1 exerts its function not only by quantitative regulation of gene expression, but also by coupling transcription and mRNA splicing [63]. It has been previously reported that the C-terminal region of PGC-1α1 is necessary for interacting with the mediator complex and the transcriptional apparatus through TRAP220/MED1 (Mediator of RNA polymerase II transcription subunit 1) [58]. The C-terminal region of PGC-1 proteins includes also two other highly conserved motifs with unknown function. One of them consists of a "DHDY" motif, which has been described as a binding site for the host cell factor (HCF) transcription factor. HCF acts in a complex with O-GlcNAc transferase to regulate PGC-1α1 stability during gluconeogenesis by inhibiting ubiquitylation of PGC-1α1 [64]. In addition, the C-terminus of PGC-

1 proteins is the interaction site for other transcription factors such as MEF-2C (Myocyte enhancer factor-2C) [43, 65], YY1 (Ying yang 1) [66, 67], and FoxO1 (Forkhead box O1) [68] as well as some coregulators such as BAF60 (BRG1 associated factor 60a) [69].

1.3.2 Regulation of PGC-1α activity

Most of the gene networks regulated by PGC-1α encode proteins involved in bioenergetically demanding metabolic pathways. The activation or inhibition of PGC-1α during these programs results in cellular adaptations to higher energy demands in response to tissue-specific physiological signals. Hence, it is important that cells keep a tight control over PGC-1α de novo synthesis, protein stability, and transcriptional activity. Many of these mechanisms are activated when BAT induces PGC-1α in response to cold exposure [40, 70, 71]. Among the signaling pathways that modulate PGC-1α1 gene expression some of the most well-characterized involve calcium-signaling [72], calcineurin A [65], cyclic AMP [73], and AMP-activated protein kinase (AMPK) [74]. In response to activation of these pathways several members of the nuclear receptor family, tissue-specific transcription factors, and other coactivators will be recruited to regulatory regions surrounding the PGC-1α gene [57]. Epigenetic modifications such as DNA methylation can limit the transcriptional regulators' approach to the proper genomic regions and thus interfere with the modulation of gene expression [75]. Some studies have reported the effects of PGC-1α promoter methylation on PGC-1α expression in different physiological and pathological situations. PGC-1α promoter methylation is decreased by physical exercise, a typical inducer of PGC-1α expression in skeletal muscle [76]. In contrast, increased PGC-1α promoter methylation has been identified in pathological situations linked with decreased level of PGC-1α expression [77, 78].

Post-translational modifications play a prominent role in modulating the activity of PGC-1α. These modifications include phosphorylation, acetylation, and ubiquitylation. Although this is not fully understood, the combination of different phosphorylation events seems to result in a code that ultimately regulates the biological activity of PGC-1α1. AMPK [79], p38 mitogen activated protein kinase (p38MAPK) [80], S6 kinase [81], protein kinase B [82], and glycogen synthase kinase 3β (GSK3β) [83] are among kinases that regulate PGC-1α1 activity. Phosphorylation by these kinases dictates association of PGC-1α1 with specific transcription factors or other coregulators [57]. The acetylation of PGC-1α1 can be altered by acetyltransferases such as GCN5 (General control of amino acid synthesis protein 5-like 2) [25] or deacetylases like Sirt1 [84, 85]. Deacetylation by Sirt1 results in

activation of PGC-1α1, while acetylation by GCN5 represses PGC-1α1 activity. It has been shown that selective chemical inhibition of PGC-1α acetylation by GCN5 can ameliorate insulin sensitivity and thus reduce blood glucose in type 2 diabetic mice. This process happens through inhibition of interaction of PGC-1α with HNF-4α and activation of gluconeogenesis in liver [86]. In addition, PGC-1α stability and cellular localization can be modified with other post-translational modifications. Ubiquitylation by specific E3 ligases such as RNF34 and SCF^{Fbw7} directly regulates PGC-1α protein stability [83, 87, 88]. Moreover, two studies have reported mitochondrial localization of PGC-1α in a complex with SIRT1 and TFAM (mitochondrial transcription factor A) [89, 90]. Mitochondrial localization of PGC-1α suggests that it could directly coordinate the transcription of nuclear-coding genes and mitochondrial genomes, to control mitochondrial biogenesis. In summary, all these events occur to ensure that cells under a particular physiological circumstance can have the correct PGC-1α activity to quickly adapt to the new biological situations.

1.3.3 PGC-1α variants

To date, numerous studies on PGC-1α structure and function have been performed on the mouse 797-amino acid long protein. It has been recently shown that transcription of the PGC-1α gene can be regulated by distinct promoter regions [91-94]. The use of several tissue-specific PGC-1α gene promoters coupled with alternative splicing leads to expression of several transcript and protein variants, which have diverse structure and function [95, 96]. Alternative splicing is a universal phenomenon which results in the production of distinct mRNAs from multi-exonic genes [97]. Our lab has originally identified and continues to focus on three of these variants known as PGC-1α2 through PGC-1α4 [96].

PGC-1α1 is the most well-known isoform and is expressed from the previously identified, canonical gene promoter [65]. Alternative promoter usage results in the expression of PGC-1α2, α3, and PGC-1α4. PGC-1α2 and α3 have different first exons but similar remaining exon/intron structure. This results in the expression of two distinct proteins with different N-terminal but overall similar domain structure (Figure 5). Moreover, PGC-1α2 is 379 amino acids long with 41.9 kDa predicted molecular weight while PGC-1α3 is 370 amino acids long and has a predicted molecular weight of 41.0 kDa. In their structure they maintain part of PGC-1α1's activation and repression domains and completely lack all the

C-terminal motifs of PGC-1α1 (i.e., the RS and RRM motifs) [96]. Despite PGC-1α4 having the same alternative exon1 with PGC-1α2, it transcribes to a different mRNA structure (Figure 5). In fact, PGC-1α4 contains a premature stop codon induced by the insertion of a 31-nucleotide between exons 6 and 7. As a consequence, PGC-1α4 has a predicted molecular weight of 29.1 kDa, encoded by a 266 amino acid protein [96]. As PGC-1α2 and α3, PGC-1α4 also lacks all the C-terminal motifs of PGC-1α1 [94].

