Identification of Phf16 and Pnpla3 as new adipogenic factors regulated by phytochemicals

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Abstract

Adipocyte differentiation is controlled by multiple signaling pathways. To identify new adipogenic factors, C3H10T1/2 adipocytes were treated with previously known antiadipogenic phytochemicals (resveratrol, butein, sulfuretin, and fisetin) for 24 hours. Commonly regulated genes were then identified by transcriptional profiling analysis. Three genes (chemokine (C-X-C motif) ligand 1 [*Cxcl1*], heme oxygenase 1 [*Hmox1*], and PHD (plant homeo domain) finger protein 16 [*Phf16*]) were upregulated while two genes (G0/G1 switch gene 2 [*G0s2*] and patatin-like phospholipase domain containing 3 [*Pnpla3*]) were downregulated by these four antiadipogenic compounds. Tissue expression profiles showed that the *G0s2* and *Pnpla3* expressions were highly specific to adipose depots while the other three induced genes were ubiquitously expressed with significantly higher expression in adipose tissues. While *Cxcl1* expression was decreased, expressions of the other four genes were significantly increased during adipogenic differentiation of C3H10T1/2 cells. Small interfering RNA–mediated knockdown including Phf16 and Pnpla3 indicated that these genes might play regulatory roles in lipid accumulation and adipocyte differentiation. Specifically, the silencing of two newly identified adipogenic genes, Phf16 or Pnpla3, suppressed lipid accumulation and expression of adipocyte markers in both 3T3-L1 and C3H10T1/2 cells. Taken together, these data showed previously uncovered roles of Phf16 and Pnpla3 in adipogenesis, highlighting the potential of using phytochemicals for further investigation of adipocyte biology.

1 | INTRODUCTION

Adipocyte serves as an energy reservoir by storing excess energy in the form of triglycerides.[1] It also acts as an endocrine organ to regulate whole-body energy homeostasis by secreting fatty acids and adipokines including leptin, adiponectin, and tumor necrosis factor α (TNF-α).[2–4] The generation of adipocytes can be dictated by transcriptional cascades including peroxisome proliferator-activated receptor γ (Pparγ) and CCAAT/ enhancer-binding proteins (C/ebpα).[5] Pparγ also regulates whole-body glucose metabolism. It is a direct target of thiazolidinediones (TZD), a class of drugs used to treat type 2 diabetes. [5] The extracellular signal-regulated kinases (ERK)/cyclin-dependent kinase 5 (Cdk5) axis also controls diabetogenic actions of Pparγ by affecting its phosphorylation status,[6–8] suggesting that selective modulation on Pparγ activity can provide therapeutic potentials against insulin resistance. Therefore, there is a need to identify new factors that act on adipogenesis to further understand adipocyte biology. Such new factors might show potential to treat type 2 diabetes.

Phytochemicals identified from natural products have been widely studied for their effects on obesity and molecular mechanisms involved in their effects.[9,10] For example, epigallocatechin gallate, a phenolic antioxidant found in green and black tea, can inhibit adipocyte proliferation and increase fatty acid oxidation.[11] Resveratrol from red wine exhibits inhibitory effects on lipid accumulation with stimulatory actions on lipid oxidation. [12] Fisetin, sulfuretin, and butein from *Rhus verniciflua* stokes have numerous biological activities including antiadipogenic effects.[13,14] They have antiadipogenic actions on adipocyte differentiation by inhibiting lipid accumulation and reducing expression of adipogenic markers in multiple adipogenic cell lines.[15] It has been demonstrated that resveratrol can mediate antiadipogenic effects by activating Pparγ through sirtuin 1 (SIRT1) and Pparγ coactivator 1α (PGC-1α).[16] Similarly, mammalian target of rapamycin complex 1 or SIRT1 targeted by fisetin and Prdm4 targeted by butein have been identified as the axis for their antiadipogenic effects. We have previously identified complement factor D as an adipogenic modulator in a transcriptional profiling assay using proadipogenic small molecules.[19] These studies indicate that antiadipogenic phytochemicals are useful tools to uncover genes involved in adipogenesis.

The aim of this study was to identify genes regulated by four antiadipogenic phytochemicals (resveratrol, butein, sulfuretin, and fisetin). Transcriptional profiling assays of these antiadipogenic phytochemicals revealed five commonly regulated genes including chemokine (C-X-C motif) ligand 1 (*Cxcl1*), heme oxygenase 1 (*Hmox1*), PHD (plant homeo domain) finger protein 16 (*Phf16*), G0/G1 switch gene 2 (*G0s2*), and patatin-like phospholipase domain containing 3 (*Pnpla3*). Of these five genes, the roles of Phf16 and Pnpla3 in adipocyte differentiation are currently unknown. Thus, we focused on Phf16 and

Pnpla3 to investigate their roles in adipogenesis. Our results showed that Phf16 and Pnpla3 regulated by antiadipogenic phytochemicals could modulate adipocyte differentiation. We found that Pnpla3 was a downstream target of Pparγ whereas Phf16 could facilitate Pparγ activity on its target promoters. These data provide evidence for unknown roles of phytochemically regulated Phf16 and Pnpla3 in adipogenesis, further suggesting the potential of using phytochemicals to understand adipocyte biology.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Mouse C3H10T1/2 (CCL-226) and 3T3-L1 (CL-173) cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco modified Eagle medium (DMEM) (SH30243.01; Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (SH30397.03; Hyclone) and penicillin/streptomycin (SV30010; Hyclone) as described previously.[20] 3T3-L1 preadipocytes were maintained in DMEM supplemented with 10% fetal calf serum (SH30401.01 l; Hyclone). C3H10T1/2 cells were maintained in DMEM supplemented with 10% FBS. To induce adipocyte differentiation of 3T3-L1 preadipocytes, cells were seeded in six-well tissue culture plates and induced to adipocytes by differentiation medium (1 μM dexamethasone [D-2915; Sigma, St Louis, MO], 0.5 mM 3-isobutyl-1-methylxanthine [I-7018; Sigma], and 5 μg/mL insulin [I-0516; Sigma] in DMEM containing 10% FBS) for 2 days after confluence. DMEM containing 10% FBS and 5 μg/mL insulin was refreshed every 2 days up to 6 to 8 days. For C3H101/2 adipocytes, 20 nM GW1929 (G5668; Sigma) was further supplemented during the differentiation period.

Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (Affymetrix, Inc, Cleveland, OH) assay. Briefly, 3T3-L1 cells were seeded into 96-well plates and cultured. After reaching confluence, cells were treated with 0.1, 1, 5, 10, 20, and 40 μM of butein, sulfuretin, resveratrol, and fisetin in triplicates. After 24 hours, MTT (5 mg/mL in phosphate-buffered saline [PBS]) was added and formazan crystals were measured at 520 nm using a microplate reader.

2.2 | Expression analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA samples were separated on agarose gels and relatively sharp 28S and 18S ribosomal RNA bands were verified. The purity and integrity of RNA samples were further assessed using a spectrophotometer. RNA samples with A_{260}/A_{280} ratios close to 2.0 (range, 1.8 to 1.9) were used in the expression analysis. Total RNA (500 ng) and random primers were reverse transcribed using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) at 37°C for 50 minutes. Complementary DNA (cDNA) was heat inactivated at 70°C for 10 minutes and amplified with a Thermal Cycler Dicer (Takara, Shiga, Japan) using THUNDERBIRD SYBR qPCR Mix (Toyobo). The cycle threshold (C_t) was used to calculate differences between target gene C_t value and control gene (Rplp0) C_t value for each sample, where C_t = C_t (target gene) − C_t (control gene). Relative expression level was calculated using $2^{-\Delta\Delta C_t}$. Gene-specific primers were described previously.[15,19]

For microarray analysis, total RNAs from C3H10T1/2 adipocytes treated with 10 μM of butein, sulfuretin, resveratrol, or fisetin for 24 hours were prepared using RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). cDNA preparation and hybridization to Affymetrix Mouse Genome Arrays (430 version 2.0) were performed by GenoChek (Ansan, Korea). Data were analyzed using GeneSpring GX 7.3 software (Agilent Technologies, Santa Clara, CA).

2.3 | Oil Red O staining

Differentiated adipocytes were fixed with 4% paraformaldehyde in PBS at room temperature for 4 hours and then stained with 0.5% Oil Red O (Sigma) in a mixture of isopropanol and distilled water (at 3:2 ratio) for 45 minutes. To quantify intracellular triglyceride content, stained cells from at least three independent experiments were resolved with isopropanol and measured with a spectrophotometer at 520 nm.

2.4 | Knockdown experiments

Small interfering RNAs (siRNA) against candidate genes were purchased from Genolution Pharmaceutical Inc (Seoul, Korea). Sequences included the following: *Pnpla3* si#1: 5′-CCGUUCUUCAACAUUAACAUU-3′, *Pnpla3* si#2: 5′-CAAAUUAUAGUUACAAAUAUU-3′; *Phf16* si#1: 5′-GCUAUUACCUCUUUACUGAUU-3′, *Phf16* si#2: 5′-CUAGAAGAAGAAUUCUAUAUU-3′; *Cxcl1* si#1: 5′-CCUAUUUAUUUAUGUAUUUUU-3′, *Cxcl1* si#2: 5′- GAGAUA GAGUUUAGUAUUAUU-3′; *G0s2* si#1: 5′-GCUAUCAC UUUGCAUUAGAUU-3′, *GOs2* si#2: 5′- GGCUUUUUAU ACAGUU AUUUU-3′; *Hmox1* si#1: 5′-CUAACUU CUGU GUGAAAUAUU-3′, and *Hmox1* si#2: 5′-CUCUGUAAGG GAGAAUCUUUU-3′. The sense sequence of nonspecific scramble RNA as control was 5′-CCUCGUGCCGUUCC AUCAGGUAGUU-3′. C3H10T1/2 or 3T3-L1 cells in serum-free media were transfected with two independent siRNAs using Lipofectamine RNAiMAX (13778-075; Invitrogen). siRNA at a concentration of 30 nM was transfected into 50% confluent C3H10T1/2 or 3T3-L1 cells. At 16 hours after transfection, medium was replaced with fresh medium containing 10% FBS. At 48 hours posttransfection, cells were differentiated into adipocytes for 6 to 8 days followed by Oil Red O staining and gene expression analysis. To repress expression of Ppar𝛾 messenger RNA, short hairpin RNAs (shRNAs) were introduced into 3T3-L1 cells using retroviral transduction as described previously.[21] Target sequences for *Ppar𝛾* were 5′-GTTTGAGTTTGCTGT-GAAGTT-3′. Phoenix cells were transfected with pBabeshGFP or shPpar𝛾 retroviral vector. Viruses were collected at 48 hours posttransfection. Target cells were infected with viruses for 24 hours and then selected with puromycin for 2 weeks.

2.5 | Luciferase assay

Ppar responsive element (3XPPRE) containing luciferase vector, Ppar𝛾, and RXR vector were cotransfected with Phf16 expressing vector or an empty vector into 293A cells using Lipofectamine 2000 (Invitrogen). For luciferase reporter assay, luciferase vector (100 ng), Ppar𝛾 vector (100 ng), RXR vector (10 ng), Phf16 expressing vector (100 ng), or empty vector, 1 μL Lipofectamine 2000, and 50 μL Opti-MEM (Gibco, Grand Island, NY) were used for transfection in each well of a 24-well plate. At 48 hours posttransfection, cells were harvested and reporter gene activity was measured using Dual-luciferase Reporter Assay

System (Promega, Madison, WI). Pparγ ligands (Rosiglitazone 0.5 μM; GW1929 100 nM) were added and incubated for 24 hours before cell harvest. Luciferase activity was normalized by Renilla luciferase activity.

