Identification and Biochemical Characterization of PGC-1β-Interacting Proteins

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ABSTRACT

Nuclear receptors (NRs) are ligand-activated transcription factors. They regulate key biological processes including homeostasis of lipophilic molecules, organogenesis, development, and reproduction. The ability of NRs to regulate gene transcription is dependent upon their ability to directly binding to specific enhancer elements within the promoters of their target genes. While NRs directly bind to DNA, they lack the capacity to modify chromatin, unwind DNA, and recruit transcriptional machinery. All of these aforementioned processes represent key steps in the regulation of gene transcription. Therefore, the full functional activity of NRs depends upon their ability to interact with protein cofactors, termed coactivator and corepressor proteins. Metabolic syndrome is an emerging global epidemic characterized by a complex disorder that affects both glucose and lipid metabolism. Peroxisome proliferator-activated receptor-gamma (PPARγ) co-activator-1alpha (PGC-1α) is a coactivator protein that is mainly expressed in tissues that require a high oxidative capacity such as fat, muscle, brain, and liver. PGC-1α plays an important role in regulating key metabolic processes involved in energy homeostasis including gluconeogenesis, mitochondrial biogenesis, adaptive thermogenesis, and β-oxidation of fatty acids. PGC-1α accomplishes this feat through its ability to interact with selected NRs and certain other transcription factor types. PGC-1β is the closest homolog of PGC-1α and also shares some similarities with PGC-1α in amino acid sequence, expression pattern, interacting protein partners, target genes, and

biochemical function. However, these two coactivator proteins also display distinct proerties. For example, PGC-1β mediates hepatic lipogenic program but not gluconeogenesis, whereas PGC-1α plays a key regulatory role in hepatic gluconeogenesis but has no effects upon lipogenesis. We therefore hypothesized that the key biological and functional differences between these two coactivator proteins is due to their distinct protein interaction profile. A comparison of the primary amino acid sequence of PGC-1α and PGC-1β revealed that both proteins contain three NR-interaction motifs (-LXXLL-) with the first two being highly conserved in their N-termini, respectively. However, PGC-1β contains an additional and unique stretch of amino acid sequence that separates the second and third NR-interaction motif that is absent from PGC-1α. We therefore fused the cDNA encoding this N-terminal region of PGC-1β to the GAL4-DNA binding domain (GAL4-NBT3) and used this protein as 'bait' to screen cDNA expression libraries from liver, brain, and embryo using the yeast two hybrid system to identify novel PGC-1β-interacting proteins. Three potential PGC-1β-interacting proteins were identified. The first is a NR protein called COUP-TF1. The second PGC-1β-interacting protein we identified is a protein phosphatase regulatory protein termed PP4R1. The third protein we identified as a PGC-1β-interacting protein is a member of the SWI/SNF family of proteins called BAF60a. We further confirmed the interaction of PGC-1β/PP4R1 and PGC-1β/BAF60a *in vitro* using the GST pull-down assay. *In vivo* analysis using immunohistochemical methods further confirms the ability of PP4R1 and BAF60a to

co-localize with PGC-1β. The identification of PP4R1 and BAF60a as PGC-1β-interacting proteins will contribute to an increased understanding of the repertoire of PGC-1β-interacting proteins. This research will also likely contribute to understanding the potential role of these proteins in regulating PGC-1β-mediated coactivation of transcription.

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Table of Contents

List of Figures

Abbreviations

PGC-1β --- Peroxisome proliferator-activated receptor gamma coactivator-1 beta

PGC-1α --- Peroxisome proliferator-activated receptor gamma coactivator-1 beta

COUP-TF1 --- Chicken ovalbumin upstream promoter transcription factor 1

PP4R1 --- Regulatory subunit of the serine/threonine protein phosphatase 4

PP4c --- Catalytic subunit of the serine/threonine protein phosphatase 4

BAF60a --- BRM/BRG1 ATPase and BRG1-associated factor

NR --- Nuclear hormone receptors

PPARγ --- Peroxisome proliferator-activated receptor gamma

NBT1/2/3 ---N-terminus of PGC-1b 1, 2, and 3

DBD --- DNA-binding domain

LBD --- Ligand-binding domain

AF1/2 --- Activation function 1or activation function 1 and 2

HAT --- Histone acetyltransferase activity

HDAC3 --- Histone deacetylase 3

SWI/SNF --- ATPase containing chromatin remodeling protein complex

SREBP --- Steroid response element bonding proteins

HNF4α --- Hepatic nuclear receptor 4α

FOXO1 --- Forkhead transcription factor O1 (FOXO1).

XAB2 --- Xeroderma pigmentosum group A (XPA)-binding protein 2

SRP55 --- Serine/arginine (SR)-rich protein 55

INTRODUCTION

Nuclear Receptors and Protein Cofactors

In higher organisms, steroid hormones such as glucocorticoid, estrogen, androgen, progesterone, retinoid, and vitamin D regulate the expression of numerous biologically important genes. These hormones function through interacting with their intracellular receptors named nuclear hormone receptors (NRs). NRs are ligand-activated transcriptional factors. They regulate gene transcription by binding directly to specific DNA sequence such as hormone response elements (HREs) in the promoter region of their target genes. To date, over 48 nuclear receptors have been identified since the discovery of the first nuclear receptor, progesterone receptor in 1985 (Steinsapir J. et al. 1985; Yamamoto K.R. 1985). Structurally, NRs have a conserved domain structure. Their signature functional domains beginning from the amino terminus to the carboxy terminus are (1) activation function 1 (AF-1), (2) the DNA-binding domain (DBD), (3) the hinge region, and (4) the ligand-binding domain (LBD) which also contains the activation function 2 (AF-2) domain (Figure 1) (Kumar R. and Thompson E.B. 1999). The AF-2 has proven to be a particular interaction surface for a variety of co-factor protein complexes that are important for transcriptional activity of NRs (Hsiao P.W. et al. 2002). NRs are involved in various important biological processes including homeostasis, organogenesis, development, and reproduction. While NRs bind to DNA, they generally lack the activities necessary for modifying chromatin, unwinding DNA, and recruiting RNA

polymerase II, key steps for transcription of a gene. Therefore, the full functional activity of NRs depends upon a number of NR-associated proteins called cofactors. These proteins usually exist in the nucleus and can be recruited to NRs in response to a variety of cellular signals. While these transcriptional cofactors do not have DNA-binding ability, they mediate transcriptional activity of NRs by being recruited to the promoters region of the target gene by NRs in a protein-protein interaction-dependent manner. Studies have shown that the ligand binding activates NRs and causes a conformational change in the AF-2 domain, which leads to the recruitment of cofactors. Based on the effects of cofactors in regulating the transactivation activity of NRs, these cofactors have been generally divided into two groups: (1) coactivators that promote NR-mediated activation of target gene expression, and (2) corepressors that promote NR-mediated repression of target gene expression.

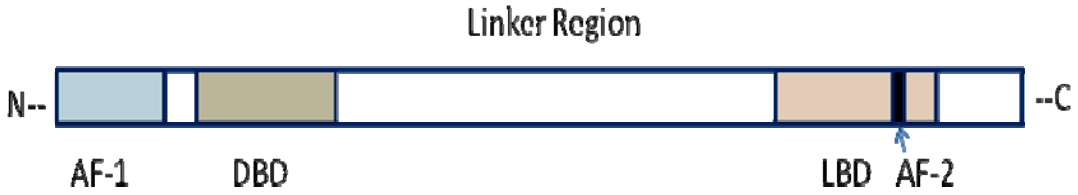

Figure 1. Schematic gene structure of the nuclear receptor. A typical nuclear receptor contains the ligand-independent transactivation domain (AF-1), the DNA-binding domain (DBD) at the NH2-terminal region and the ligand-binding domain (LBD) that contains the ligand-dependent transactivation domain (AF-2) at COOH-terminal region. In the middle region, there is a linker region that connects DBD and LBD domain.

Transcriptional Corepressor and Coactivator Proteins

Transcriptional corepressor proteins are a group of protein cofactors involved in the suppression of transcription. The corepressor proteins suppress transcription by being recruited to the promoters of the various NR-target genes in the absence of ligand and/or in the presence of NR antagonists. Nuclear hormone receptor corepressor (N-CoR) and silencing mediator of retinoid and thyroid hormone receptor (SMRT) were the first identified corepressor proteins (Davie J.K. and Dent S.Y. 2004). The N-CoR/SMRT corepressor proteins can recruit histone deacetylase proteins (HDACs), a core component of repression complexes, to the NR-bound DNA-response elements through the repression domain located in their N-terminus (Nagy L. et al. 1997). It is well known that acetylation of histone is closely associated to gene activation via unwinding of the DNA. HDACs repress the transcription of target genes through deacetylation (removal of acetate group from the histone core) and subsequent compaction of the surrounding chromatin. It is believed that HDAC complex and histone acetyltransferase activity (HAT) targeted to the promoter region mediate the level of acetylation (hyperacetylation or hypoacetylation) that is correlated to the transcription activation and repression.

Coactivator proteins are another group of protein cofactors that cooperate with NRs and regulate transcription. In contrast to the repressive role of corepressor proteins, coactivator proteins enhance transactivation activity of NRs by interacting with NR in a ligand-dependent manner. Following identification of the first

coactivator, steroid receptor coactivator 1 (SRC-1) in 1995, over two hundred coactivator proteins have been discovered. Structural research revealed that a key feature of the most coactivator proteins is an -LXXLL motif, where -L- is leucine and -X- is any amino acid (Heery D.M. et al. 1997). The -LXXLL- motif is responsible for the interaction of coactivator proteins with the AF-2 domain of NRs, therefore it is also called the NR box (Torchia J. et al. 1997). Different -LXXLL- motifs within different coactivator proteins selectively interact with different NRs. Based on their role in mediating transcriptional activation of NRs, coactivator proteins have been broadly classified into two types: (1) those involved in chromatin remodeling, and (2) those that directly bind to NRs, transcriptional machinery, and mRNA splicing factors serving as adaptor proteins.

The identification of coactivator and corepressor proteins provided important insights into the mechanism of the transactivation and transrepression function of NRs. The research reviewed thus far indicates that NR-associated protein cofactors serve as a transcriptional "switch" from gene repression to gene activation. Generally, NRs are associated with HDAC-containing corepressor multi-protein complexes in the absence of ligands. The binding of a ligand to NRs results in the dissociation of the multiprotein corepressor complex and subsequent recruitment of coactivator protein complexes to the NR-bound promoter of their target genes. Thereafter, coactivator proteins coactivate NRs through multiple steps. First, some coactivator proteins, such as SRC-1, directly bind to NRs serving an adaptor or scaffold function.

The adaptor/scaffold coactivator proteins in turn recruit additional coactivator proteins such as CBP, p300, and p/CAF that have intrinsic and various enzymatic activities. The different enzymatic activities contained within the coactivator multiprotein complexes (e.g. chromatin modification, chromatin remodeling, transcription initiation, RNA elongation, and RNA splicing) work together to modify histones, chromatin, and other proteins, thereby positively regulating the transcription of NR-target genes.

Chromatin Remodeling by SWI/SNF

Genomic DNA is packed into a highly dense structure named chromatin. The nucleosome is the basic unit of chromatin, which is formed by coiling 147 bp of DNA around a histone core. During the process of gene expression, transcription requires that DNA can be accessible to transcription factors and RNA polymerase. However, this highly dense chromatin structure forms a barrier to gene activation. Modifying chromatin is necessary for the binding of transcription factors and initiation of the transcription. NRs themselves do not have the intrinsic ability to modify chromatin. Following activation, NRs must therefore recruit coactivator proteins that subsequently modify the structure of chromatin. The coactivator proteins recruited by NRs to modify chromatin can be classified into two distinct classes: (1) those that function through covalent modification of histone proteins using their intrinsic histone acetyltransferase activity (HAT), and (2) those that remodel the chromatin structure in an ATP-dependent manner. The role of acetylation of the core histone on

lysine residues has been well established and strongly correlated with gene activation. Some coactivator proteins including cyclic AMP response element-binding protein (CREB)-binding protein and p300 (CBP/p300) contain intrinsic acetyltransferase activity (HAT) (Bannister A.J. and Kouzarides T. 1996). These HAT-containing coactivator protein complexes catalyze the addition of acetate groups to lysine residues in the core histone, unwinding chromatin structure, thereby facilitating the binding of transcription factors to promoters. However, the major question regarding how this acetylation modification changes the structure of chromatin remains unclear.