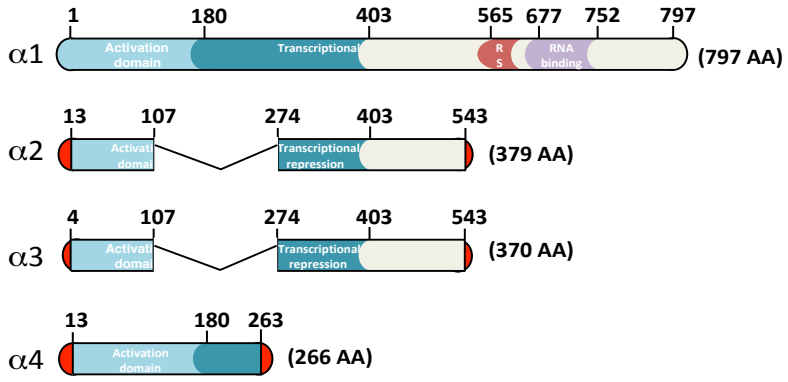

Figure 5. Members of PGC-1α family. Three newly identified PGC-1α isoforms are expressed from an alternative gene promoter. All new PGC-1α isoforms lack the C-terminus motifs of PGC-1α1 but retain part of the activation and repression domains.

Induction of PGC-1α2 and PGC-1α3 by physical exercise has been reported to be associated with VEGF (Vascular endothelial growth factor) expression and promotion of estrogen-related receptor (ERR)α-dependent angiogenesis [93]. Surprisingly, PGC-1α4 expression in skeletal muscle leads to robust muscle hypertrophy [96] by upregulation of IGF-1 (Insulin-like growth factor-1) expression, which regulates muscle mass [98] and decreasing myostatin level, a negative regulator of muscle growth [99, 100]. Moreover, high expression of PGC-1α4 levels in mouse skeletal muscle causes resistance to cancer-cachexia and disuse-induced atrophy [96]. In addition, hypoxia activates skeletal muscle PGC-1α4 to increase angiogenesis through ERRα and increase in VEGF gene transcription [101]. The mechanism of action of PGC-1α4 still needs to be further elucidated. The biological functions of PGC-1α2 and PGC-1α3 are under investigation.

Changes in the expression of different PGC-1α coactivators in skeletal muscle can have several systemic consequences with even crosstalk between skeletal muscle and other tissues (Figure 3) [56]. Recently, several studies report that elevating specific PGC-1α variant in skeletal muscle can affect other tissues by secreting circulating factors (myokines). Among these myokines are irisin and BAIBA (β-aminoisobutyric acid) [102],

both under PGC-1α1 control, and the PGC-1α4-regulated myostatin and meteorin-like proteins [103, 104]. These molecules are secreted from skeletal muscle, transferring specific metabolic signals to, for example, adipose tissue. In this manner, overexpression of either PGC-1α1 or PGC-1α4 in skeletal muscle results in browning of adipose tissue through diverse mechanisms and pathways. Furthermore, we have recently reported that activation of skeletal muscle PGC-1α1 can protect from stress-induced depression [105] by skeletal muscle detoxification of the tryptophan metabolite kynurenine, which is converted to kynurenic acid. Moreover, Mills *et al* [106] show that skeletal muscle transduction with PGC-1α1 isoform but not PGC-1α4 stimulates secretion of neurturin, which is sufficient and necessary to increase NMJ formation and size. Neurturin was identified as a myokine that mediates this retrograde messaging from muscle to motor neurons.

1.3.4 PGC-1α stability

PGC-1α1 protein has a short half-life of approximately 30 minutes. Several studies have demonstrated that PGC-1α1 is a substrate for degradation by the ubiquitin-proteasome system [83, 87, 88, 107, 108]. Ubiquitin-mediated proteolysis is an important regulatory mechanism involved in different cellular processes such as development, cell cycle control, and transcription [109]. The E3 ubiquitin ligases recognize substrates for ubiquitylation. So far, different laboratories have reported some E3 ubiquitin ligases, which can target PGC-1α1 for degradation. The function of E3 ligases in the ubiquitylation system is to determine substrate specificity via interaction with target proteins and transfer of ubiquitin to them. Different E3 ligases bind to different PGC-1α1 regions to target it for degradation. Some of these ligases recognize a domain in the N-terminus of PGC-1α1 that targets this protein for degradation and has a code for ubiquitylation. Some other ligases recognize a C-terminal domain as a code to process ubiquitylation.

It has been shown that the multi-subunit E3 ligase SCF^{Fbw7} complex promotes PGC-1α1 ubiquitylation and degradation. SCF^{Fbw7} recognizes a phosphodegron consisting of four amino acids in the N-terminus of PGC-1α1 protein. This phosphodegron is phosphorylated by GSK3β and p38MAPK [83] and is conserved in the PGC-1 family, suggesting that SCF^{Fbw7} might also be an E3 ligase for PGC-1β. However, the importance of this ubiquitylation by Fbw7 in PGC-1α target gene expression and energy metabolism is still not clear. RNF34 is another E3 ligase controlling PGC-1α degradation with regulation of the endogenous PGC-1α protein levels, target gene expression, and cellular respiration

capacity in brown fat cells. The effects of RNF34 are independent of change in PGC-1α expression and disappear with expression of a mutant RNF34 that misses its ligase activity [88]. In contrast to SCF^{Fbw7}, the C-terminal half (amino acids 350 to 797) of PGC-1α interacts with RNF34 and targets this protein for degradation.

Although PGC-1α1 is an intrinsically disordered protein (IDP) that degrades fast by the 20S proteasome [110], some studies have shown that binding of PGC-1α1 to some of its protein partners like ERRγ can induce conformational changes in PGC-1α1 structure that might rescue this protein from degradation [111].