2.6 | Statistical analysis

All statistical analyses were conducted using PASW Statistics 17 (SPSS Inc, Chicago, IL). Data are presented as mean ± SD or SEM. Statistical differences of gene expression and lipid accumulation were analyzed using one-way analysis of variance or two-tailed unpaired Student t test (PASW Statistics 17, SPSS Inc., Chicago, IL). Statistical significance was defined at $P < 0.05$.

3 | RESULTS

3.1 | Identification of adipogenic genes regulated by phytochemicals

We performed MTT assays to select optimal doses of four antiadipogenic phytochemicals (butein, sulfuretin, resveratrol, and fisetin) in C3H10T1/2 cells (Figures 1A and 1B). Treatments with these phytochemicals up to 20 μM for 24 hours did not significantly affect cell viabilities. All four phytochemicals at 40 μM exhibited slight but significant cellular toxicity (Supporting Information Figure S1). Based on these data, concentration of 20 μM or lower was chosen to analyze actions of these phytochemicals in adipogenesis.

To identify new adipogenic genes, we performed transcriptional profiling analysis for C3H10T1/2 adipocytes treated with 10 μM of four known antiadipogenic phytochemicals (resveratrol, butein, sulfuretin, and fisetin) for 24 hours (Figure 1C). Numerous genes controlled by a single compound were identified but *Pparγ* expression is not affected by any of the phytochemicals in this analysis (Figures 1D and 1E). Multiple signaling pathways are known to be involved in the regulation of adipocyte differentiation. Thus, we hypothesized that genes commonly controlled by these four phytochemicals might have direct effects on adipocyte differentiation. Three genes, *Cxcl1*, *Hmox1*, and *Phf16*, were induced by at least 1.7 folds and two genes, *G0s2* and *Pnpla3*, were downregulated by at least 0.6 folds by any of the four antiadipogenic compounds (Figure 1D). We then verified the differential expression of these five genes by real-time quantitative polymerase chain reaction (qPCR) in C3H10T1/2 adipocytes (Figure 1E).

3.2 | Expression profiles of these phytochemically regulated genes

We considered that if these genes were involved in the regulation of adipocyte differentiation, expression of these genes would change over the period of differentiation. Thus, we investigated the expression of these five genes during adipocyte differentiation of C3H10T1/2 adipocytes. We found that expression levels of *Hmox1*, *Phf16*, *G0s2*, and *Pnpla3* were increased during adipocyte differentiation whereas the expression level of *Cxcl1* was decreased at the beginning of differentiation but moderately increased during the remaining differentiation period of C3H10T1/2 adipocytes (Figure 2A). We also examined tissue distribution of these genes. *Hmox1*, *Phf16*, *G0s2*, and *Pnpla3* were expressed in adipose tissues, showing the highest expression in inguinal white adipose tissue (iWAT). Expression of *G0s2* and *Pnpla3* were found to be highly specific to adipose depots (iWAT,

epididymal WAT [eWAT], and brown adipose tissue [BAT]) while the other three induced genes were ubiquitously expressed, yet with significant expression in adipose tissues. Expression of *Cxcl1* in adipose tissue showed relatively lower expression levels than the other four genes (Figure 2B).

3.3 | Roles of Cxcl1, Hmox1, Phf16, G0s2, and Pnpla3 in adipocyte differentiation

To investigate the roles of Cxcl1, Hmox1, Phf16, G0s2, and Pnpla3 in adipocytes, we silenced their expression in 3T3-L1 using siRNA and assessed lipid accumulation based on Oil Red O staining (Figure 3A). Silencing of *Phf16*, *G0s2*, *Pnpla3*, and *Cxcl1* knockdown reduced lipid accumulation but *Hmox1* knockdown induced lipid accumulation, validating our approach for identifying genes involved in adipogenesis by using phytochemicals as tools (Figure 3A and 3B)

3.4 | Proadipogenic functions of Phf16 and Pnpla3 during adipocyte differentiation

Roles of Phf16 and Pnpla3 in adipogenesis are currently unknown. Thus, we further investigated the function of Phf16 and Pnpla3 in adipocyte differentiation. Our data showed that silencing of *Phf16* and *Pnpla3* in 3T3-L1 prevented adipocyte differentiation (Figure 3). To further address whether Phf16 and Pnpla3 could contribute to adipocyte differentiation, we used siRNAs to knockdown *Phf16* and *Pnpla3* expression in C3H10T1/2 cells. Consistent with their effects in 3T3-L1 cells, both *Phf16* and *Pnpla3* knockdown in C3H10T1/2 cells reduced differentiation compared with control (nonspecific control siRNA transfection) (Figures 4A and 4B). Furthermore, expression levels of *Pparγ*, the master regulator of adipogenesis, and its target genes cluster of differentiation 36 (*Cd36*), fatty acid binding protein 4 (*Fabp4*), *C/ebpα*, lipoprotein lipase (*Lpl*), and adiponectin were also reduced in Phf16 and Pnpla3 siRNA–transfected cells (Figures 4C and 4 D). These data demonstrate that Phf16 and Pnpla3 are new adipogenic factors.

3.5 | Pnpla3 is a Pparγ downstream gene

Since Pparγ is a coordinator of adipogenesis, it is reasonable to suspect that Phf16 and Pnpla3 might be transcriptional target genes of Pparγ. To this end, we stimulated C3H10T1/2 adipocytes with Pparγ agonists and measured expression levels of *Phf16* and *Pnpla3* along with *Fabp4,* a direct target of Pparγ. As expected, *Fabp4* was significantly induced either by GW1929 or TZD treatment. Interestingly, TZD treatment also resulted in significantly reduced expression of *Pnpla3*, suggesting that *Pnpla3* might be another Pparγ downstream gene. However, the expression of *Phf16* was not affected by GW1929 or TZD treatment (Figure 5A). To complement this, we compared gene expression in Pparγ shRNA–expressing cells and control shRNA–expressing (ctrl) cells. Similar to adipose selective expression, the expression of *Pnpla3* and *Fabp4* was reduced in Pparγ-deficient cells. However, *Phf16* expression was not affected in the absence of Pparγ (Figure 5B). These results suggest that Pnpla3, but not Phf16, is an adipogenic gene acting downstream of Pparγ cascades.