In the past few years, the critical role of SWI/SNF complex has been discovered and it represents the second class of the chromatin remodeling coactivator proteins. This ATPase-containing multi-protein coactivator complex remodels the chromatin using energy derived from the hydrolysis of ATP. The net effect of these proteins is the repositioning of the nucleosomes on the DNA by sliding the histone core to adjacent DNA segments, thereby facilitating the access of the transcriptional machinery (Langst G. et al. 1999; Gutierrez J. et al. 2007). Research has revealed that SWI/SNF complexes interact with and coactivate many transcription factors. For example, the SWI/SNF complex interacts with estrogen receptor alpha (ERα) on the promoter of estrogen-responsive genes in a ligand-dependent manner. The SWI/SNF multi-protein complex coactivates glucocorticoid receptor (GR) and PPARγ through direct protein-protein interaction. An important question raised is how SWI/SNF recognizes the specific chromatin to unwind DNA? Recent research has indicated

that SWI/SNF exerts its effect on the specific chromatin region through highly functional cooperation with HAT-containing multi-protein complexes. It has been postulated that HATs first acetylate histones, which construct a code as well as an anchor on the histone proteins, then the SWI/SNF complex recognizes this acetylated lysine residue on histones and attaches to it on the promoter region of target genes. SWI/SNF breaks the connection between the DNA and histones, unwinds DNA for the access of basal transcription factors, thus turning the gene into the 'on' position (Hassan A.H. et al. 2002; Singh M. et al. 2007).

Transcriptional Regulation during Metabolic Syndrome

Metabolic syndrome is an emerging global epidemic characterized by a complex disorder that affects both glucose and lipid metabolism (Eckel R.H. et al. 2005). It is a cluster of metabolic abnormalities, including impaired glucose metabolism, dyslipidemia (high level of serum triglycerides and low serum high density lipoprotein), hypertension, and obesity (Sutherland J.P. et al. 2004; Grundy S.M. 2005; Deedwania P.C. and Gupta R. 2006), that ultimately leads to an increased risk of developing cardiovascular diseases and diabetes. This syndrome currently affects a large number of people, and its prevalence is increasing dramatically. Some studies estimate that over 47 million adults in the United States (almost 25 percent) have metabolic syndrome, and the numbers are continuing to grow. The chance of developing metabolic syndrome is closely linked to obesity and a sedentary lifestyle. Clinically, patients presenting with at least three out of the five following symptoms

are diagnosed as having the syndrome: (1) increased insulin resistance and higher than normal fasting levels of blood glucose; (2) higher than normal blood pressure; (3) central obesity; (4) lower than normal serum high density lipoprotein levels; and (5) higher than normal serum levels of triglycerides. Diseases and signs associated with metabolic syndrome include elevated uric acid levels, fatty liver that progresses to non-alcoholic fatty liver disease, polycystic ovarian syndrome, hemochromatosis, and acanthosis nigricans.

Although the precise mechanisms of metabolic syndrome have not been elucidated, it has been recognized that some transcription factors play crucial roles in the development of metabolic syndrome, such as PPARs (PPARα, PPARβ, and PPARδ), SREBPs (SREBP1a, SREBP1c, and SREBP2), LXR, and HNF4 (Nagao K. and Yanagita T. 2008). These transcription factors generally function through interacting with specific cofactors including coactivator and corepressor proteins. The transcriptional coactivators peroxisome proliferator-activated receptor gamma coactivator-1alpha and beta (PGC-1α and PGC-1β) are pivotal coactivator proteins for these transcription factors, especially for PPARs and SREBPs in energy metabolism (Feige J.N. and Auwerx J. 2007). Therefore, by interacting with transcription factors PGC-1α and PGC-1β are thought to be profoundly involved in the development of metabolic syndrome (Sookoian S. et al. 2005; Finck B.N. and Kelly D.P. 2006). For instance, dysfunctions of PGC-1α protein have also been associated with insulin resistance and diabetes (Ek J. et al. 2001; Hara K. et al. 2002;

Barroso I. et al. 2006), obesity (Esterbauer H. et al. 2002; Muller Y.L. et al. 2003), and hypertension (Oberkofler H. et al. 2003; Vimaleswaran K.S. et al. 2008), key features of metabolic syndrome. While PGC-1β has also been associated with diabetes and obesity (Andersen G. et al. 2005), the direct role that the PGC-1β may play in metabolic syndrome remains to be determined.

The PGC-1 Family of Transcriptional Coactivator Proteins

The PGC-1 family has three members including PGC-1α, PGC-1β, and PGC-1-related coactivator (PRC). PGC-1α was originally identified as a coactivator protein for PPARγ in brown adipose tissue (BAT) library using the yeast two-hybrid screening system in the presence of the agonist troglitizone (Puigserver P. et al. 1998). As the first identified family member, PGC-1α has been well characterized, and most knowledge about PGC-1 family known so far came from the research of PGC-1α-associated proteins. Sequence analysis revealed that PGC-1α has an activation domain (AD), three - LXXLL- motifs at the N terminus, a negative regulatory domain in the central region, an arginine/serine rich domain (RS), and an RNA-recognition motif (RRM) at the C-terminus. The PGC-1α protein interacts directly with multiple NRs through the three separate -LXXLL- motifs contained within its N-terminal region including estrogen receptor-α, estrogen related receptor (ERR), thyroid hormone receptor (TR), glucocorticoid receptor (GR), as well as PPARγ (Savkur R.S. et al. 2004). While the PGC-1α protein itself has no histone acetyltransferase activity,

it can recruit other protein cofactors such as SRC-1, CBP, and p300 that harbor this activity (Scarpulla R.C. 2002). Moreover, PGC-1α associates directly with RNA polymerase II. Additional research suggests that PGC-1α is involved in mRNA splicing via the RRM motif in the C-terminus (Monsalve M. et al. 2000). It has been observed that PGC-1α is highly induced in response to environmental stimuli. The expression level of PGC-1α is extremely low in brown fat tissue, however it increases dramatically when mice are exposed to thermal stimulation such as cold temperature. Moreover, PGC-1α function is regulated in muscle and liver by additional external environmental stimuli including exercise, fasting, and caloric restriction (Liang H. and Ward W.F. 2006). Since PGC-1α is recruited by PPARγ to the promoter of uncoupling protein 1 (UCP-1), it induces the expression of UCP-1 in brown fat cells but not in fibroblasts (Puigserver P. et al. 1998). This indicates that PGC-1α is a tissue- and promoter-specific coactivator protein. PGC-1α is mainly expressed in tissues that need a high oxidative capacity: brown adipose tissue, muscle, heart and liver. By interacting with multiple NRs and other transcription factor types in these tissues PGC-1α plays a critical role in coordinately regulating multiple aspects of energy homeostasis in mammals including gluconeogenesis (Rhee J. et al. 2003), mitochondrial biogenesis (Gleyzer N. et al. 2005), adaptive thermogenesis, and fatty acid β-oxidation. (Lin J. et al. 2005; Handschin C. and Spiegelman B.M. 2006; Feige J.N. and Auwerx J. 2007).

Similarities between PGC-1α and PGC-1β

Identified in 2002, PGC-1β is the closest homolog of PGC-1α (Lin J. et al. 2002). PGC-1β shares high sequence identity with PGC-1α in the N-terminal activation domain (50%), a central regulatory domain (35%), and a C-terminal RNA binding domain (48%) (Figure 2) (Lin J. et al. 2002). PGC-1β, like PGC-1α, is also expressed in tissues with high oxidative capacity, such as heart, skeletal muscle, and brown adipose tissue (Lin J. et al. 2002). They both interact with and coactivate NRs such as thyroid receptor (TR), PPARα, PPARγ, HNF-4α, and non-NR transcription factor-nuclear respiratory factor 1 (NRF-1) (Meirhaeghe A. et al. 2003). These similarities suggest that, like PGC-1α, PGC1β also plays a role in regulating some aspects of energy metabolism and may be involved in the development of metabolic syndrome. In supporting this hypothesis, PGC-1β has been shown to induce important genes involved in mitochondrial biogenesis and oxidative energy metabolism in adipose tissue and skeletal muscle through coactivation of estrogen-related receptors (ERRs), PPARs, and nuclear respiratory factors (NRFs) (Sonoda J. et al. 2007).

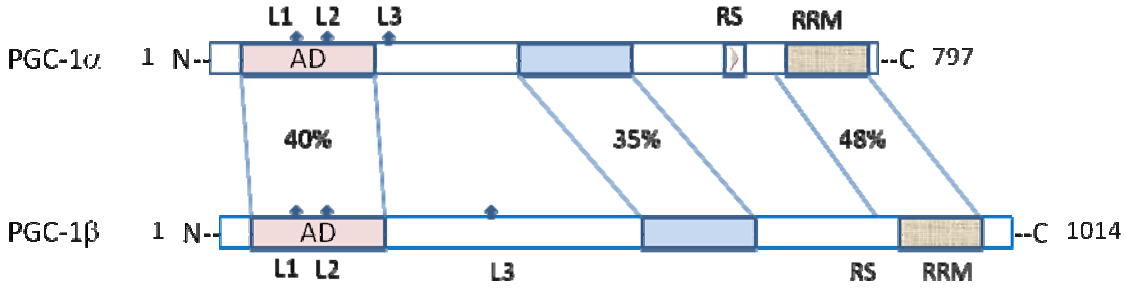

Figure 2. Comparison of gene structure of PGC-1α and PGC-1β. Protein sequence alignment of PGC-1α and PGC-1β and the degree of sequence identity between two proteins are shown. Conserved domains and motif are shown in the same color boxes. AD stands for activation domain, L1, L2, and L3 represent three -LXXLL- motif, RS represents the arginine/serine rich domain, and RRM indicates the RNA recognition motif.

The Distinct Functions of PGC-1β

While PGC-1β shares similarities with PGC-1α, PGC-1β also displays distinct biological functions. For example, in liver, PGC-1α is a key regulator of gluconeogenesis, an important process of glucose homeostasis, whereas PGC-1β has almost no effect on it (Yoon J.C. et al. 2001; Lin J. et al. 2003). On the other hand, it is well known that lipogenesis is closely associated with at least two important symptoms of metabolic syndrome, obesity and dislipidemia. PGC-1β is a key mediator in the regulation of lipogenesis and very low-density lipoprotein secretion in liver through co-activation of SREBPs (Lin J. et al. 2005), whereas PGC-1α does not affect this process (Lelliott C.J. et al. 2007). This functional difference between PGC-1α and PGC-1β is most likely due to their distinct protein-protein interaction profile. For instance, PGC-1α regulates gluconeogenesis through interacting with and coactivating hepatic nuclear receptor 4α (HNF4α) and forkhead transcription factor O1 (FOXO1). These two critical transcription factors mediate the activation of gluconeogenic gene expression, however they interact with PGC-1β with very low affinity (Lin J. et al. 2003). On the other hand, PGC-1β regulation of hepatic lipogenesis is through coactivating SREBPs, which is not a PGC-1α-interacting protein partner. Therefore, we hypothesized that this functional difference between PGC-1α and PGC-1β is most likely due to their distinct interacting protein profile.

While much is known regarding the biological function of PGC-1α, relatively little is known regarding the function of PGC-1β during metabolic syndrome. Most

of what is currently known regarding the biological function of PGC-1α was initially learned through the identification of the multiple transcription factors and accessory proteins with which it interacts. To investigate the biological function of PGC-1β and the underlying mechanism of its coavtivation in metabolic syndrome, we therefore sought to identify additional PGC-1β-interacting proteins using the yeast two-hybrid screening system. Since the -LXXLL- motifs at N-terminus of PGC-1α are essential for interaction between this coactivator protein and transcription factors, we designed the bait using the N-terminus of PGC-1β (amino acids 82-426). This bait contains certain amino acid sequence unique to PGC-1β's and three -LXXLL- motifs, one of which is novel in comparison with PGC-1α. We used this PGC-1β bait to screen cDNA libraries from liver, brain, and fat tissues. We identified three PG-1β-interacting proteins, (1) a novel splice variant of the nuclear receptor superfamily member- chicken ovalbumin upstream promoter transcription factor 1 (COUP-TFI), (2) a core component of the SWI/SNF complex, previously described nuclear receptor coactivator-BAF60a, and (3) the regulatory subunit of the serine/threonine protein phosphatase 4 (PP4R1) in yeast two-hybrid screening system. We also preliminarily confirmed that PGC-1β interacts with PP4R1 and Baf60a in some *in vitro* and *in vivo* studies. The identification of PP4R1 and BAF60a may help us reveal a general mechanism of PGC-1β coactivation in regulation of PGC-1β-mediated transcription.