1.4 THE ROLE OF PGC-1α IN METABOLIC REGULATION IN DIFFERENT TISSUES

1.4.1 PGC-1α function in skeletal muscle

Skeletal muscle (about 40% of the body weight of a lean individual) is a tissue with fundamental role in mobility, energy balance and metabolism in the body. Interestingly, PGC-1α expression increases both in human and rodent muscle after exercise [112, 113]. A summary of PGC-1α function in skeletal muscle in response to different physiological cues is shown in Figure 6. In skeletal muscle especially oxidative slow-twitch fibers, all PGC-1α isoforms can be expressed in response to exercise to increase mitochondrial content and oxidative phosphorylation capacity. It has also been shown that increasing muscle PGC-1α expression (driven by muscle creatine kinase promoter) will protect from sarcopenia (loss of muscle mass and strength) and metabolic diseases during aging. This protection happens through increase in mitochondrial gene expression and improved metabolic responses such as increased insulin sensitivity in aged mice. Moreover, overexpression of PGC-1α in mouse model leads to fiber type switch toward slow-twitch fibers rich in mitochondria [43], increased angiogenesis [114] and improvement in muscle function and motor coordination [115]. All these studies emphasize the importance of PGC-1α in the adaptation of skeletal muscles to physical activity. On the other hand, PGC-1α skeletal muscle-specific knockout mice show a shift from oxidative fibers toward more glycolytic fibers and exhibit reduced endurance capacity and increased inflammation markers after treadmill running [116]. Nevertheless, there are some other studies that suggest PGC-1α expression in muscle is not strictly required for adaptation to exercise [93, 113]. These studies were done using global PGC-1α knockout or skeletal muscle specific PGC-1α knockout mice. These mice can perform similar voluntary exercise (not forced exercise) and run the same distance as their

wild-type controls. This discrepancy in the results can be due to the differences in targeting strategy in generation of the whole body and muscle specific PGC-1α knock out mice. The global PGC-1α knockout mice show a complex phenotype since these animals are characterized by elevated metabolic rates and hyperactivity [71]. Moreover, we should also consider the functional redundancy between PGC-1α and PGC-1β in maintaining skeletal muscle function.

Besides physical activity, calorie restriction is another challenge that will alter oxidative metabolism in muscle. This stimulus also leads to the induction of PGC-1α expression that is necessary for increase in mitochondrial mass [117].

All these beneficial effects of PGC-1α in metabolic control in the muscle has promoted this protein as a good candidate for treatment of metabolic disorders and muscle wasting disorders such as sarcopenia and Duchene muscular dystrophy (DMD) [46] or cancer. Overexpression of PGC-1α can ameliorate DMD and is associated with improvement of muscle injury. This phenomenon happens through increase in mitochondrial biogenesis and oxidative phosphorylation [118]. Moreover, overexpression of Sirt1, PGC-1α activator, also ameliorates DMD in the mouse model with this disease (mdx mice) by increasing mitochondrial biogenesis, fiber type switching toward oxidative fibers, and inducing genes regulating neuromuscular junction [119].

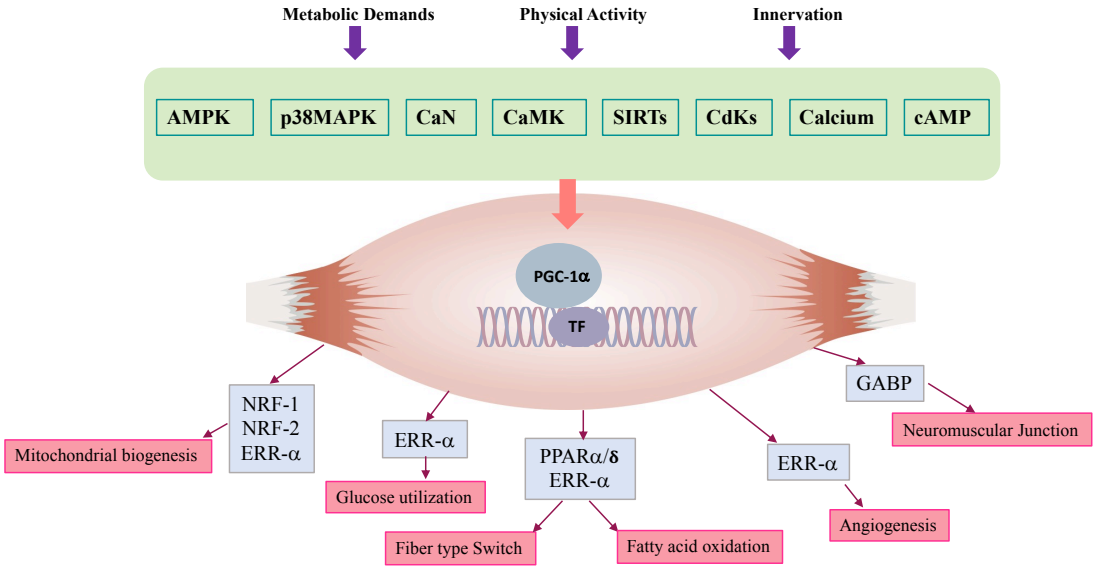

Figure 6. PGC-1αs regulatory function in skeletal muscle. Different physiological cues trigger specific gene programs to modulate cellular adaptation in skeletal muscle to diverse challenges. These gene programs modulate mitochondrial biogenesis, glucose utilization, fiber type switching, angiogenesis and NMJ properties to improve skeletal muscle function and performance. CaN (Calcineurin), CaMK (Calmodulin-dependent protein Kinase), CdK (Cyclin-dependent Kinase).

1.4.2 PGC-1α function in liver

PGC-1α expression is induced in liver upon fasting. This coactivator shows a pivotal function in the regulation of gluconeogenesis and hepatic expression of mitochondrial genes in liver. This regulation by PGC-1α occurs through interactions with transcription factors such as FOXO1 [68], HNF-4α [73] and glucocorticoid receptors and increases in the transcription of genes such as phosphoenolpyruvate carboxy kinase (PEPCK) and glucose 6-phosphatase (G6Pase). PGC-1α induction by fasting happens both through glucagon and glucocorticoids [73, 120], which are the main signaling hormones during fasting and deacetylation by Sirt1, which leads to increase in its transcriptional activity [121]. This tight regulation balances the hepatic glucose production according to fuel supply and demands. Impaired control over gluconeogenesis will affect the whole-body glucose homeostasis. Indeed, elevated hepatic glucose production is recognized as one of the consequences of hepatic insulin resistance [122]. Reduced hepatic insulin sensitivity results in increased hepatic lipogenesis and hepatic steatosis [123].