3.6 | Phf16 acts as a coactivator of Pparγ

Phf16 contains a zinc finger motif often found in transcriptional regulators. This observation suggests that Phf16 might regulate adipocyte differentiation by acting as a cofactor for Pparγ. To investigate transcriptional coactivation by Phf16, 293A cells were transfected with 3XPPRE containing luciferase reporter, Pparγ, and RXR. Cells were then exposed to Pparγ ligands (GW1929 and rosiglitazone) in the presence or absence of a Phf16 expressing vector. Cells transfected with Pparγ and RXR expression vectors showed increased luciferase activity in the presence of Phf16 (Figure 6A) or Pparγ ligands (Figure 6B). Reporter activity was more robustly enhanced when both Phf16 and Pparγ ligands are present (Figure 6B). Similarly, Phf16 showed enhanced transcription when a 5.4 kb Fabp4-luciferase reporter was used in the assay, indicating that Phf16 could stimulate Pparγ in the synthetic promoter and upstream promoter region of Fabp4 (Figure 6C). These results strongly suggest that Phf16 may coactivate Pparγ activity to induce target gene expression.

4 | DISCUSSION

Bioactive compounds from natural products including resveratrol, sulfuretin, fisetin, fustin, kaempferol, gallic acid, quercetin, protocatechuic acid, and butein have been widely investigated for their beneficial effects on obesity.[9] In this study, *Cxcl1*, *Hmox1*, *Phf16*, *G0s2*, and *Pnpla3* were found to be genes commonly regulated by four antiadipogenic phytochemicals sulfuretin, fisetin, resveratrol, and butein. In agreement with our findings, previous studies have shown that enhanced Hmox1 expression has a beneficiary effect on adiposity by increasing preadipocytes and reducing enlarged adipocytes.[22,23] G0s2 expression is upregulated during adipocyte differentiation in 3T3-L1 cells while suppression of endogenous G0s2 can reduce adiposity.[24–26] Other reports indicate that Cxcl1 is a dominant chemokine in preadipocytes and downregulated during adipogenesis.[27,28] Cxcl1 can be induced by epidermal growth factor, a secreted peptide closely related to obesity.[29] Pnpla3, a member of patatin-like phospholipases, is upregulated during adipocyte differentiation.[30–32] Phf16 is a member of a family of large proteins containing plant homeo domain (PHD)-type zinc fingers.[33] However, functions of Pnpla3 and Phf16 in adipocyte differentiation remain unclear. Therefore, further analyses may provide insights into the roles of Pnpla3 and Phf16 genes in adipocyte differentiation.

It has been demonstrated that Pnpla3 can function as both lipase and transacylase and that its expression is tightly regulated by nutritious status.[30,34] Although Pnpla3 is involved in lipid metabolism and expressed in the adipose tissue at the highest level,[35] functions of Pnpla3 in adipocyte differentiation have not been reported yet. Here, we observed that Pnpla3 was upregulated during adipocyte differentiation and that silencing Pnpla3 reduced lipid accumulation. Moreover, the expression of Pnpla3 was responsive to Pparγ agonizts, consistent with previous publication.[36,37] However, direct regulation of Pparγ on Pnpla3 transcription should be carefully performed in promoter analysis.

Further, our data suggest that Pnpla3 regulated by Pparγ can also control adipogenesis. There are precedents that a set of Pparγ target genes can also behave as suppressors or inducers of Pparγ and adipocyte differentiation. For example, C/EBPα is a direct target of Pparγ. It can also increase Pparγ expression and adipogenesis. In addition, other Pparγ

target genes such as Hrasl3, Dlk (dual leucine zipper-bearing kinase), adiponectin, G0s2, and chemerin can similarly increase adipogenesis and Pparγ expression.[5,38] Alternatively, since Pnpla2 and Pnpla3 share high homology, they may exhibit similar effects on Pparγ activation. G0s2, an inhibitor of Pnpla2, is a regulator of lipid droplet formation. It can also increase adipocyte differentiation and Pparγ expression.[39] CGI-58, an activator of Pnpla2, promotes adipogenesis. Pnpla3 also induces a reduction in LD size upon coexpression with ABDH5/CGI-58.[39] However, Pnpla2 and Pnpla3 homologous genes are usually regulated in the conflicting direction from each other, suggesting nonredundant or divergent actions of these related genes. Our results showed that Pnpla3 was suppressed by Pparγ activation while its deficiency in cells decreased adipocyte differentiation. By contrast, Pnpla2 is transcriptionally induced by Pparγ.[40] It has been shown that lipolytic products of Pnpla2 can activate Ppar family members in response to cyclic adenosine monophosphate stimulation in brown adipocytes.[41] Furthermore, knockdown of G0s2 or Pnpla3 in this study exhibited similar antiadipogenic effects, implying that G0s2 may not inhibit Pnpla3 activity, unlike its regulatory effects on Pnpla2.[42] Thus, Pnpla3 and Pnpla2 may possess different regulatory effects on Pparγ and adipocyte differentiation. Together, it is obvious that Pparγ regulation by various growth, transcription factors, and lipid metabolic enzymes including Pnpla3 is complicated. This should be further elucidated in the future.