MATERIALS AND METHODS

Compounds and plasmids. Unless otherwise stated, all chemical compounds were purchased from Sigma Chemical Co. (St Louis, MO). pCMV6-hPGC-1β plasmid was commercially provided by OriGene Technologies (Cat. #SC305937). To generate hPGC-1β-PGBKT7, the bait for yeast two hybrid screening, we first performed PCR for fragments of amino acids 82-426 and 82-361 of N-terminal PGC-1β using pCMV6-hPGC-1β as template. The right primer for PCR is 5' GAC GGC GAA TTC TCA GCT TCG CCT GCC CCC TCA TCT GCA 3' with engineered EcoR1 site, and the left primer is 5' GAC GGC GGA TCC CTA CAG TCT GGC AGG CCG GCG GAC CTC 3' or 5' GAC GGC GGA TCC CTA CAG ACG GTA GGG TTT GCT GAC ATC 3' with engineered BamH1 site. PCR products were inserted in PCR4-TOPO vector (Invitrogen Cat. K4575-02) at the sites of EcoR1/BamH1. PGC-1β fragments were then cut out from PCR4-TOPO-PGC-1β and subcloned into in PGBKT7 vector (Clontech, Cat. 630303) at the sites of EcoR1/BamH1 for GAL4-hPGC-1β baits. pGEX-hPGC-1β is generated by inserting the PGC-1β fragments at sites of EcoR1/Sal1 in pGEX-4T-1 vector for GST-hPGC-1β fusion proteins. 3xFLAG- PGC-1β was generated by subcloning full length 3xFLAG-PGC-1β cut from pShuttle-hPGC-1β into the pCDNA 3.1 (-) vector (Invitrogen, Cat. V795-20) at the site of NotI/HindIII. BAF60a-PACT2 plasmid is obtained from cDNA library. BAF60a-pGEX-5x-1 is generated by inserting BAF60a

fragment cut from BAF60a-PACT II plasmid at the sites of BamH1 and Xho1 in pGEX-5x-1 vector. BAF60a-pRSET is generated by insert BAF60a fragment at the sites of BamH1/Xho1 in the pRSET A vector. 3xmyc-BAF60a was generated by inserting the BAF60a fragment into pCMV3-Tag 2B (Stratagene, Cat. 240196) at the sites of BamH1 and Xho1. FLAG- PP4R1 was kind gift from Dr. Wadzinski's lab.

Yeast two-hybrid screening. Amino acids 82-426 of N-terminal PGC-1β were clone in-frame into the GAL4 DNA-binging domain expression vector PGBKT7. cDNA liberary containing cDNA constructed in the GAL4 activation domain plasmid PACT is commercially available in CLONTECH. Yeast two hybrid screening was performed as described in the CLONTECH Matchmaker yeast two-hybrid system protocol. Briefly, PGKT7-PGC-1β (GAL4-NBT3) was transformed into Y153 yeast cells by the lithium acetate method and maintained by selection in Leucine- plates. cDNA libraries were transformed into Y153-GAL4-PGC-1β pre-transformed Y153 yeast cells and plated on selective medium lacking amino acids tryptophan, leucine, and histidine. Positive clones were assayed for β-galactosidase activity in a filter assay as described in the CLONTECH protocol. Qualitative standards for primary yeast two-hybrid screen: (1) +++ strong positive: < 30 min, (2) ++ positive: 30 ~ 60 min, (3) + weak positive: > 60 min, (4) - negative: no blue. Screening Procedures include (1) primary screen with GAL4-NBT3, (2) secondary screening (cell cloning), (3) rescue positive clones from yeast → E. coli (shuttle plasmid), (4) re-transform putative positives with GAL4-NBT3 to confirm, (5) 'criteria for specificity of

interaction' test: "prey" plasmid alone and "prey" plasmid in the presence of non-specific control baits, PAS1-OCTN1 and PAS1-OAT1 cDNA. After three round of screening, the cDNA inserts of positive clones were sequenced. Quantitative liquid assay was performed using AH109 yeast strain. COUP-TF-1, BAF60a, and PP4R1 were co-transformed with PGC-1β bait (NBT1 or NBT3) or a non-specific baits (OCTN1 or OAT1) into yeast strain AH109. The transformed cells were cultured in appropriate liquid medium until the cells were in mid-log (OD600 if 1ml=0.5-0.8). The assay was performed using ONPG as the substrate and β-galactosidase activities were measured by the spectrophotometer and calculated according to the protocol (Yeast Protocols Handbook, CLONTECH).

Isolation of GST-PGC-1β and GST-BAF60a fusion proteins. The N-terminus of human PGC-1β (NBT3) was cloned into EcoR1/Sal1 sites of the pGEX-4T-1 vector to produce plasminds encoding glutathione-S-transferase (GST) fusion protein. PGC-1β fragments were subcloned into the EcoR1/Sal1 site of pGEX-4T-1. BAF60a fragments were inserted at BamH1/Xho1 site in pGEX-5x-1 vector for the expression of GST-BAF60a fusion protein. GST fusion protein expression vectors were transformed into BL21DE3 cells (Novagen #69450-1), which contain the T7 polymerase stably intergrated under the control of an IPTG induciable promoter, and plated on LB-amp (100ug/mL) plates. A single colony was used to inoculate a 10 mL LB liquid culture containing 100ug/mL ampicillin. This culture was grown at 37°C with shaking for 6 hours. A 15% glycerol stock was prepared

from the 10mL culture in a final volume of 0.5 mLs. Transformants were inoculated and grown as before for 10 hours. A 50 mL culture was inoculated with the 50mL culture and grown as before for 2-3 hours. The culture was then induced to express the fusion protein with 0.5 mM IPTG (0.24g/L) for 4-6 hours. The cells were pelleted and resuspended in 5.0mls NETN (NETN=100mM NaCl, 20 mM tris pH=8.0, 1 mM EDTA, 0.5% NP-40). The cells were sonicated 3 times, on a medium setting, for 1 minute each on ice. The sonicated cell lysates were centrifuged at 12,000 rpm for ten minutes at 4°C. The supernatant was made 10% in glycerol and frozen at - 80°C for future use. 25 µl of glutathione agarose beads (Pharmacia #17-0756-01) was added to the 300 µl supernatant of the -80°C frozen supernatant after thawing and incubated with shaking at 4°C for 30minutes in a 1.5 mL eppendorf tube. The beads were pelleted for 1 minute and then washed three times in a 1mL NETN.

GST pull-down assay. ^{35}S-Labeled PGC-1β, BAF60a, and PP4R1 were synthesized in the TNT coupled in vitro transcription/translation reticulocyte lysate system (Promega #L4610) using pRSET-PGC-1β, pRSET-Baf60a, and pCDNA-PP4R1 expression vectors. In the pull-down reaction, after immobilizing GST fusion proteins to the beads and washing the beads extensively with NETN, ^{35}S-labeled PGC-1β, BAF60a and PP4R1 were added in incubation buffer (50mM KCl, 40 mM HEPES pH 7.5, 5 mM mercaptoethanol, 0.1% Tween-20, 0.5% non-fat dry milk). The slurry was allowed to rock at 4°C for 1 hour. The beads were pelleted and washed 3 times in 1 mL of NETN and 5 times in 1 ml of cold incubation buffer

containing 100 mM KCl. 30 µl of 2x SDS-PAGE sample buffer was added to beads and the samples were resuspended. After the samples were boiled for 10 minutes, the eluted proteins were analyzed by 10% polyacrylamide gels. The gels were dried and autoradiography was performed overnight at -80°C.

Cell culture and transient transfection of CV-1 cells. CV-1 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplied with 10% fetal bovine serum. Transfections were performed with using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instrucrion.

Immunocytochemistry. CV-1 cells were cultured on coverslips and grown for 24 hours. Cells were cotransfected with 3x-FLAG-tagged PGC-1β and 3xmyc tagged PP4R1. 48 hours later, transfected CV-1 cells were washed with 1mL of PBS and fixed in 4% paraformaldehyde/PBS for 10 minutes. Then cells were briefly washed with PBS and permeabilized with 0.5 mL of 0.5% Triton X-100 in PBS for 5 minutes. Following 2 washes with PBS (5 minutes each), cells were incubated with monoclonal anti-FLAG M2-cy3 antibody and monoclonal anti-c-myc-FITC antibody (Sigma, Cat.A9594 and Cat.F2047 respectively) at a dilution of 1:250 for 1 hr in blocking solution (0.5% Trton X-100 and 2.5% BSA in PBS). Cells were then washed 3 times (5 minutes each) with PBS and incubated with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 5 minutes at a dilution of 1:1000. After

briefly wash with PBS, cells were mounted and ready for immunofluoresence microscopy.

RESULTS

Design of GAL4-N-terminal PGC-1β 'Bait'.

The N-terminal activation domain of PGC-1α is responsible for the interaction between PGC-1α and many of its protein partners. In order to design feasible PGC-1β baits for screening, we compared the primary amino acid sequence of the N-terminal domain of PGC-1β with that of PGC-1α (Figure 3A). The sequence analysis shows that both PGC-1α and PGC-1β contain three -LXXLL- motifs known as interacting surface for nuclear receptors. The first two -LXXLL- motifs within PGC-1α and PGC-1β share a similar orientation and position, which might be the reason that these two coactivators share some interacting partners. However, the third -LXXLL- motif within PGC-1β is significantly distinct from PGC-1α in two aspects. First, the third -LXXL- motif of PGC-1β appears to be in a reverse orientation, i.e. in PGC-1β it is -LXXLL-, whereas in PGC-1α it is -LLXXL-; second, there are some additional amino acids flanking the third -LXXLL- motif of PGC-1β (aa 238-287 and 236-266) as compared with that of PGC-1α (shown as shielded parts in Figure 3A). These differences make the third -LXXLL- motif of PGC-1β unique. Therefore, we rationalized that the third -LXXLL- motif and those additional amino acids may generate a distinct profile of PGC-1β-interacting protein partners. This is the reason that we selected this region of the N-terminal PGC-1β to design the "bait". Our previous study indicated that baits containing first 80 amino

acids resulted in auto-activation (data not shown), therefore we designed the "bait" starting from amino acids 82. Moreover, as the amino acids from 427-450 are repeated glutamine and glutamic acid (Figure 1A), we thought these amino acids might be also associated with auto-activation. Therefore we used amino acids from 82 to 426 to make GAL4-PGC-1β DNA-BD "bait", named NBT3, by fusing them with GAL4 DNA-binding domain (BD). Considering a shorter "bait" may lead to more specific interacting proteins of PGC-1β, we also designed two smaller baits using amino acids 82-361 (named NBT1) and 126-361 (named NBT2) (Figure 3B). All three "baits" contain -LXXLL- motifs and the unique surrounding amino acids (Figure 3C). We used these "baits" to identify PGC-1β-interacting proteins using a yeast two hybrid screening system.