1.4.3 PGC-1α function in brain

PGC-1α is expressed in different cell types of the brain, such as dopaminergic cells [124]. In addition to regulating mitochondrial biogenesis and oxidative phosphorylation, this coactivator is also implicated in the activation of antioxidant defense genes in the brain [48]. Since neural tissues are very dependent on mitochondrial function, a lack of PGC-1α expression has been suggested to contribute to many neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases [52, 125, 126]. In all of these disorders, reduced mitochondrial function due to reduced levels of PGC-1α expression has been reported, making PGC-1α a potential therapeutic target for neurodegenerative diseases. Moreover, we have recently reported that activation of skeletal muscle PGC-1α1 can have protective effect against stress-induced depression [105]. The mechanism that mediates this muscle to brain crosstalk is through skeletal muscle detoxification of kynurenine, which is in this way converted to kynurenic acid. Kynurenine aggregation in the brain has been connected to neuroinflammation and depression. Activation of the PGC-1α1-PPARα/δ pathways in skeletal muscle induces kynurenine aminotransferases expression, the enzymes responsible for the conversion of kynurenine to kynurenic acid. Since kynurenic acid cannot freely cross the blood-brain barrier, it remains in the periphery thus reducing central kynurenine accumulation and associated effects.

1.5 MOLECULAR ASPECTS OF ADIPOSE TISSUE

Vertebrates are able to store energy in the form of lipid droplets in specialized cells known as adipocytes [127]. From a broad metabolic perspective, adipocytes tend to be grouped into 3 types: white adipocytes, brown adipocytes, and beige/brite adipocytes. White adipocyte store lipids, which can later be released as fatty acids. On the other hand, brown adipose tissue (BAT) stores lipids as fuel for thermogenesis [128, 129]. BAT is highly vascularized and contains multilocular lipid droplets. Brown adipocytes exert their function in thermogenesis through uncoupling protein 1 (UCP1), which is located in the mitochondria. Prolonged cold exposure or adrenergic signaling induces other $UCP1^{+}$ cells located in white fat depots. These cells are called beige/brite adipocytes and have similar morphology to brown adipocytes but distinct gene expression profiles and have lower basal level of UCP1 expression [130]. Additionally, beige/brite adipocytes have been shown to promote thermogenesis through UCP1-independent mechanisms [131].

1.5.1 Developmental origins of brown adipose tissue

Brown adipocytes exist in both rodents and newborn humans in paraspinal, intrascapular and interscapular regions. In rodents, brown adipocytes that are located in the interscapular regions develop during embryogenesis. BAT share the same precursor cells with skeletal muscle and they both derive from $PAX7^{+}/Myf5^{+}$ stem cells during development [132, 133] and their divergence occurs between days 9.5 and 12.5 of mouse gestation [134] (Figure 6). In contrast, white and beige/brite precursor cells derive from $PAX7^{-}/Myf5^{-}$ stem cells. It has been shown that beige/brite adipocytes that have appeared in response to cold exposure can return to the morphology and gene expression pattern of white adipocyte after exposure to warm condition through trans-differentiation [135].

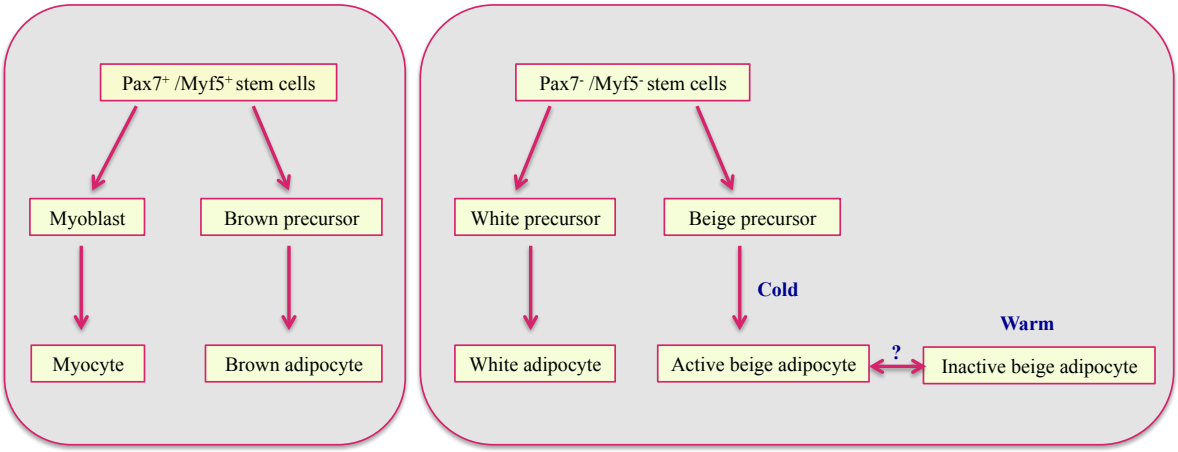

Figure 7. Origin of different adipocytes. Brown adipocyte has the same origin as myocyte and comes from $Pax7^{+}$ /$Myf5^{+}$ stem cells. White and beige adipocytes drive from $Pax7^{-}$ /$Myf5^{-}$ stem cells through distinct precursor cells. Beige precursor cells differentiate to active beige adipocytes by cold exposure and when the challenge is removed they become inactive again with morphology of white adipocytes.

1.5.2 Transcriptional regulation of thermogenesis through UCP1

β-adrenergic signaling and cAMP are the main regulators of thermogenesis in brown adipocytes via strong induction of UCP1 expression and activity. Indeed, cellular cAMP variations are sensed by protein kinase A (PKA) and result in the activation of p38MAPK and CREB [136-138]. This in turn leads to the activation of PGC-1α1, which causes a subsequent increase in UCP1 expression.