It was expected that antiadipogenic chemicals would reduce the expression of proadipogenic genes. However, we observed that Phf16 induced by antiadipogenic chemicals acted as an adipogenic gene. Of note, all four antiadipogenic compounds utilized in the current study have been shown to be able to affect Wnt or transforming growth factor β (TGF-β) signaling pathways. It is thus possible that the four antiadipogenic compounds might exert effects on TGF or Wnt signaling pathways followed by induction of Wnt or Tgf-target genes. Large numbers of known Wnt-induced target genes including Axin, Sfrp, chemokine receptor 1, and Dickkopf-1 can also act as Wnt suppressors to inactivate Wnt signaling and increase adipogenesis.[43] Similarly, TGF-induced Smad7 negatively controls TGF receptor TβRI activity.[44] Interestingly, Phf16 has been recently shown as a Wnt-induced gene,[45] suggesting that it may either directly or indirectly affect Wnt pathways. Therefore, Phf16 induction by antiadipogenic chemicals may negatively act on Wnt or TGF-signaling pathways, which may in turn affect adipocyte differentiation and Pparγ. In addition to this possibility, Phf16 is a general transcriptional regulator. As such, chemically induced Phf16 may affect a wide range of transcriptional activators or repressors that direct transcriptions toward stimulation of adipogenesis. Additional studies are needed to provide mechanistic explanations for action of Phf16 in adipocyte differentiation.

We also demonstrated that Phf16 controlled adipocyte differentiation. Phf16 (JADE family PHD zinc finger 3) encoding 823-amino acids belongs to a family of small JADE proteins. Roles of Phf14 (JADE1) in cytokinesis and regulation of histone acetylation have been reported.[46–48] JADE1 functions through interaction with other transcriptional partners. Phf16 has PHD zinc finger domains found in transcription regulating proteins.[49,50] Thus, we speculated that Phf16 might act as a coactivator of Pparγ in adipocytes. We found that coexpression of Phf16 with Pparγ and RXR increased Pparγ driven transcription activity, which could be further enhanced in the presence of Pparγ ligand. These data demonstrate that Phf16 could be a component of the protein complex that works cooperatively with Pparγ

to stimulate adipocyte differentiation. A probable direct physical interaction and specificity with Pparγ need to be determined in the future to provide mechanistic explanations for action of Phf16 in adipocyte differentiation.

In summary, we identified previously uncovered roles of Phf16 and Pnpla3 in adipogenesis. Results of this study further highlight the potential use of phytochemicals as tools to further dissect adipocyte biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Abbreviations:

|	
BAT	brown adipose tissue
Cd36	cluster of differentiation 36
Cdk5	cyclin-dependent kinase 5
C/ebpα	CCAAT/enhancer-binding protein α
Cxcl1	chemokine (C-X-C motif) ligand 1
ERK	extracellular signal-regulated kinases
eWAT	epididymal white adipose tissue
Fabp4	fatty acid binding protein 4
G0s2	G0/G1 switch gene 2
Hmox1	heme oxygenase 1
iWAT	inguinal white adipose tissue
Lpl	lipoprotein lipase
Phf16	PHD (plant homeo domain) finger protein 16
Pnpla3	patatin-like phospholipase domain containing 3
Pparγ	peroxisome proliferator-activated receptor γ

| **siRNA** | small interfering RNA |
| **TNF-α** | tumor necrosis factor α |

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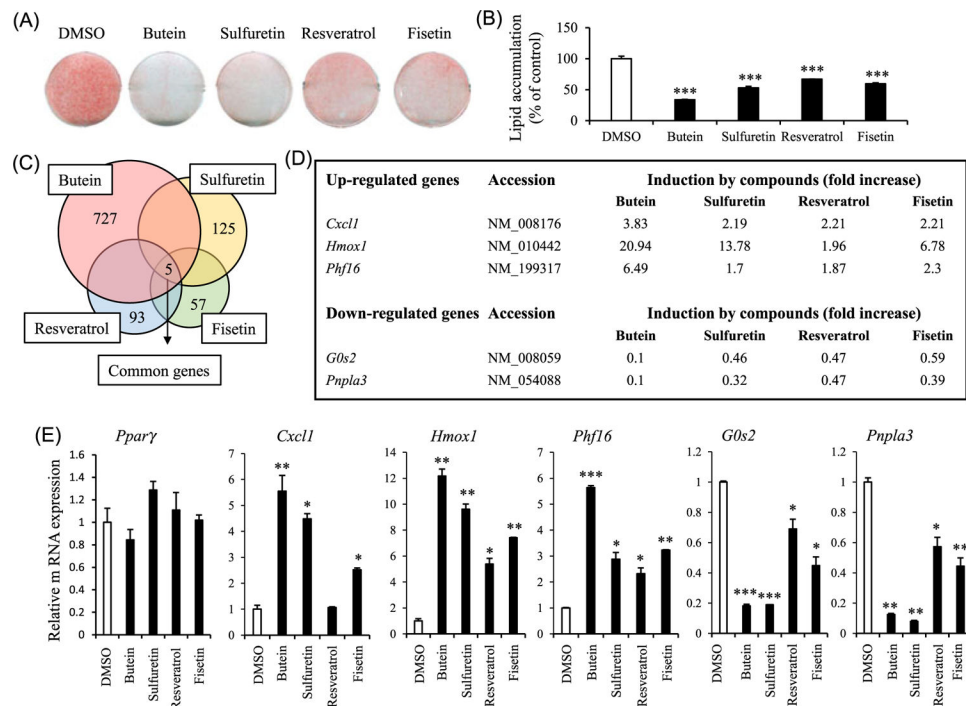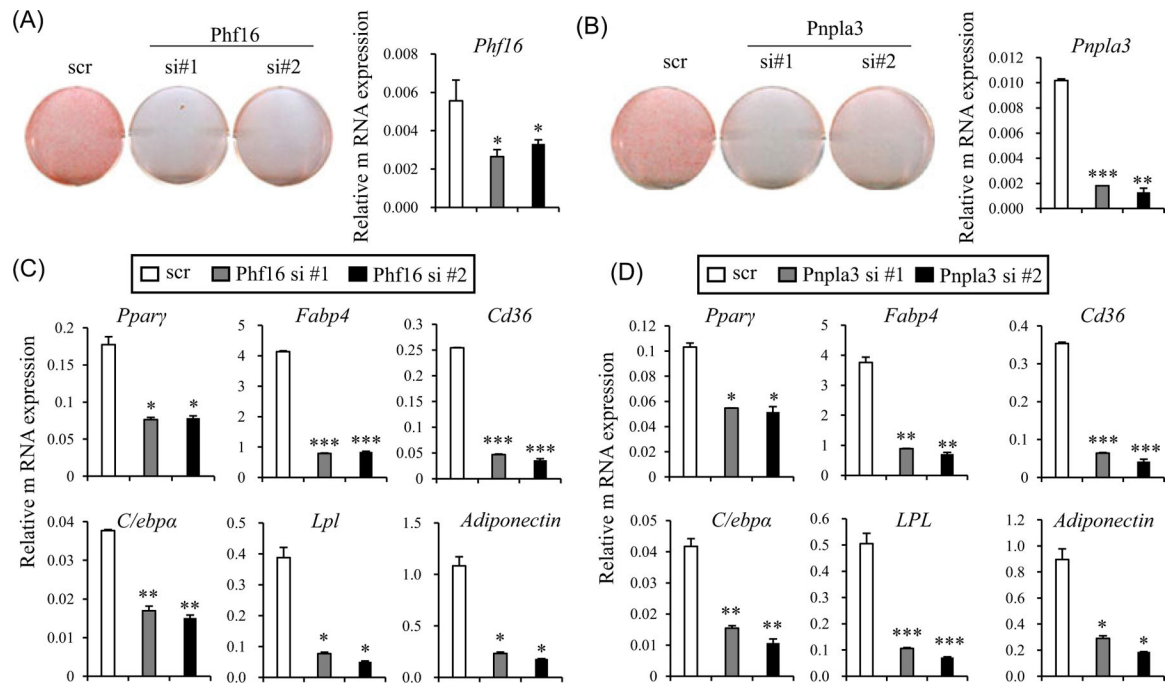
FIGURE 1.