3-A

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PGC1alpha   MAWDMCNQDSESVWSDIECAALVGE------DQPLCPDLPELDLSELDVNDLDTDSFLGG 54
PGC1beta    MAGNDCGALLDEELSSFFLNYLADTQGGGSGEEQLYADFPELDLSQLDASDFDSATCFGE 60
            ** : *.   :.  *.:    *..       :: * .*:******:**..*:*: : :*
PGC1alpha   LKWCSDQSEIISNQYNNEPSNIFEKIDEENEANLLAVLTETLDSLPVDEDGLPSFDALTD 114
PGC1beta    LQWCPENSETEPNQYSPDDSELFQIDSENE--ALLAELTKTLDDIPEDDVGLAAFPALDG 118
            *:**.::**  .***. : *::*:  .*::   *** **:***.:* *: **.:* ** .
PGC1alpha   GDVTTDNEASPSSMPDG--------TPPPQEAEEPSLLKKLLLAPANTQLSYNECSG--- 163
PGC1beta    GDALSCTSASPAPSSAPPSPAPEKPSAPAPEVDELSLLQKLLLATSYPTSSSDTQKEGTA 178
            **. : ..***:. .           :.*. *.:* ***:***.:. .  * :  .
PGC1alpha   --------------LSTQNHANHNHRIRTNPAIVKTENSWSNKAKSICQQQKPQRRPCS- 208
PGC1beta    WRQAGLRSKSQRPCVKADSTQDKKAPMMQSQSRSCTELHKHLTSAQCCLQDRGLQPPCLQ 238
                      :.:::.  :::  :  . :   **     .: . * *::  : **
PGC1alpha   ------------------------------------------------ELLKYLTTND 218
PGC1beta    SPRLPAKEDKEPGEDCPSPQPAPASPQDSLALGRADPGAPVSQEDMQAMVQLIRYMHTYC 298
                                                             :*::*: *
PGC1alpha   DPPHTKPTENRNSSRDKCTSKKKSHTQSQSQHLQAKP---------------------- 255
PGC1beta    LPQRKLPPQTPEPLPKACSNPSQQVRSRPWSRHHSKASWAEFSILRELLAQDVLCDVSKP 358
             * :. *.:. :.   . *:. .:.   .    .: ::*.
PGC1alpha   --------TTLSLPLTPESPNDPKGSPFENKTIERTLSVELSGTAGLTPPTTPPHKANQD 307
PGC1beta    YRLATPVYASLTPRSRPRPPKDSQASPGRPSSVEEVRIAASPKSTGPRPSLRPLRLEVKR 418
                  ::*:    *..*:*.:.** . .:::*..   .   . ::*  *.  * :   :
PGC1alpha   NPFRASPKLKSSCKTVVPPPSKKPRYSESSGTQGNNSTKKGPEQSELYAQLSKSSVLTGG 367
PGC1beta    EVRRPARLQQQEEEDEEEEEEEEEEEKEEEEEWGRKRPGRGLPWTKLGRKLESSVCPVRR 478
            :  *.:   :.. :      .:: . .*..   *.: . :*   ::*  :*..*   .
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3-B

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MAGNDCGALL DEELSSFFLN YLADTQGGGS GEEQLYADFP ELDLSQLDAS 50
DFDSATCFGE LQWCPENSET EPNQYSPDDS ELFQIDSENE ALLAELTKTL 100
DDIPEDDVGL AAFPALDGGD ALSCTSASPA PSSAPPSPAP EKPSAPAPEV 150
DELSILQKLL LATSYPTSSS DTQKEGTAWR QAGLRSKSQR PCVKADSTQD 200
KKAPMMQSQS RSCTELHKHL TSAQCCLQDR GLQPPCLQSP RLPAKEDKEP 250
GEDCPSPQPA PASPRDSLAL GRADPGAPVS QEDMQAMVQL IRYMHTYCLP 300
QRKLPPQTPE PLPKACSNPS QQVRSRPWSR HHSKASWAEF SILRELLAQD 350
VLCDVSKPYR LATPVYASLT PRSRPRPPKD SQASPGRPSS VEEVRIAASP 400
KSTGPRPSLR PLRLEVKREV RRPARLQQQE EEDEEEEEEE EEEEKEEEEE 450
WGRKRPGRGL PWTKLGRKLE SSVCPVRRSR RLNPELGPWL TFADEPLVPS 500
EPQGALPSLC LAPKAYDVER ELGSPTDEDS GQDQQLLRGP QIPALESPCE 550
SGCGDMDEDP SCPQLPPRDS PRCLMLALSQ SDPTFGKKSF EQTLTVELCG 600
TAGLTPPTTP PYKPTEEDPF KPDIKHSLGK EIALSLPSPE GLSLKATPGA 650
AHKLPKKHPE RSELLSHLRH ATAQPASQAG QKRPFSCSFG DHDYCQVLRP 700
EGVLQRKVLR SWEPSGVHLE DWPQQGAPWA EAQAPGREED RSCDAGAPPK 750
DSTLLRDHEI RASLTKHFGL LETALEEEDL ASCKSPEYDT VFEDSSSSSG 800
ESSFLPEEEE EEGEEEEEDD EEEDSGVSPT CSDHCPYQSP PSKANRQLCS 850
RSRSSSGSSP CHSWSPATRR NFRCESRGPC SDRTPSIRHA RKRREKAIGE 900
GRVVYIQNLS SDMSSRELKR RFEVFGEIEE CEVLTRNRRG EKYGFITYRC 950
SEHAALSLTK GAALRKRNEP SFQLSYGGLR HFCWPRYTDY DSNSEEALPA 1000
SGKSKYEAMD FDSLLKEAQQ SLH                              1023
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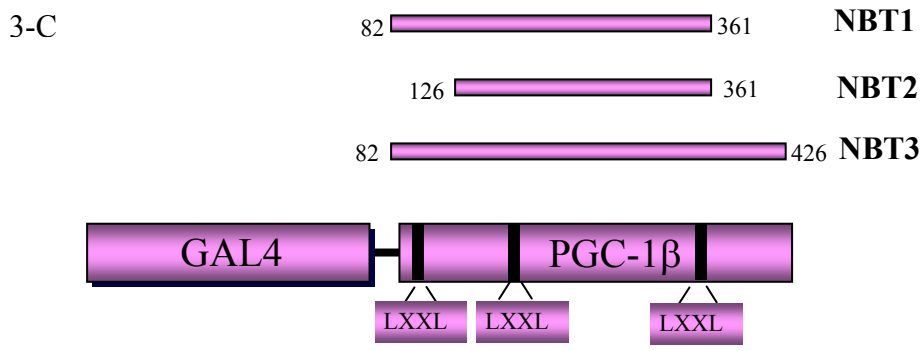


Figure 3. Design of GAL4-N-terminal PGC-1β "bait". A. Comparison of the primary amino acid sequence of N-terminal PGC-1α and PGC-1β. Dark shielded parts indicate additional amino acid sequence of PGC-1b compared to PGC-1a. Light shield part represents repeated "glutamine" and "glutamic acid". Red boxes indicate –LXXLL- motif B. Amino acid sequence for GAL4-N-terminal PGC-1β "baits": NBT1: 82-361; NBT2: 126-361; NBT3: 82-426. It contains three –LXXLL- motifs (shown in box). C. Structure of GAL4-N-PGC-1β 'bait'. NBTs represent different GAL4-PGC-1β DNA-BD "baits" made from N-terminal PGC-1β. Bait contains amino acid sequence of N-terminal PGC-1β 82-361 named NBT1, 126-361 named NBT2, and 82-426 named NBT3.

Protein expression of GAL4-N-terminal PGC-1β "bait" in yeast strains.

After design of GAL4-PGC-1β bait using N-terminus of PGC-1β, we tested whether these baits were able to be correctly expressed in yeast strains. We transformed all three GAL4-N-termianl PGC-1β "baits" NBT1, NBT2, and NBT3 into two yeast strains, AH109 and Y153, and cultured them in proper selective medium. Proteins in the cell lysates were separated by SDS-PAGE and transferred to the nitrocellulose membrane, which was later probed for anti-GAL4 antibody to detect the presence of GAL4-PGC-1β-BD fusion proteins. Immuno blotting showed that NBT1 and NBT3 were well expressed in both yeast strains AH109 and Y153 among the three "baits" (Figure 4). NBT3 appeared to be larger in size (70 kD) compared with NBT1 (55 kD), which is consistent with their amino acid sequence. However, NBT2 protein did not express in yeast. The result from sequence analysis showed that it was out of the reading frame. Therefore, the baits NBT1 and NBT3 were used for further experiments.

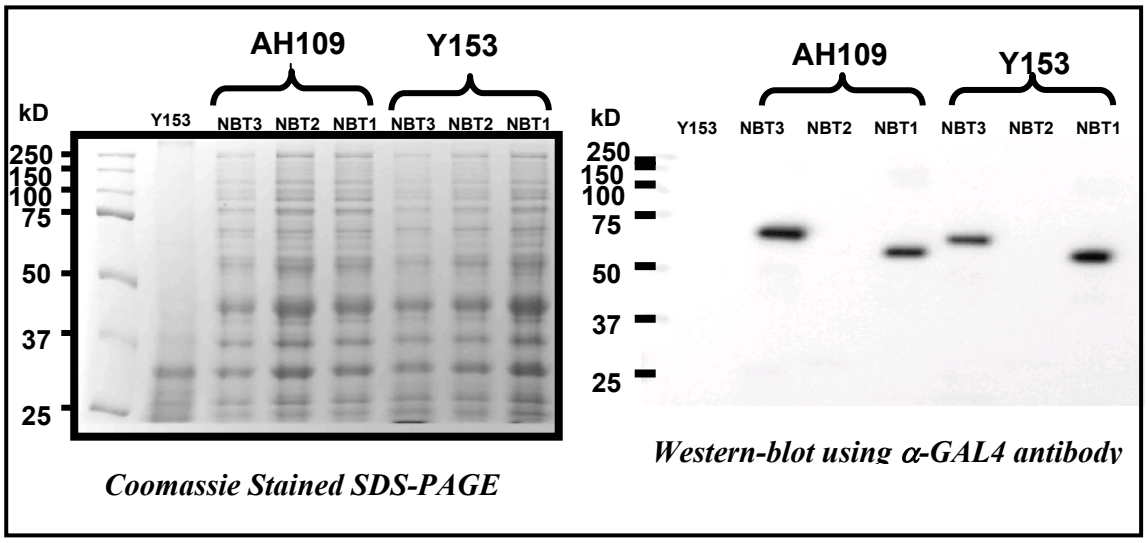


Figure 4. Protein expression of GAL4-N-terminal PGC-1β "baits" in yeast strains. Three GAL4-N-terminal PGC-1β "baits" were transformed into two yeast strains AH109 and Y153 respectively and incubated at 30°C overnight. Cells were lyzed by vortexing with glass beads in RIPA lysis buffer containing protease inhibitor for 5 minutes. Cell lysates were analyzed in 10%SDS-PAGE gel stained with coomassie blue staining (left). GAL4-PGC-1β fusion protein was detected by immunoblotting with antibody against GAL4 protein (right). NBTs represent different GAL4-PGC-1β DNA-BD "baits" made from N-terminal PGC-1β. Bait contains amino acid sequence of N-terminal PGC-1β 82-361 named NBT1, 126-361 named NBT2, and 82 to 426 named NBT3.

GAL4-PGC-1β "bait" NBT3 did not auto-activate in auto-activity test.

To determine whether these PGC-1β "baits" could be auto-activated in yeast cells, the auto-activation potency test was performed for all three "baits". In this experiment, NBT1, NBT2, and NBT3 were independently transformed into two yeast strains and cultured in selective medium for 3 days. The β-galactosidase colony-lift filter assay was performed to detect auto-activation events. The results showed that NBT1 displayed slight auto-activation in both AH109 and Y153 yeast strains (blue color clononies) (Figure 5). This result that NBT1 is not ideal for the yeast two-hybrid screening. NBT2 and NBT3 did not show any auto-activation potency; however, since NBT2 could not express the protein correctly in yeast strains, it cannot be used as a bait for screening. NBT3 protein was well expressed in both AH109 and Y153 yeast strains, and did not display any auto-activation. Therefore, NBT3 was the only feasible bait for yeast two-hybrid screening. Since NBT1 only displayed a slightly auto-activation after a longer incubation period, we still used it in some downstream experiments for confirmation using a relative short incubation time.