In response to cold exposure, PGC-1α1 regulates gene expression of transcription factors such as PPARα [139], PPARγ [40], Retinoid X receptors (RXR), NRF1 and thyroid receptors [140] to positively regulate thermogenesis via UCP1. Furthermore, applying specific agonists for some of these transcription factors (that are nuclear receptors) will lead to induction of UCP1 in adipose tissue. For example, rosiglitazone, a PPARγ agonist, induces PGC-1α1 expression and causes PRDM16-mediated adipose tissue browning [139]. Finally, another mechanism that results in activation of adaptive thermogenesis is mediated by the induction of PPARα/δ signaling pathways by lipolytic products [141].

1.5.3 PGC-1α function in thermogenesis in brown adipose tissue

PGC-1α expression is highly induced in brown adipocytes during cold exposure to positively control the expression of genes participating in thermogenic program such as UCP1 and deiodinase-2 (Dio2) [40]. Indeed, PGC-1α acts at the promoter of these genes in a complex with diverse transcription factors and nuclear receptors such as thyroid hormone receptors, PPARγ, CREB or activating transcription factor-2 (ATF-2) to activate thermogenesis [137, 142]. In line with this, one of the available PGC-1α knockout mouse models [71] shows cold-intolerance and cannot induce UCP1 expression in response to cold exposure. In contrast, a different PGC-1α knockout mouse model [70] responds to cold exposure normally through increased UCP1 expression. The differences in the phenotypes observed in these two models are likely to be the result of different gene targeting strategies. The strategy used by Lin *et al* [71] deletes exons 3-5 of the PGC-1α gene. The gene continues to be transcribed, but the resulting mRNA encodes (at most) for a relatively

short peptide encoded by exons 1 and 2. In addition, this strategy interferes with the expression of most known PGC-1α isoforms. On the other hand, in the model generated by Leone *et al* [70] there is no exon deletion but a duplication of exon 3. This leads to a frame shift in the resulting mRNA, that still encodes a protein remarkably similar to NT- PGC-1α, one of the PGC-1α isoforms. Importantly, NT- PGC-1α has been shown to be sufficient to activate thermogenesis [143].

Increasing adaptive thermogenesis has been suggested as an attractive anti-obesity therapeutic strategy. To this end, induction of PGC-1α activity can be a useful tool to protect against obesity. Identifying mechanisms and molecules that can increase PGC-1α activity is an interesting subject for development of new therapies.

2 AIMS OF THE THESIS

The overall goal of the work presented in this thesis was to characterize the structure/function relationship in the PGC-1α family of transcriptional coactivators. We aimed to identify the contribution of these proteins to transcriptional regulation especially in skeletal muscle and brown adipocytes.

Specific aims were as follow:

- To identify protein partners for the different PGC-1α isoforms in order to elucidate how PGC-1α coactivators regulate target gene expression and understand the molecular pathways that they might regulate.

- To identify target genes of each PGC-1α isoform and delineate the molecular pathways that they regulate in skeletal muscle.

- To identify molecules that activate PGC-1α1 in brown adipocytes.

3 RESULTS AND DISCUSSION

3.1 IDENTIFICATION OF PGC-1α ISOFORM-ASSOCIATED PROTEINS (PAPER I)

Transcription of a single PGC-1α gene produces different isoforms (e.g. PGC-1α1 to α4) with different biological functions. The mechanism of action of PGC-1α1 is well known and it interacts with different chromatin remodeling factors, transcription factors and the mediator complex through different structural domains (Figure 4). In paper I, we characterized the nuclear interactome for each PGC-1α isoform, with an emphasis on transcriptional regulators that they recruit to regulate gene expression. Interestingly, we observed despite the differences in the structure of different PGC-1α isoforms, they can recruit versions of the mediator, histone acetyltransferase, and splicing complexes. For some of these interactions we noticed isoform specificity. For example, we identified that KAT8 (member of the HAT complex) has the preference to interact with novel PGC-1α isoforms or that TADA1 just interacts with PGC-1α4. To date, no histone deacetylase proteins have been shown to interact with PGC-1α1. However, for the first time we show that all PGC-1α coactivators can associate with histone deacetylases and related repressor complexes. This finding provides a novel mechanism to explain the negative effect of PGC-1α isoforms on gene expression [95, 96, 144].

Moreover, we found that all PGC-1s were able to efficiently interact with the mediator complex even in the absence of the RS/RRM domain of PGC-1α1 that was reported to be necessary for this interaction [58]. Furthermore, we identified members of the spliceosome complex in association with the PGC-1α isoforms. This data is in line with our previous study demonstrating that all PGC-1α variants can affect target gene splicing [95]. We identified some of the SRSF (serine/arginine splicing factors) factors in all PGC-1α complexes. Some of these SRSF factors were previously reported just interacting with PGC-1α1 [63]. We also found the transcription factors interacting with each PGC-1α variant. Some of these TFs, such as TFAM, HCF, and YY1 proved to interact with all PGC-1α variants. Additional TFs with high affinity for all PGC-1α isoforms were PBX1 (pre-B-cell leukemia transcription factor), SP1, SP2, and ATF1 (activating transcription factor).

To elucidate how PGC-1α isoforms regulate target gene expression in skeletal muscle, we performed an integrated analysis of each PGC-1α interactome and transcriptome. With this approach, we could identify several novel isoform-associated transcription factors with binding sites present in the promoters of the corresponding target genes, and therefore

propose novel modes of action for each PGC-1α isoform. From the multi-omics analysis, we determined an overlap between the PGC-1α-interacting TFs and the corresponding predicted TFBS (transcription factor binding sites) in their target gene promoters. We could validate this approach by the identification of several previously known PGC-1α-interacting TFs (e.g. RXR, ERR, HNF4), and also identify additional TF partners. Most notably, among the predicted TFs for each PGC-1α isoform, the functional relationship between CREB and PGC-1α1 has been extensively reported [45, 120], but the interaction between these two proteins had never been shown. CREB was also present in the PGC-1α3 and PGC-1α4 complexes. Interestingly, PGC-1α4 has been shown to be downstream of beta-adrenergic signaling in skeletal muscle [96], which also results in CREB activation [145].