Identification of genes controlled by antiadipogenic phytochemicals. A, Antiadipogenic effects of butein, sulfuretin, resveratrol, and fisetin at 20 μM in C3H10T1/2 cells. B, Lipid accumulation was quantified by measuring the extracted Oil Red O dye at 520 nm. Data shown represent the mean ± SEM from three independent experiments. C, Diagram showing genes commonly induced by antiadipogenic compounds. C3H10T1/2 adipocytes were treated with previously known antiadipogenic phytochemicals (butein, sulfuretin, resveratrol, and fisetin at 10 μM) for 24 hours and commonly regulated genes were identified by transcriptional profiling analysis. D, *Cxcl1*, *Hmox1*, and *Phf16* were induced at least 1.7 folds and *G0s2* and *Pnpla3* were down regulated at least 0.6 folds by the four antiadipogenic compounds. E, Changes in expression of *Pparγ*, *Cxcl1*, *Hmox1*, *Phf16*, *G0s2*, and *Pnpla3* by phytochemicals were verified in C3H10T1/2 cells. C3H10T1/2 adipocytes were treated with compounds for 24 hours and then mRNA expression level was assessed by real-time PCR. Data are expressed as means ± SEM and statistical analysis was performed using one-way ANOVA and Student *t* test (*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$). ANOVA, analysis of variance; Cxcl1, chemokine (C-X-C motif) ligand 1; DMSO, dimethyl sulfoxide; G0s2, G0/G1 switch gene 2; Hmox1, heme