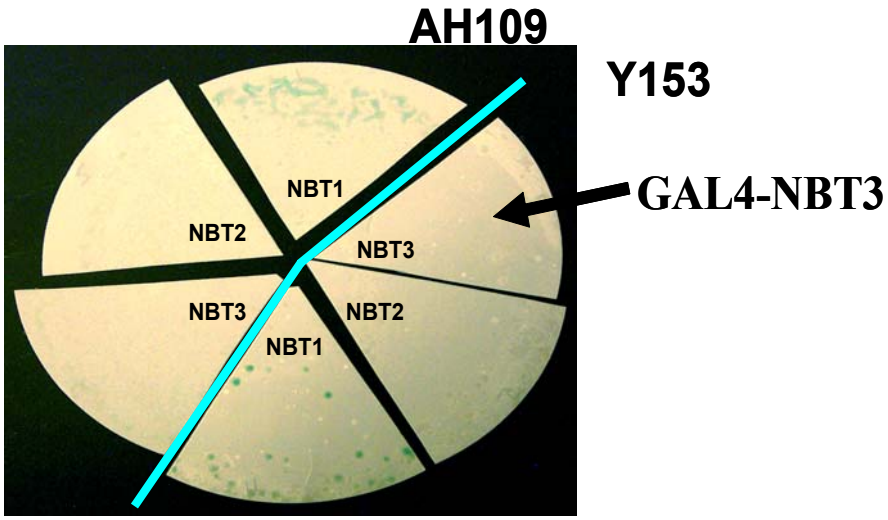


Figure 5. Auto-activity test for GAL4-N-PGC-1β "baits". Three GAL4-N-PGC-1β "baits": NBT1, NBT2, and NBT3 were independently transformed into strain AH109 and Y153 using-small scale yeast transformation protocol (Two-Hybrid User Manual, CLONTECH). Transformants were selected on the appropriate medium for 3 days, and assay for activation of LacZ reporter gene using the β-galactosidase colony-lift filter assay. NBTs represent different GAL4-PGC-1β DNA-BD "baits" made from N-terminal PGC-1β. Bait contains amino acid sequence of N-terminal PGC-1β 182-361 named NBT1, 126-361 named NBT2, and 82 to 426 named NBT3. Blue colonies indicate auto-activation events for baits. Blue line separated the image into two parts. Upper part is the auto-activation potency test in AH109 yeast strain and lower part is in Y153 yeast strain.

Screening cDNA library using GAL4-PGC-1β "bait" in a yeast Two-hybrid system.

It is known that PGC-1β is expressed in oxidative tissues including fat, brain, and liver. In order to identify its interacting protein partners, we screened cDNA libraries from fat, brain, and liver using GAL4-PGC-1β "bait", NBT3. In yeast two-hybrid system, the N-terminal-PGC-1β as the "bait", was fused with the GAL4-binding domain (DNA-BD) that binds to a specific enhancer-like sequence of LacZ. cDNAs from a specific tissue were fused with a transcriptional activation-domain (AD) and were termed "prey". Both the DNA-BD and the AD are required to activate the transcription of LacZ; however, they are physically separated by recombinant DNA technology and thus cannot directly interact with each other, and consequently cannot activate the responsive genes alone. The interaction between a bait protein (PGC-1β protein fused to the DNA-BD) and a prey (library proteins fused to the AD) creates a novel transcriptional activator that can activate transcription of the reporter gene LacZ in yeast (Figure 6). The protein expression of LacZ encoding β-galactosidase turns colonies blue in the presence of X-GAL substrate. In the screen, positive colonies were graded according to the time required for a colony to turn blue. They are: (1) strong positive (+++): < 30 min, (2) positive (++): 30 ~ 60 min, (3) weak positive (+): > 60 min, (4) negative (-): no blue. The whole process for the two-hybrid screen includes: (1) primary screen with GAL4-NBT3, (2) secondary screening (cell cloning), (3) rescue positive clones from yeast to

E. coli (shuttle plasmid), (4) re-transform putative positives with both GAL4-NBT3 and GAL-NBT1 to confirm the interaction, and (5) 'criteria for specificity' test using non-specific baits OCTN1 and OAT1.

About six million transformants in total were screened and 650 positives were picked from primary screen. A secondary screen was conducted within these 650 primary positives, among which 119 positives were identified and rescued from yeast to E. coli. We tested the specificity for all these 119 clones. After this test, 32 clones (including 19 "+++" and "13 ++") were selected and re-tested at least three times for confirmation. All 32 clones were then sequenced for their identity.

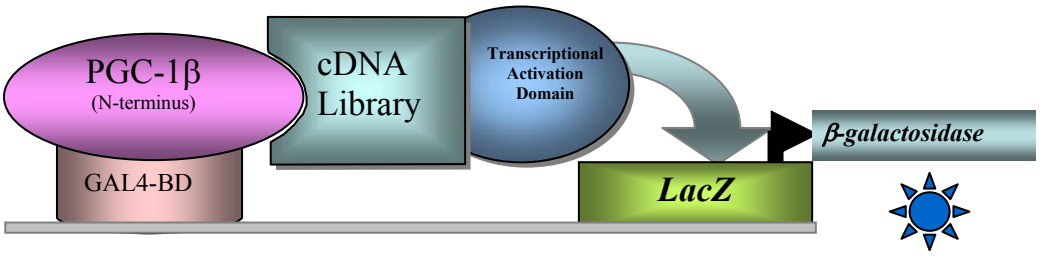


Figure 6. Scheme of GAL4-PGC-1β "bait" screening cDNA library. In yeast two-hybrid system, the N-terminal-PGC-1β as the "bait", was fused with the GAL4-binding domain (DNA-BD) that binds to a specific enhancer-like sequence of LacZ. cDNAs from a specific tissue were fused with a transcriptional activation-domain (AD) and were termed "prey". Both the DNA-BD and the AD are required to activate the transcription of LacZ. When "bait" protein and "prey" protein interact, it will bring BD and AD together and form a novel transcriptional activator that can activate transcription of the reporter gene LacZ in yeast. The protein expression of LacZ encoding β-galactosidase turns colonies blue.

COUP-TF1, PP4R1, and BAF60a protein were identified interacting with PGC-1β in yeast two-hybrid system.

Among thirty-two sequenced clones, most of them are structural proteins and non-specific proteins such as β-actin, which are not associated with regulation of transcription, therefore these proteins are not the target proteins we are looking for. Interestingly, three proteins we identified have started being linked to the regulation of transcription recently by interacting with some transcription factors and cofactors, but their interactions with PGC-1b have been demonstrated. Three interesting proteins are **(1)** a novel splice variant of the nuclear receptor superfamily member-chicken ovalbumin upstream promoter transcription factor 1 (COUP-TFI), **(2)** a core component of the SWI/SNF complex, previously described nuclear receptor coactivator-BAF60a, and **(3)** the regulatory subunit of the serine/threonine protein phosphatase 4 (PP4R1). In addition, there are several other proteins might also associated with the transcription or mRNA splicing process such as Xeroderma pigmentosum group A (XPA)-binding protein 2 (XAB2), the serine/arginine (SR)-rich protein 55 (SRP55), however, they might be our project for the second stage since we are going to start our research from three most interested proteins.

In order to investigate the specificity of the interaction between PGC-1β and these three prey proteins, we co-transformed COUP-TF1, PP4R1 and BAF60a with a PGC-1β bait (NBT1 or NBT3) or with a non-specific bait OCTN1 into yeast strain Y153. The results showed that cells cotransformed with prey and NBT3 turned dark

blue within 30 minutes, which indicated that all three proteins strongly interact with PGC-1β. In addition, NBT1 also confirmed the interaction between these three proteins and PGC-1β. There were no interactions between the non-specific bait OCTN1 and PGC-1β, suggesting that the interactions between PGC-1β and COUP-TF1, PP4R1, and BAF60a were specific and strong (Figure 7).

To further investigate the specificity and intensity of the interaction between these three proteins and PGC-1β, quantitative liquid assays were performed using ONPG (o-nitrophenyl β-D-galactopyranoside) as a substrate for β-galactosidase activity. In the quantitative assay, COUP-TF1, PP4R1, and BAF60a were co-transformed with PGC-1β baits (NBT1 or NBT3) or the non-specific bait (OCTN1 or OAT1) into the yeast strain AH109, and the transformants were cultured in an appropriate selective liquid medium. The results showed that the β-galactosidase activity in transformants co-transformed with COUP-TF1 and PGC-1β bait, PP4R1 and PGC-1β bait, and BAF60a and PGC-1β were significantly higher than in transformants with the non-specific bait (Figure 8). This indicated that the interactions between PGC -1β and COUP-TF1, PP4R1, and BAF60a were not only specific but also intense.

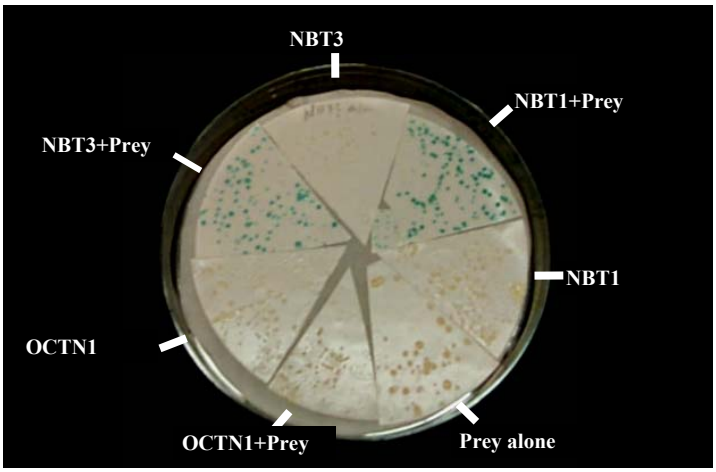


Figure 7. Identification of COUP-TF1, PP4R1, and BAF60a as the PGC-1β-interacting proteins in yeast two-hybrid system. Preys, COUP-TF1, PP4R1, and BAF60a, were co-transformed with GAL4-N-PGC-1β "bait", NBT1 and NBT3, or nonspecific "bait" OCTN1 into Y153 yeast strain**.** NBT1, NBT3, and OCTN1 represented these three baits were transformed alone. Transformants were cultured on appropriate medium for 3 days. Transformants were assayed for activation of LacZ reporter gene using the β-galactosidase colony-lift filter assay. Blue colonies represent interaction and white represents no interaction. NBT represent different GAL4-PGC-1β DNA-BD "baits" made from N-terminal PGC-1β. Bait contains amino acid sequence of N-terminal PGC-1β 82-361 named NBT1, 126-361 named NBT2, and 82 to 426 named NBT3.

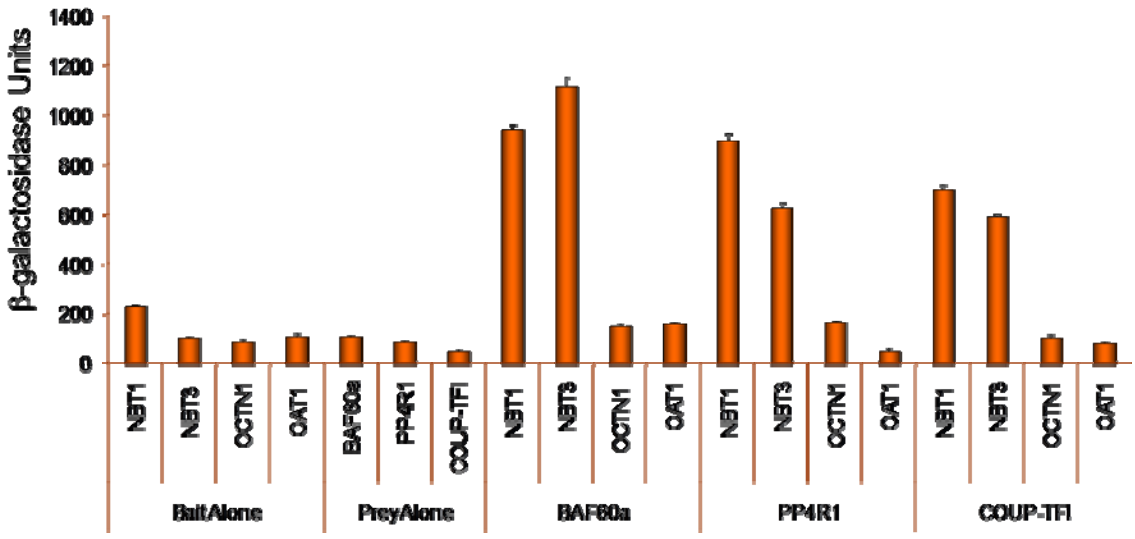


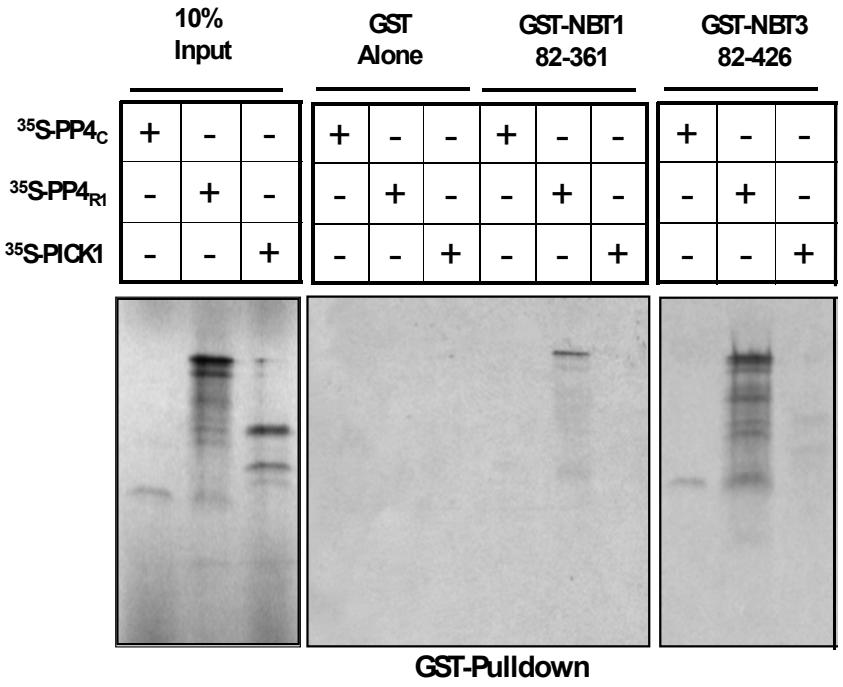
Figure 8. Quantitative Assessment for interaction between PGC-1β and COUP-TF1, PP4R1, and BAF60a.