3.2 PGC-1α1 FORMS HETERODIMERS WITH ALL PGC-1α ISOFORMS (PAPER I)

PGC-1α1 has been shown to homodimerize that leads to protein stabilization and enhanced recruitment of protein partners [59, 111], and we also observed the presence of hPGC-1α in all PGC-1α isoform complexes. These observations inspired us to investigate the existence of isoform heterodimers. To this end, we expressed and purified FLAG-PGC-1α1 in insect cells and used it to perform protein-protein interaction assays with GST-fusions of all isoforms. Strikingly, apart from the expected FLAG-PGC-1α1: GST-PGC-1α1 homodimerization, we determined FLAG-PGC-1α1 heterodimerizes with PGC-1α2, α3, and α4 (Figure 7). Although we don't know the interaction interfaces in formation of the different heterodimers, these results indicate the existence of previously unknown PGC-1α dimers.

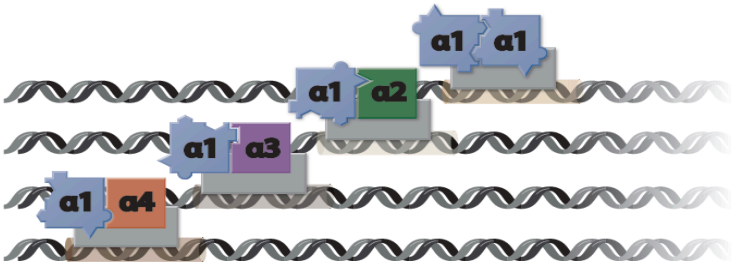

Figure 7: PGC-1α isoforms dimerization. PGC-1α1 can heterodimerize with novel PGC-1α isoforms.

3.3 REGULATION OF TARGET GENE EXPRESSION BY PGC-1α1 DEPENDS ON THE OTHER ISOFORMS (PAPER I)

We performed global analysis of gene expression upon ectopic PGC-1α1 expression in wild-type myotubes or myotubes with genetic deletion of all PGC-1α variants to determine to what extent PGC-1α1 is able to regulate target gene expression in muscle cells. Interestingly, we observed that muscle development, muscle cell differentiation and myofibril assembly are processes regulated only in the presence of all PGC-1α isoforms (WT background). On the other hand, PGC-1α1 alone without the help of other isoforms regulates processes linked to energy metabolism and protein transport. Strikingly, these results suggest that interaction of PGC-1α1 with the other isoforms mainly drives gene programs affecting skeletal muscle cell differentiation and development, while PGC-1α1 alone functions in cellular energy metabolism.

From the multi-omics analysis, we have predicted the interaction of PGC-1α2 with FOX family of transcription factors (FOX-M, FOX-N and FOX-K). These transcription factors are involved in the regulation of muscle cell proliferation and muscle development [146, 147] that supports our observation that novel PGC-1α isoforms have a role in regulation of genes related to muscle development.

Taken together, our results provide novel mechanisms by which PGC-1α coactivators can regulate gene expression. It will be interesting to characterize if different PGC-1α heterodimers have novel biological activities or if their heterodimerization illustrate some of the known biological effects of PGC-1α in distinct tissues.

3.4 PGC-1α ISOFORMS DISPLAY DISTINCT PROTEIN STABILITY (PAPER II)

PGC-1α1 has a short half-life and is degraded fast by ubiquitin-proteasome system. To determine whether the same degradation pathway targets all PGC-1α isoforms, myotubes with ectopic expression of each PGC-1α isoforms were treated with proteasome inhibitor MG132. Interestingly, all PGC-1α proteins accumulate to different levels after MG132 treatment. This means they all degrade through the ubiquitin-proteasome pathway. Strikingly, we observed different degradation patterns for PGC-1α isoforms after inhibition of protein synthesis by cycloheximide. PGC-1α2 and PGC-1α3 exhibited a half-life similar to PGC-1α1 (~ 30 min), but PGC-1α4 showed higher stability with a remarkably long half-life (~240 min).

3.5 IDENTIFICATION OF REGULATED GENE TARGETS/PATHWAYS BY EACH PGC-1α ISOFORMS (PAPER II)

We performed global gene expression analysis using exon arrays in myotubes expressing each PGC-1α isoform to identify genes regulated by each isoform and identified a large number of specific PGC-1α isoform target genes. We found the highest number of coregulated genes between isoforms PGC-1α1 and α4 and between PGC-1α2 and α3. Since PGC-1α2 and PGC-1α3 lack part of the activation and repression domains present in the canonical isoform (PGC-1α1) (Figure 5), they produce a distinct cluster of target genes from PGC-1α4 that shows more similarity to PGC-1α1. This suggests that the transcriptional activity of PGC-1α isoforms is mainly dictated by the conservation of the N-terminal activation domain compared to the RS/RRM motif.

In addition, we performed a comprehensive pathway and network analysis to predict biological functions linked to the transcriptional programs regulated by each PGC-1α isoform. We validated some of the selected genes identified from each pathway by qRT-PCR. Once again, this analysis showed the involvement of PGC-1α1 in mitochondrial biology and oxidative metabolism. PGC-1α2 target genes in myotubes were associated with cholesterol biosynthesis and regulation of inflammatory processes. The pathways identified for PGC-1α3 were linked to development and cell cycle control. Finally, PGC-1α4 regulates muscle mass and hypoxia-driven angiogenesis in skeletal muscle. We also found PGC-1α4 as a negative regulator of hypoxia-inducible factor-1α signaling (and also involved in cancer signaling). Because PGC-1α isoforms have distinct protein accumulation (for similar mRNA levels), we were concerned that the different target gene sets could be a reflection of the differences in PGC-1α isoform stability. Therefore, we validated target gene specificity upon increased expression of each PGC-1α variant in myotubes. We observed increased protein accumulation of PGC-1α1, PGC-1α2 and PGC-1α3 but not PGC-1α4. Subsequently, we observed the specificity of PGC-1α target genes remained unaffected.