oxygenase 1; mRNA, messenger RNA; PCR, polymerase chain reaction; Phf16, PHD (plant homeo domain) finger protein 16; Pnpla3, patatin-like phospholipase domain containing 3; Pparγ, peroxisome proliferator-activated receptor γ
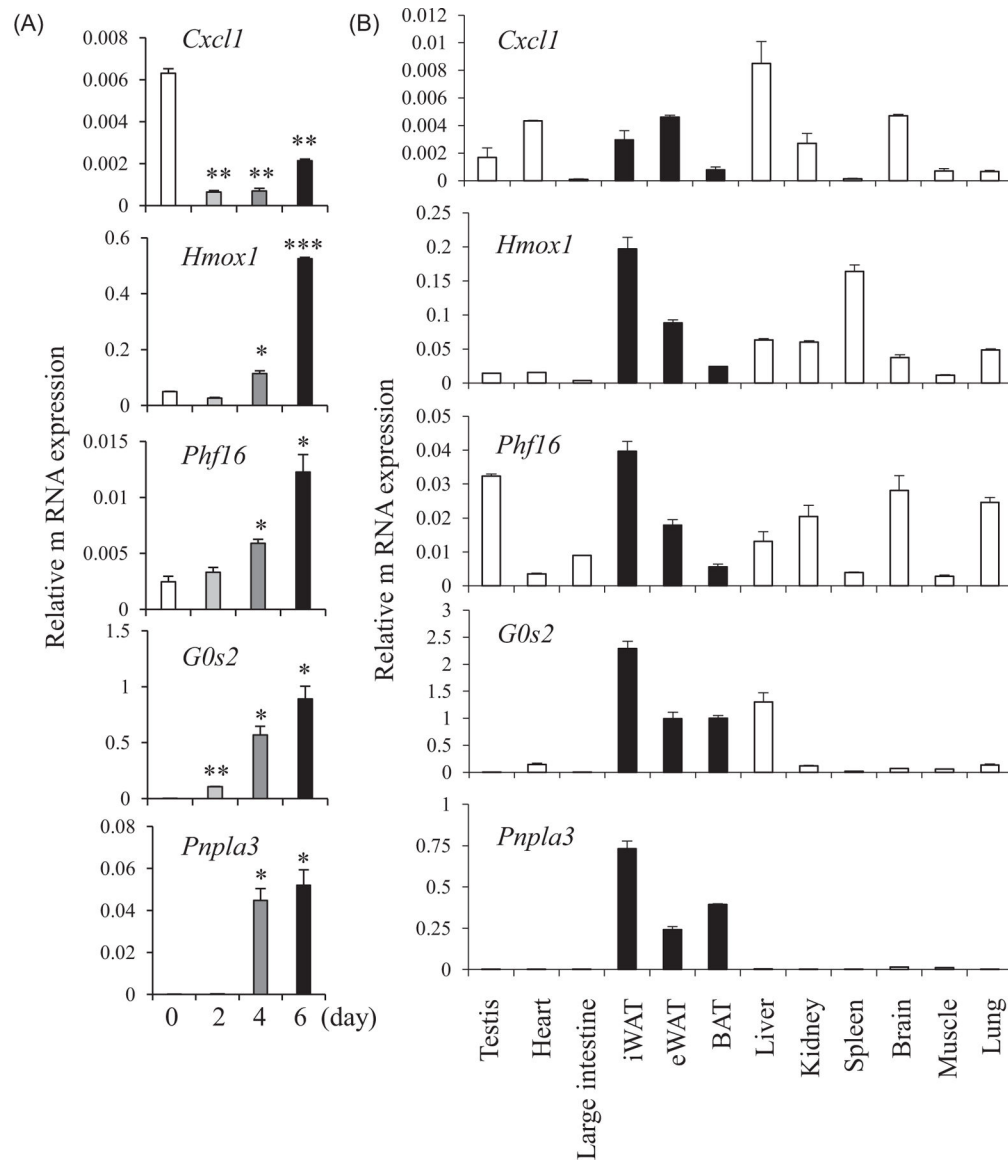
FIGURE 2.
Expression profiles in tissues and during adipocyte differentiation. A, Induction of expression during differentiation of C3H10T1/2 cells treated with differentiation cocktail including dexamethasone, IBMX, and insulin. The mRNA levels were assessed by real-time PCR at various time points for 6 days after treatment. B, Tissue distributions of *Cxcl1*, *Hmox1*, *Phf16*, *G0s2*, and *Pnpla3*. Relative mRNA expression was quantified by real-time PCR ($n = 3$). Data are expressed as means ± SEM and statistical analysis was performed using one-way ANOVA and Student *t* test (*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$). ANOVA, analysis of variance; BAT, brown adipose tissue; Cxcl1, chemokine (C-X-C motif) ligand 1; G0s2, G0/G1 switch gene 2; Hmox1, heme oxygenase 1; iWAT, inguinal white adipose tissue; eWAT, epididymal white adipose tissue; mRNA, messenger RNA; PCR, polymerase chain reaction; Phf16, PHD (plant homeo domain) finger protein 16; Pnpla3, patatin-like phospholipase domain containing 3
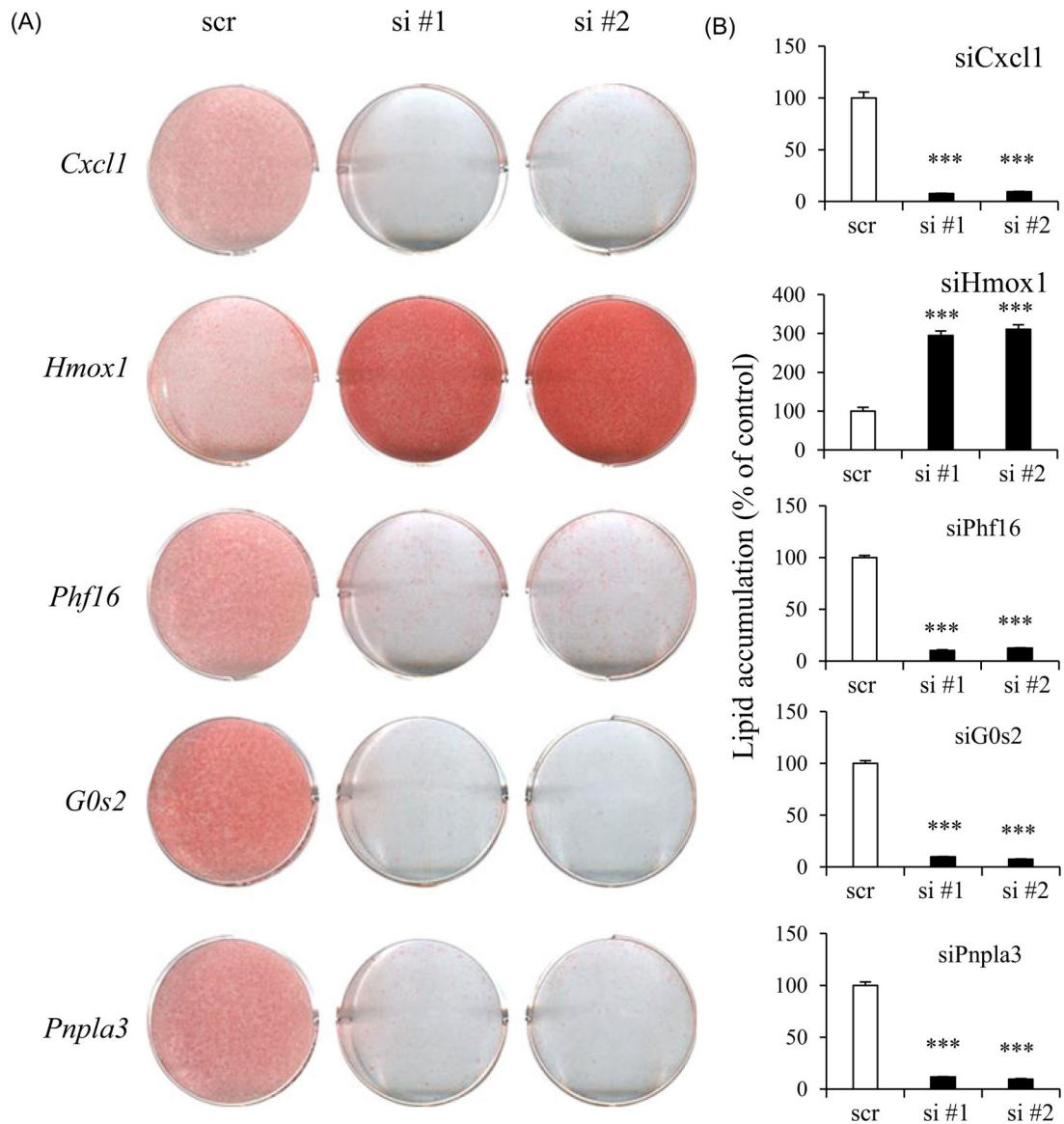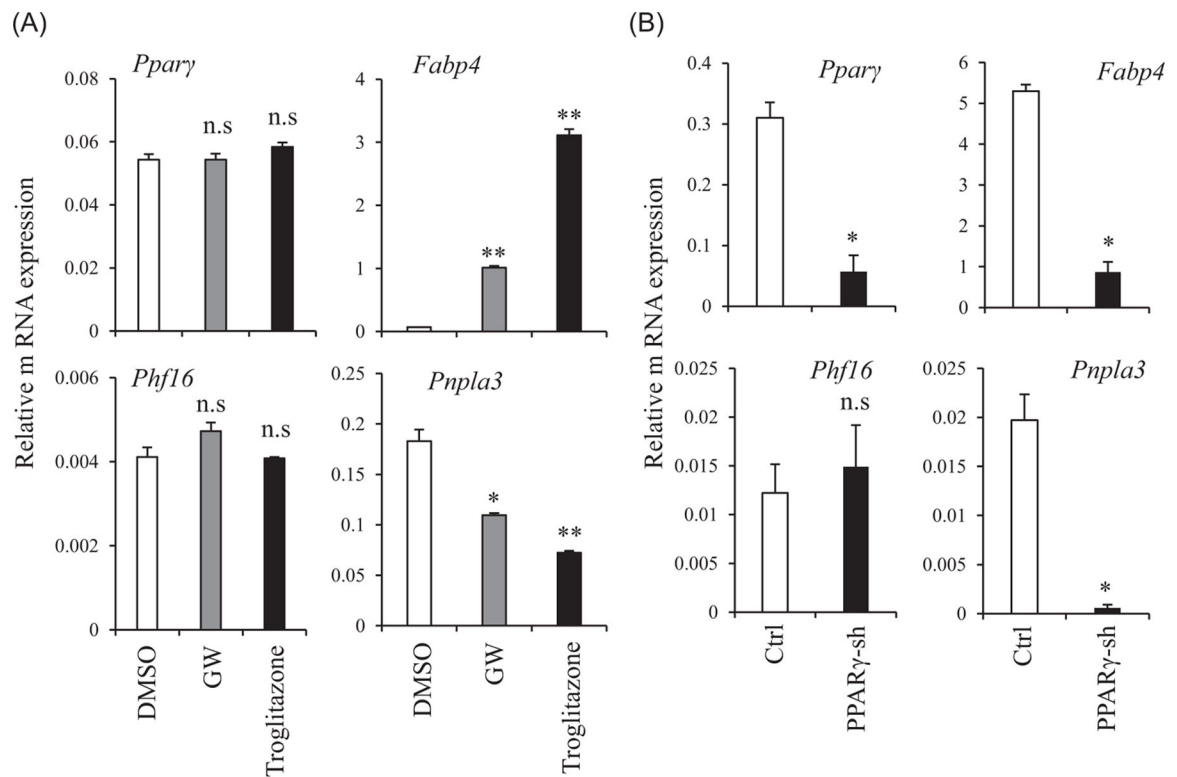
FIGURE 3.