In this assay, PP4R1 were co-transformed with PGC-1β bait (NBT1 or NBT3) or a non-specific baits (OCTN1 or OAT1) into yeast strain AH109. The transformed cells were cultured in appropriate liquid medium until the cells were in mid-log (OD600 if 1ml=0.5-0.8). The assay was performed using ONPG as the substrate and β-galactosidase activities were measured by the spectrophotometer and calculated according to the protocol (Matchmaker yeast two-hybrid system Protocol, CLONTECH). The results are from three duplicate experiments.

PGC-1β physically interacts with PP4R1 in vitro.

After using the yeast two-hybrid screen to obtain this preliminary data of PGC-1β interacting with COUP-TF1, PP4R1, and BAF60a, we expected confirmed the interaction between PGC-1β and these three proteins using a different system. GST pull-down assay is a method that is robust and easy to use. It can capture proteins that even are weak interactions due to the high concentration of the purified target proteins, and even in the absence of signaling to induce the interaction. Therefore, we chose GST pull-down system as the first step of our confirmation. We used GST-NBT1 fusion protein and GST-NBT3 fusion protein (containing amino acids 82-361 and 82-426 of N-terminal PGC-1β respectively) to capture ^{35}S-PP4R1 and ^{35}S-PP4c proteins, the catalytic subunit of the serine/threonine protein phosphatase 4 (PP4). The result shows that both GST-PGC-1β fusion proteins (GST-NBT1 and GST-NBT3) interact with PP4R1 but not control GST protein alone and ^{35}S-PICK protein (Figure 9). We also detected the involvement of catalytic subunit of PP4, PP4c. It shows that PP4c also interacts with both GST-PGC-1β fusion proteins. However, the PGC-1β protein containing 82-426 amino acids displays much stronger interaction with PP4R1 and PP4c compared with those containing 82-361 amino acids, which may indicate amino acids sequence from 361 to 426 are responsible for high binding affinity for PP4. These data indicated that PGC-1β physiologically interacts with PP4 *in vitro*.

9-A


10% Input
GST Alone
GST-NBT1 82-361
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35S-PP4C
35S-PP4R1
35S-PICK1
GST-Pulldown

9-B

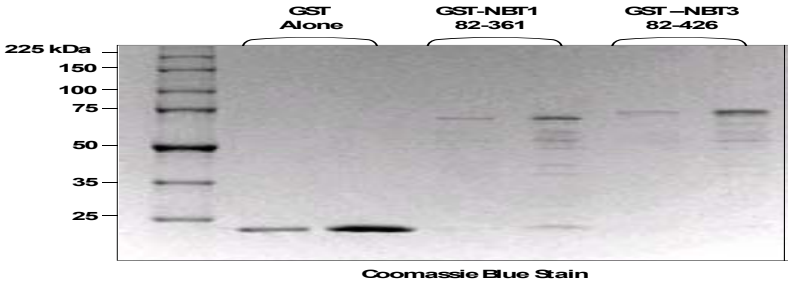

GST Alone
GST-NBT1 82-361
GST –NBT3 82-426
225 kDa
150
100
75
50
35
25
Coomassie Blue Stain

Figure 9. GST pull-down assay for the interaction of PGC-1β and PP4 *in vitro*.

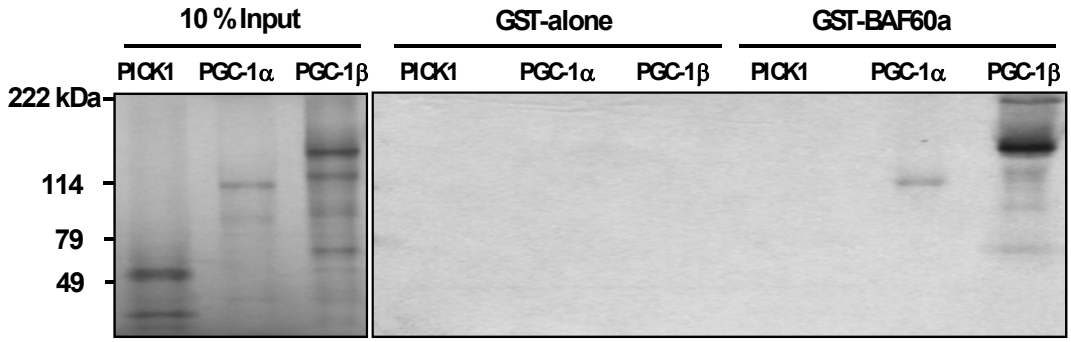
A. In GST pull-down assay, 300ul of cell lysates containing the control GST protein alone and the GST- PGC-1β fusion proteins, GST-NBT1 and GST-NBT3 (containing N-terminal PGC-1β amino acid sequence 82-361 and 82-426 respectively) were incubated with 20ul of radio-labeled ^{35}S-PP4R1, ^{35}S-PP4c, and ^{35}S-PICK (^{35}S-PPARα) , which were produced by *in vitro* transcription/translation reactions, in an incubation buffer (50mM Kl, 40mM HEPES PH=7.5, 5mM β-mercaptoethanol, 1% Tween-20, 1% non-fat dry milk) for 90 minutes at 37°C, then 25ul of glutathione-sepharose beads were added and incubated for 60 min at 4°C with gentle mixing. The beads were harvested at 2000rpm, washed 3 times with incubation buffer and 5 times with wash buffer. Beads were then resuspended in 30ul of 2xSDS-PAGE sample buffer containing 50mM DTT. Samples were heated at 100°C for 10min and loaded on a 10% SDS-PAGE gel for electrophoresis. After electrophoresis, the gel was dried and exposed to an X-ray film for an autoradiograph.

B. Expression of GST-NBT1 and NBT3 fusion protein stained with coomassie blue.

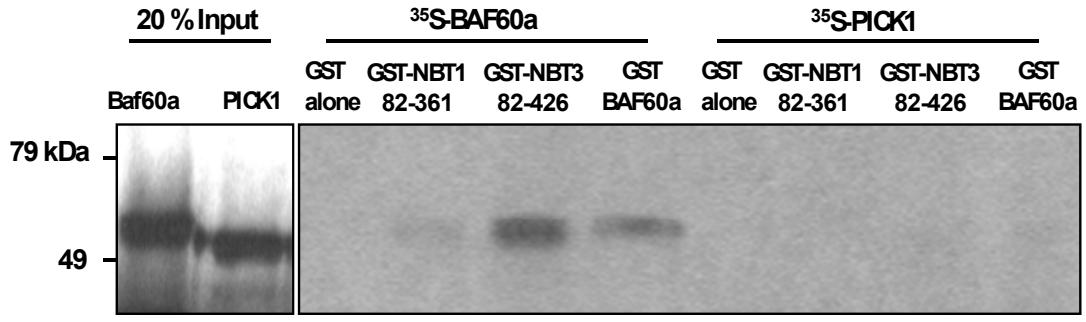
PGC-1β interacts with BAF60a in vitro.

We also performed GST pull-down assay to confirm the interaction between PGC-1β and BAF60a. In order to rule out a false positive interaction due to a conformational change of the GST-fusion protein, two GST pull-down assay were performed. First, we used GST-BAF60a fusion protein to capture radio-labeled ^{35}S-PGC-1β, and ^{35}S-PICK as control; we then used GST-PGC-1β fusion proteins, GST-NBT1 and GST-NBT3, to capture ^{35}S-BAF60a and control ^{35}S-PICK. As shown in Figure 10, the interaction between PGC-1β and BAF60a was detected in both GST pull-down assays. In addition, we also detect the interaction between PGC-1α and BAF60a, which has also been confirmed by other research group recently (Li S. et al. 2008). Interestingly, we also detected BAF60a interacts with itself (Figure 10B). The dimerization of BAF60a has not been described in the literature and its biological indication is unclear.

10-A


10 % Input
GST-alone
GST-BAF60a
PICK1
PGC-1α
PGC-1β
PICK1
PGC-1α
PGC-1β
PICK1
PGC-1α
PGC-1β
222 kDa
114
79
49

10-B


20 % Input
35S-BAF60a
35S-PICK1
Baf60a
PICK1
GST alone
GST-NBT1 82-361
GST-NBT3 82-426
GST BAF60a
GST alone
GST-NBT1 82-361
GST-NBT3 82-426
GST BAF60a
79 kDa
49

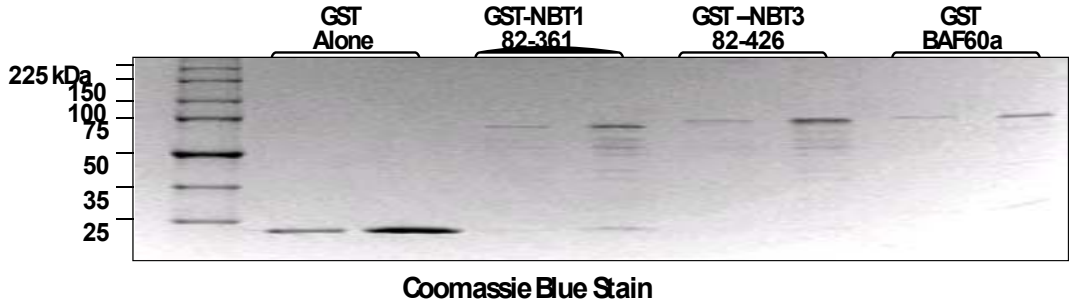
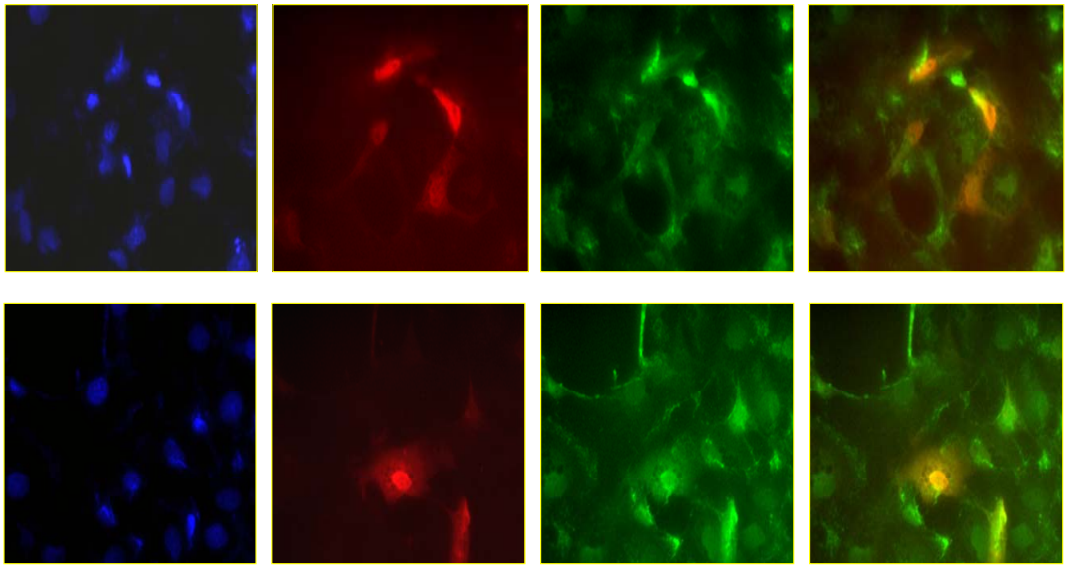