When we compared the results in our study with previously published PGC-1α variant target gene data [96], we found both studies (exon array and Affymetrix gene array) were equally efficient in the identification of PGC-1α1 target genes. However, this was different for other isoforms especially PGC-1α2 and PGC-1α3, for which we identified over 1000 regulated genes by either coactivator compared to less than 100 regulated genes identified by Affymetrix gene array. For PGC-1α4 the top canonical pathways observed in our study did not show a significant overlap with Affymetrix gene array.

3.6 PGC-1α ISOFORMS SELECTIVELY AFFECT THE SPLICING EVENTS IN MYOTUBES (PAPER II)

In paper II, we observed that expressing each PGC-1α isoform leads to a different pattern of exon alignment for some target genes. This indicates that PGC-1α isoforms can affect target gene splicing. We used several complementary bioinformatics tools and determined a large number of genes with different splicing patterns modulated by different PGC-1α variants. Later, we validated some of these different splicing events under control of PGC-1α isoforms via targeted qRT-PCR. For example, we show that PGC-1α2 and PGC-1α3 affect Ddx27 isoform expression, while PGC-1α1 and PGC-1α4 regulate Osbpl1a and Ndrg4 isoform expression.

We found that the splicing events by PGC-1α4 in myotubes were similar to what we observed for PGC-1α1. Splicing events regulated by PGC-1α2 were also similar to PGC-1α3. Therefore, we suggest that conservation of the activation domain in their structures can impact defining both gene programs and also the splicing process during mRNA maturation.

Furthermore, we could validate some of the splicing procedures, which we observed *in vitro* for PGC-1α1 and PGC-1α4 *in vivo* on muscle-specific transgenic mouse lines for these two isoforms. We observed changes in both gene expression and protein levels of short C-terminal isoform of Osbpl1a in skeletal muscle of both transgenic models. Specific expression of PGC-1α1 promoted increase in the mRNA level of Ndrg4 variants derived from the internal promoter region while in PGC-1α4 transgenic mice we observed the increase in all Ndrg4 isoforms regardless of the promoter of origin. In contrast to the results from transcript level, the protein content of Ndrg4 short isoforms in muscle of both transgenic mice was reduced. The precise mechanism of how coactivators regulate co-transcriptional splicing is under investigation.

Taken together our results in paper II show that PGC-1α isoforms regulate target gene splicing and affect the expression of specific exon composition of their corresponding transcripts.

3.7 IDENTIFICATION OF SMALL MOLECULE PGC-1α1 STABILIZERS/ACTIVATORS IN BROWN ADIPOCYTES (PAPER III)

As we discussed in this thesis, PGC-1α1 is one of the main modulators of metabolism in a tissue-specific manner. Several studies have tried to increase the expression of PGC-1α1

and use it as a therapeutic approach in treatment of metabolic disorders [148, 149]. But the short half-life of this protein is an obstacle to overcome. In Paper III, we developed a cell-based screening system to identify stabilizers of PGC-1α1. To this end, we generated a stable cell line expressing an EGFP-PGC-1α1 fusion protein and performed high-throughput screening using a small compound library. After treating cells with compounds, changes in fluorescence localization and intensity of the fusion protein were analyzed using a fully automated microscopy system. Among the 114 primary hits we decided to continue with the validation of 79 compounds in brown adipocytes. After treatment of fully differentiated brown adipocytes with different compounds, DMSO (negative control) and isoproterenol (positive control), primary validation was done by qRT-PCR analysis of PGC-1α1 target gene activation. Validation by qRT-PCR was done for some of the known PGC-1α1 target genes involved in mitochondrial biogenesis, browning, non-shivering thermogenesis, antioxidant defenses and oxidative phosphorylation. We included the general proteasome inhibitor MG132 in our treatments to compare compound effects with maximal protein stabilization. Brown adipocytes treated with MG132 showed reduced expression of most of the genes we analyzed. This difference in gene expression pattern by MG132 from our selected compounds indicates that these compounds were not general inhibitors of the proteasome. With this approach we excluded compounds with general proteasome inhibitory effects that have no specific impact on PGC-1α1 activation and biological effects. From all the target genes we analyzed, after treatment with the different compounds, UCP1 showed the highest induction. 19 compounds, among all the validated compounds, showed at least 2-fold induction in UCP1 expression.

Next, we performed a second validation of compounds in brown adipocytes, on endogenous PGC-1α1 protein level by western blot. We found that 45 compounds induced PGC-1α1 accumulation. In summary, after both validations we found that 17 of the validated compounds increased both PGC-1α1 protein accumulation and UCP1 expression. We chose five of these compounds (AM31, AM73, AM79, AM80 and AM89) that induced UCP1 expression over 5-fold and also stabilized PGC-1α1 protein for further analysis.

To explore if the selected compounds can impact biological activity of PGC-1α1, we measured cellular respiration rate in brown fat cells after treatment with these compounds. In agreement with the observed increase in UCP1 expression we reported an elevation in basal level of mitochondrial respiration for all select compounds except AM89. The basal respiration increase remained even after addition of an inhibitor of ATP synthase (oligomycin). This indicates an increase in uncoupled respiration rather than ATP turnover,

likely through increase in UCP1 expression. Only AM89 shows slight increase in maximal respiration, which could be the effect of more mitochondria and/or mitochondrial protein.

To understand the mechanism of action of selected compounds, we performed a broad gene analysis in brown adipocytes treated with AM73 and AM80. Interestingly, we found regulated pathways by AM73 were canonical PGC-1α1-regulated pathways such as BAT cells differentiation, response to hypoxia, insulin signaling and carbohydrate metabolism. We performed a DiRE analysis to identify the transcriptional pathways modulating genes differentially expressed upon treatment with AM73 or AM80. We found the transcription factor binding sites for many previously known PGC-1α1 protein partners including C/EBP, MEF2, PPARα, HNF, NRF2, XBP1, and SREBP1 proteins. All these transcription factors are expressed in adipocytes and it is possible that PGC-1α1 is the key factor in modulating their respective target genes.