GST Alone
GST-NBT1 82-361
GST –NBT3 82-426
GST BAF60a
225 kDa
150
100
75
50
35
25
Coomassie Blue Stain

Figure 10. Confirmation of the physiological interaction of PGC-1β and BAF60a *in vitro*. In GST pull-down assays, 300ul of control GST protein alone and various GST fusion proteins were incubated with 20ul of ^{35}S-labeled proteins generated by in vitro transcription/translation assay. A. Control GST protein and GST-BAF60a fusion protein were incubated with ^{35}S-PGC-1β, positive control ^{35}S-PGC-1α, and negative control ^{35}S-PICK respectively for 1 hr. 10% input served as loading control. B. GST protein, two GST-PGC-1β fusion proteins: GST-NBT1 and GST-NBT3, and GSTBAF60a were incubated with ^{35}S-BAF60a and negative control ^{35}S-PICK respectively for 1hr. Bottom panel is coomassie blue staining for loading control for fusion proteins, GST-NBT1, GST-NBT3, and GST-BAF60a.

PGC-1β co-localizes with PP4R1 and BAF60a respectively in nucleus.

To determine the possible biological relevance of the interaction of PGC-1β with PP4R1, or BAF60a. CV-1 cells were transiently co-transfected with flag-PGC-1β and myc-PP4R1. We performed immunocytochemistry to determine whether PGC-1β protein co-localizes with PP4R1 and BAF60a protein in nucleus. As shown in Figure 11A, fluorescent microscopy revealed that both PGC-1β and PP4R1 were expressed in nucleus, and their expression highly overlapped. Ihis indicated that PGC-1β co-localizes with PP4R1 in nucleus upon over-expression. Meanwhile, using the same approaches, we confirmed that PGC-1β co-localized with BAF60a (Figure 11B). This is consistent with the co-immunoprecipitation of PGC-1β and BAF60a from another group (Li S. et al. 2008). These data suggested that PGC-1β interacts with both PP4R1 and BAF60a respectively in nucleus and their interactions may be biologically relevant.

11-A



11-B

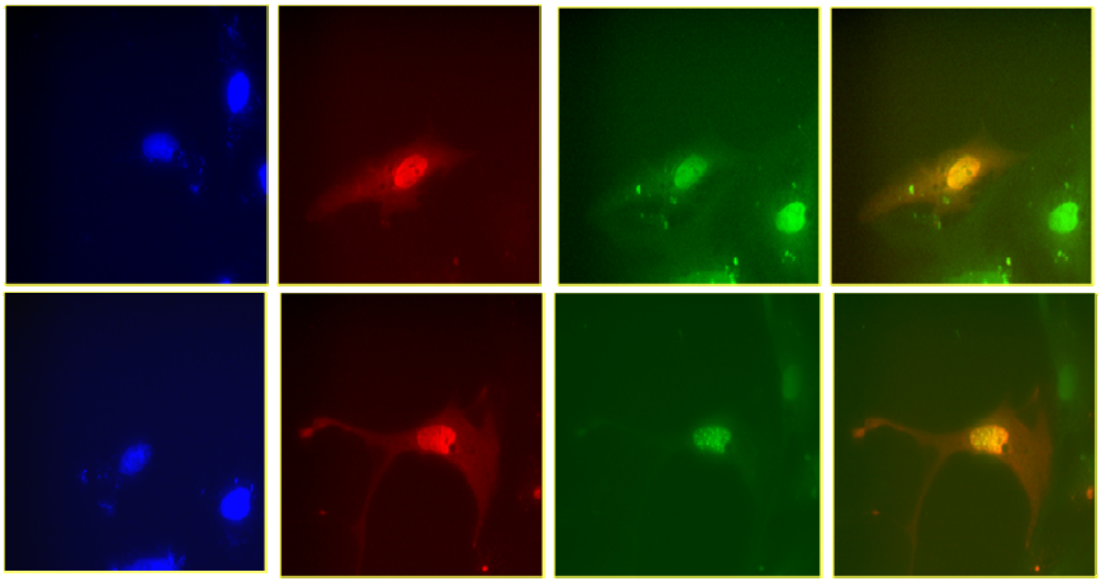


Figure 11. Immunocytochemistry staining for the colocalization of PGC-1β with PP4R1 and BAF60a in nucleus in CV-1 cell line. A. Co-localization of PGC-1b and PP4R1. Flag-PGC-1β and myc-PP4R1 were cotransfected into CV-1 cells on coverslips. 24 hours later, CV-1 cells were washed and fixed in 4% paraformaldehyde for 10 minutes. Then cells were briefly permeabilized for 5 minutes. Following 2 washes with 1xPBS, cells were incubated with monoclonal anti-flag M2-cy3 antibody and monoclonal anti-c-myc-FITC antibody for 1 hour. Cells were then washed 3 times (5 minutes each) with 1XPBS and incubated with DAPI 1 for 5 minutes for nuclei. Then cells were analyzed using immunofluoresence microscopy. Blue indicates nuclei, red represented PGC-1β, green represented PP4R1, and yellow indicated co-localization. B. Co-localization of PGC-1b and BAF60a. Blue indicates nuclei, red represented PGC-1β, green represented BAF60a, and yellow indicated co-localization.

DISCUSSION

It has been estimated that about 25% of adults in USA have metabolic syndrome. Metabolic syndrome greatly increases the risk of coronary heart disease and type II diabetes. People with metabolic syndrome increase four times the risk of heart disease and seven times the risk of diabetes compared with those without the syndrome (*www.sciencedaily.com*). The PGC-1α is known to be involved in the development of metabolic syndrome. The knowledge of a role of PGC-1α in metabolic syndrome came from the studies of interaction of PGC-1α and its protein partners. As a member of PGC-1 family, PGC-1β has also shown to play a role in the regulation of energy metabolism including mitochondrial biogenesis, fatty acid β-oxidation. Importantly, PGC-1β plays a critical role in the regulation of hepatic lipogenesis, a process closely associated with two important symptoms in metabolic syndrome, obesity and dislipidemia. Although it has been found that PGC-1β regulates lipogenesis by coactivating SREBPs, the molecular mechanisms underlying the regulation and function of PGC-1β in the lipogenic process are largely unknown. In our yeast two-hybrid screen, three interesting PGC-1β interacting-proteins, COUP-TF1, PP4R1, and BAF60a were identified. This identification will help us to investigate how PGC-1β coactivates SREBPs in regulating SREBP-mediated lipogenic genes. Although only a few of experiments have been done to confirm

interaction of these three proteins with PGC-1β, we believed that they highly likely interact with PGC-1β based on our data and the literatures reviewed.

The identification of COUP-TF1 proved the validity of our screen system. As mentioned previously, PGC-1β-baits we designed contain three -LXXLL- motifs, and we anticipated to identify nuclear receptors as the PGC-1β-interacting protein. COUP-TF1 is what we expected, an orphan nuclear receptor. This identification technically proved that our screening system is valid. In addition, the PGC-1β "bait" contained the first two of -LXXLL- motifs similar within two coactivator proteins, which could result in the identification of interacting nuclear receptors shared by PGC-1α and PGC-1β. COUP-TF1 might be one of interacting nuclear receptor partners shared by PGC-1α and PGC-1β. COUP-TF1 belongs to the steroid/thyroid hormone receptor superfamily. COUP-TF1 is involved in the regulation of several important biological processes, such as neurogenesis, organogenesis, and metabolic homeostasis. COUP-TF1 generally served as a repressor in the regulation of transcription. COUP-TF1 represses transcription through at least three mechanisms: (1) by competitively binding to a number of nuclear receptor response element; (2) by forming a nonproductive complex by heterodimerizing with retinoid X receptor, which is an essential heterodimer partner of a number of nuclear hormone receptors; (3) by directly binding to the ligand-binding domain of nuclear receptors such as thyroid receptor, vitamin D receptor, hepatocyte nuclear factor 4 (HNF4), and PPARγ (Robinson C.E. et al. 1999). Although COUP-TF1 mainly functions as

negative regulator in transcription, it was originally identified as an activator of chicken ovalbumin gene expression.

Interestedly, like PGC-1α, COUP-TF1 is also involved in various metabolism enzymes such as phosphoenopyruvate carboxykinase (PEPCK), a key enzyme in hepatic gluconeogenesis, and mitochondrial 3-hydroxy-3-methyglutaryl-CoA synthase. Research has found that it functions as an activator by directly binding to DNA elements on the promoter of PEPCK and that PGC-1α is a coactivator of COUP-TF1 in the regulation of PEPCK under chronic fasting condition (Herzog B. et al. 2004). This research has determined that both PGC-1α and COUP-TF1 bind to the glucocoticoid reponse unit (GRU) in the chromatin immunoprecipitation experiment (CHIP), however they did not perform further experiments to determine the physiological interaction between them. It is likely that PGC-1α coactivates COUP-TF1 by a direct protein-protein interaction manner. PGC-1α likely interacts with COUP-TF1 through its -LXXLL- motifs shared by PGC-1β and COUP-TF1 might be a novel interacting nuclear receptor shared by PGC-1α and PGC-1β.

PP4R1 is another PGC-1β-interacting protein we identified. PP4R1 is a regulatory subunit of the serine/threonine phosphatase4. The holoenzyme of the serine/threonine protein phosphatase 4 (PP4) consists of two different subunits, a regulatory subunit (PP4R1) and the catalytic subunit (PP4C). Regulatory subunits PP4R1 and PP4R2 are thought to target PP4 to the specific protein complex on the

promoter by interacting with both the catalytic subunit of PP4 and nuclear receptors. PP4 modifies proteins through dephosphorylation and thus regulates their biological functions. The identification of PP4R1 from our screen indicates the involvement of phosphorylation and dephosphorylation in the regulatory function of PGC-1β.

It is well known that phosphorylation of proteins on serine or threonine residues by protein kinases is an important mechanism for the regulation of many cellular processes including metabolism, gene expression and functions (Sefton B.M. and Shenolikar S. 2001; Diradourian C. et al. 2005). There is growing evidence that transcription factors and cofactors are primary targets of a variety of kinase-mediated cellular signaling pathways. PGC-1α has been shown to be regulated by phosphorylation. It was phosphorylated on serine and threonine residues by p38 mitogen-activated protein kinase (p38 MAPK). Phosphorylation of PGC-1α results in greatly increased protein stability by tripling its half-life (Knutti D. et al. 2001; Puigserver P. et al. 2001) and enhancing transcriptional activity by releasing its interaction with corepressors (Fan M. et al. 2004). These data may also indicate that phosphorylation might be involved in the regulatory function of PGC-1β due to high sequence similarity between these two proteins. In support of this indication, recent study of protein phosphorylation using high accuracy mass spectrometry has determined that PGC-1β is phosphorylated on Serine 524 and Tyrosine 990 (Dephoure N. et al. 2008)(www.phosphosite.org/proteinAction.do?id=10906&showAllSites=true),

although it has not been demonstrated by *in vivo* studies. In addition, studies have shown that phosphorylation is involved in many aspects of PGC-1β-mediated hepatic lipogenesis indicating the association of PGC-1β and phosphorylation. For example, transcription factor steroid response element bonding proteins (SREBPs), the PGC-1β interacting protein, are phosphorylated on serine residues by p38 MAPK, and extracellular signal-regulated kinases, ERK1 and ERK2 in the process of growth factor-induced lipogenesis. The phosphorylation of SREBPs increases their transcriptional activity, which is partially mediated by decreased recruitment of HDAC3 (Kotzka J. et al. 2004; Arito M. et al. 2008), a core component of corepressor complexes of SREBPs. Furthermore, HDAC3 has also been found to be phosphorylated by casein kinase at Serine 424 and phosphorylation of HDAC3 increases its enzymatic activity.

On the other hand, it is also well known that most phosphorylation is reversible. The phosphorylation status of the serine/threonine phosphorylated proteins is a result of the balance between the activities of protein kinases and serine/threonine protein phosphatases, such as PP1, PP2, and PP4. Phosphorylation modification of proteins have been well studied, however, the importance of protein phosphatases has not gained attention until recently. The identification of PP4R1 provide us an opportunity to investigate whether PGC-1β-containing protein complexes are affected by phosphorylation and dephosphorylation and how this may mediate the transcriptional function of PGC-1β.

The physiological interaction between PGC-1β and PP4R1 has been preliminarily confirmed by GST pull-down assay and immunohischemistry staining in our laboratory. In addition to these preliminary data, the possibilities of the interaction of PGC-1β and PP4R1 are also supported by some recent research. PP4, like PGC-1β, is mainly expressed in the nucleus. Research has shown that PP4 interacts with transcription factors c-Rel and RelA (NF-kb p65), members of the NF-kB family, and activates NF-kB-mediated transcription (Yeh P.Y. et al., 2004; Hu M.C. et al., 1998). Interestingly, PP4 has recently been found to interact with and dephosphorylate HDAC3 which decreases its enzymatic activity and may also alter the ability of HDAC3 to interact with other proteins (Zhang X. et al. 2005). It has also been observed that the recruitment of PGC-1s leads to the release of corepressors including HDAC and that recruitment of coactivators SRC-1 and p300 which contain HAT activity (Puigserver P. et al. 1999; Knutti D. et al. 2001). However, the precise molecular mechanism by which PGC-1s replace corepressors with coactivators is unclear. Taken together these data and our results on PGC-1β and PP4R1, we propose that activation and phosphorylation of transcription factors such as SREBP leads to recruitment of PGC-1β, which further recruits PP4. Recruited PP4 may dephosphorylate and inactivate HDAC3 resulting in the disassociation of HDAC3 from the protein complex, which is required for the recruitment of HAT activity containing coactivators such as SRC-1 and p300. Recruitment of HATs acetylate histone within chromatin and ultimately results in an increased transcriptional

activation. However, as reviewed in the introduction, HATs add acetate groups to histone proteins within chromatin, but they do not possess energy to break bonds that connect histone and DNA together. How does this transcriptional protein complex unwind DNA for the access of the transcriptional machinery? This question might be answered by another protein identified in our screen, BAF60a.

BAF60a is a subunit of SWI/SNF protein complex, an ATPase containing chromatin remodeling family. Mammalian SWI/SNF complex consist of about 15 subunits and fall into two classes: catalytic subunit BRM/BRG1 ATPase and BRG1-associated factors (BAFs) bound to ATPase subunit. BAFs, such as including BAF57, BAF 60a, and BAF60c, have been indicated to be involved in targeting of SWI/SNF complex to specific promoter in specific tissue via interaction with nuclear receptors or transcription factors. For instance, SWI/SNF coactivates GR and PPARα through BAF60a (Hsiao P.W. et al. 2003) and PPARγ through BAF60c (Debril M.B. et al. 2004). BAFs recruitment to specific promoters is not only through interaction with transcription factors, but also through direct interacting with coactivator proteins. For example, SWI/SNF coactivates ERα through BAF57 recruited to the promoter of estrogen-responsive genes by transcriptional coactivator SRC (Belandia B. et al. 2002). Importantly, very recent research has identified that BAF 60a interacts with PGC-1α in genome-wide coactivation screen for globally identifying transcriptional partners of PGC-1α (Li S. et al. 2008). They demonstrated that BAF60a serves as a molecular link between SWI/SNF and hepatic lipid metabolism. PGC-1α mediates

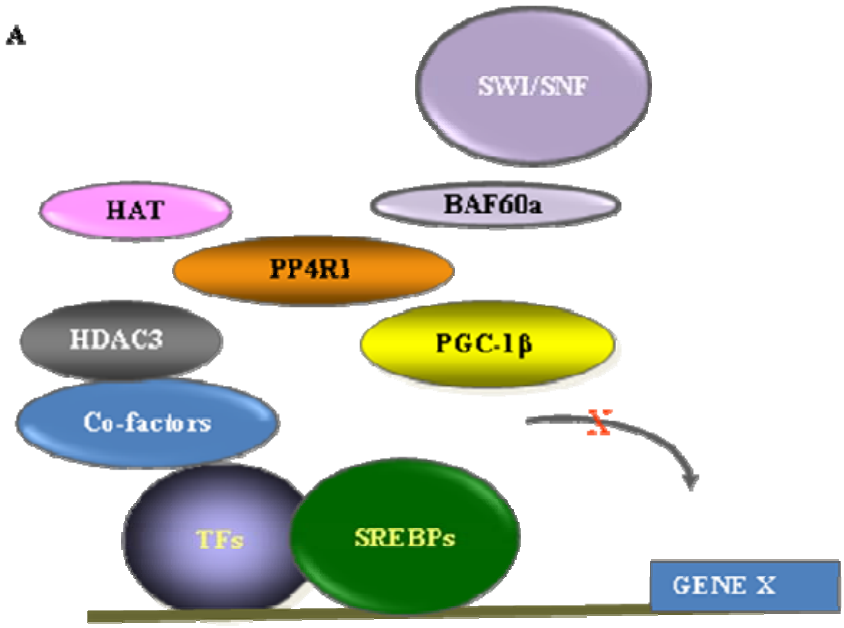
the recruitment of BAF60a to PPARα transcriptional protein complex, leading to increased transcription of PGC-1α-mediated hepatic lipid metabolic genes. In addition to interaction of PGC-1α and BAF60a, they also detected the interaction between PGC-1β and BAF60a in co-immunoprecipitation experiments. This research strongly supports that our screen system is valid and that the interaction of PGC-1β and BAF60a is biologically relevant. The interaction of PGC-1β and BAF60a may further reveal the mechanism of how PGC-1β unwinds DNA after histone acetylation.

The PGC-1β coactivator has been indicated to be a crucial regulator in energy metabolism and diverse biological processes. However the underlying mechanism of PGC-1β coactivating nuclear receptors or transcription factors to regulation gene transcription is unclear. Our data of the identification of PP4R1 and BAF60a might reveal a general mechanism of PGC-1β coavtivation. In case of SREBP-mediated lipogenesis, research has suggested that insulin-like growth factor-1 (IGF-1) induces phosphorylation of SREBPs, which decreases the recruitment of an HDAC3 corepressor complex and results in increased lipid uptake and synthesis (Arito M. et al. 2008). It is known that PGC-1β is a key coactivator of SREBP in the regulation of lipogenesis. The transcriptional activity of SREBPs is inhibited by HDAC3 (Arito M. et al. 2008), and HDAC3 is inactivated and dephosporylated by PP4 (Zhang X. et al. 2005), which suggests that PP4 might be a partner of SREBP-HDAC3 complex. PGC-1α can release corepressors including HDAC and recruit HAT-containing

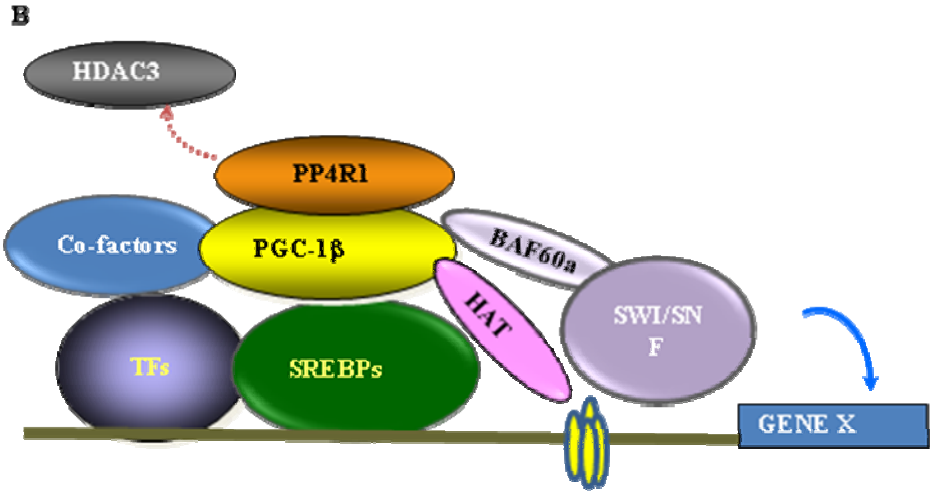
coactivators including SRC-1 and p300. The SWI/SNF complex can recognize and attach to acetylated lysine residues in histone core and unwind DNA using the energy they generated to facilitate the access of transcription factors. Our preliminary data fills these gaps since PGC-1β potentially interacts with SREBPs, PP4R1, and BAF60a. Taken together, we proposed a model shown in Figure 12. It is likely that transcriptional factors such as SREBP is associated with HDAC3 when it is inactivated during conditions such as fasting. When it is activated by hormones such as insulin or other signals, SREBPs recruit PGC-1β, which in turn recruits phosphatase PP4 via a protein-protein interaction manner. PP4 may then dephosphorylate HDAC3 and decrease its activity or even cause the release of HDAC3 from the complex. Consequentially, the release of HDAC3 leads to increased recruitment coactivators including HATs (SRC and p300) and SWI/SNF (BAF60a), which modify and remodel the chromatin structure leading to the increase of the transcriptional activity of SREBP.

Our data suggest that PGC-1β interacts with PP4R1 and BAF60a. PP4 is known to regulate the activity of a transcriptional corepressor HDAC3, a key inhibitory component of many transcription complexes. Modification of HDAC3 by PP4 switches transcriptional complexes from the inhibitory state to the active state by recruitment of HATs and SWI/SNF. Understanding the molecular mechanisms by which PGC-1β interacts with PP4R1 and BAF60a and the biological functions of their interaction may lead to a comprehensive understanding of the mechanism and

the biological function of PGC-1β and its role in metabolic syndrome. This research will facilitate finding potential new therapeutic targets for the management of metabolic syndrome.



12-A. Before PGC-1β is recruited



12-B. After PGC-1β is recruited

Figure 12. A proposed model for the interaction of PGC-1β, PP4R1, and BAF60a, and its effects on the transcriptional function of PGC-1β. A. Before PGC-1β is recruited. Before PGC-1β is recruited to the activated transcription factor, the inactivated form of transcription factors such as SREBP was interacted with HDAC-containing repressor complex, which suppress the transcriptional activity of SREBPs. B. After PGC-1β is recruited. After the transcription factor was activated, PGC-1β was recruited to the activated SREBPs. PGC-1β further recruits PP4, which interact with and dephosphorylate HDAC3 leading in the release of repressor complexes and the recruitment of HATs and SWI/SNF complex, which remodel histones and chromatin and turn transcription on.

FUTURE DIRECTIONS

In the future, we are going to (1) further confirm the physical interaction of PGC-1β and PP4R1, and that of PGC-1β and BAF60a using co-immunoprecipitation; (2) determine the role of PP4R1 and BAF60a in the regulating the transcriptional function of PGC-1β, such as determine whether PP4R1 and BAF60a participates the function of PGC-1β in the induction of its target genes' expression using a reporter gene assay; determine whether endogenous PP4R1 and BAF60a regulates the transcriptional function of PGC-1β and the expression of its target genes by silencing endogenous PP4 of primary hepatocytes; determine the role of PGC-1β/PP4-R1 interaction in regulating the activity of HDAC3; (3) determine the changes of the phosphorylation status of HADAC3 and PGC-1β, and the sites of serine/threonine on HDAC3 or PGC-1β dephosphorylated by PP4; (4) determine the downstream biological functions such as the altered lipid accumulation in hepatocytes, and (5) investigate the signaling pathway that induce the PGC-1β to recruit PP4 and BAF60a. For interaction of PGC-1β and COUP-TF1, experiments to detect their physical interaction and to discover the functional role of this interaction might also help us to understand some physiological importance of PGC-1β and its associated diseases.

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