Moreover, to compare the gene expression profile differences upon treatment of AM73/AM80 with another known inducer of browning, we used the previously published data from cAMP-treated brown adipocytes. cAMP is a classical inducer of thermogenic program in brown adipocyte [150]. We analyzed the regulated pathways with each reagent separately, the coregulated genes/pathways with both cAMP and AM73, and contra-regulated genes/pathways by these two reagents. For example, we found both cAMP and AM73 induce blood vessel development. Strikingly, brown fat cell differentiation and oxidation/reduction were the main regulated pathways with AM73 alone. We performed the same analysis for AM80 as well. The specific gene signature for AM73 and AM80 compare to cAMP indicates these compounds exert their functions distinct from cAMP induced pathways.

The exact mechanisms of action of PGC-1α1 activators are still unclear. Although we show that the select compounds are not just general inhibitors of the ubiquitin-proteasome system (through different gene expression profile than MG132), they still might be general inhibitors of E3-ubiquitine ligases, which are not specific to PGC-1α1. Therefore, they could also be stabilizers of other proteins leading to unwanted effects. Moreover, some compounds that are just PGC-1α1 protein stabilizers, had small or no impact on target gene expression, which might induce PGC-1α1 stabilization by post-translational modifications (PTMs). In contrast, some compounds that are just UCP1 expression inducers, with no changes in PGC-1α1 protein accumulation, might induce UCP1 expression via PGC-1α1-independent mechanisms or PTMs. This results in a more target gene activation without increase in total PGC-1α1 protein accumulation. We should also consider the fact that

PGC-1α1 is among intrinsically disordered proteins that degrades fast [151]. In theory the interaction of PGC-1α1 with small compounds could stabilize PGC-1α1 protein by conformational changes that make it less susceptible for degradation [152].

Finally, we should consider that systemic activation of PGC-1α1 might not have beneficial effects in treatment of all types of metabolic disorders. In liver, increase in PGC-1α1 activity leads to higher glucose output, which is not a desirable effect in treatment of type 2 diabetes and obesity. However, the specificity of PGC-1α1 function depends also on the presence of specific transcription factors and on post-translational modifications of PGC-1α1 in tissue specific manner. Although this quickly confuses the prediction of general activation of PGC-1α1, it also provides the possible activation of a particular gene program such as mitochondrial genes and fatty acid oxidation whereas leaving other networks like gluconeogenesis in the liver unaffected. Furthermore, development of chemicals that could be targeted to particular tissues (and not the liver) might be another solution to avoid the unwanted effect of PGC-1α1 activation in the liver. Indeed, a recent study [86], used chemical inhibition of PGC-1α acetylation by GCN5 to reduce activity of PGC-1α as a tool to reduce blood glucose levels in type 2 diabetic mice.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

The general aim of the studies included in this thesis was to investigate the structure/function relationships in the PGC-1 family of transcriptional coactivators. To this end, we have used biochemical and molecular techniques to identify the protein partners of different PGC-1α isoforms, their respective target genes, and to characterize their mechanisms of action. We observed that all PGC-1α isoforms can play a positive role in the regulation of gene expression at the transcriptional level (paper I). All the isoforms interact with transcriptional coregulators such as the mediator complex, histone acetyltransferases, and different splicing factors (albeit to different degrees and in an isoform-specific manner). As it was presented in the results of paper I and paper II, we demonstrate all PGC-1α isoforms are involved in regulating gene-splicing events. Distinct PGC-1α isoforms promote a diverse exon composition of their target transcripts (paper II). In this thesis we used bioinformatic tools for the prediction of target genes and pathways, which are regulated by each PGC-1α variant (paper II). We further combined the identified target genes in this study with obtained proteomics data from paper I to exploit the mechanism of function of these proteins. Taken together, the observations from paper I and paper II helped us identify the predicted transcription factors for each PGC-1α isoform. Strikingly, for the first time we identify the heterodimerization of PGC-1α1 with PGC-1α2, α3 and α4 (paper I). This finding might change the way we evaluate PGC-1α1 function in diverse tissues because some of the known biological effects of PGC-1α1 might be through its interaction with other isoforms. For example, our findings in paper I indicate that PGC-1α1 function on the broad pathway of skeletal muscle development, only works in the presence of other isoforms.

Furthermore, we observed that PGC-1α isoforms have different protein stability (paper II) and in paper III we developed a screening platform to increase PGC-1α1 accumulation and activity in brown adipocyte. We identified several compounds that increase PGC-1α1 protein levels and the expression of some of its target genes. These small molecules also increased basal mitochondrial respiration in brown adipocytes through induction of UCP1 expression. These findings could be important for developing new therapeutic tools for the treatment of metabolic disorders and skeletal muscle wasting.

As it has been broadly shown, PGC-1αs are important transcriptional regulators in metabolism to balance the energy supply and demand. According to the prominent role of PGC-1αs in these cellular processes, increasing PGC-1α activity is gaining great attention for treatment of metabolic disorders. Inducing PGC-1α activity in brown fat and switching

higher lipid storage to more heat generation might be an interesting tool in treatment of obesity. Furthermore, the effect of PGC-1α in the regulation of mitochondrial biogenesis, oxidative phosphorylation and glucose metabolism renders this molecule as a target for treatment of neurodegenerative disorders (Parkinson disease, Huntington and Alzheimer diseases), cancer, type 2 diabetes and skeletal muscle wasting diseases such as Duchene muscular dystrophy.

In developing new therapies by targeting PGC-1α activation, we should consider that systemic activation of PGC-1α might not have beneficial effects in all tissues. Tissue specific function of PGC-1α should be considered in designing new therapies. Our findings about protein partners and regulated target genes by each PGC-1α isoform can be a useful resource for understanding the mechanism of function of PGC-1 family and improve the methods, which will be applied in clinical research in the future.

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