Adipogenic potential of Cxcl1, Hmox1, Phf16, G0s2, and Pnpla3. 3T3-L1 cells were transfected with control siRNA or two independent siRNAs against each gene. A, Differentiation levels were assessed by Oil Red O staining. B, Oil Red O staining of cells on day 6 was quantified by measuring extracted dye at 520 nm. Data are expressed as means ± SEM and statistical analysis was performed using Student t test (***$P < 0.0005$). Cxcl1, chemokine (C-X-C motif) ligand 1; G0s2, G0/G1 switch gene 2; Hmox1, heme oxygenase 1; Phf16, PHD (plant homeo domain) finger protein 16; Pnpla3, patatin-like phospholipase domain containing 3; siRNA, small interfering RNA
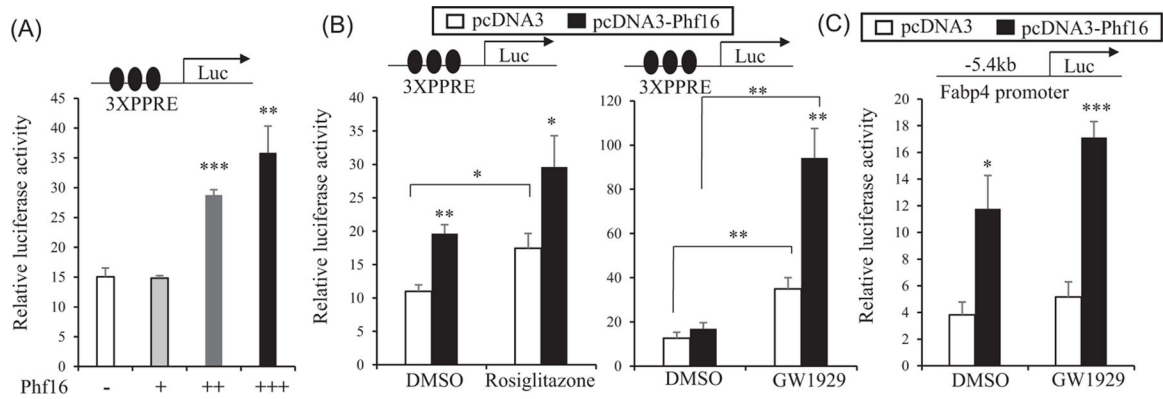
FIGURE 4.

siRNA-mediated silencing of Phf16 and Pnpla3 inhibits adipocyte differentiation. A, C3H10T1/2 cells were transfected with control siRNA or two independent siRNAs against Phf16. Differentiation levels were assessed by Oil Red O staining (left) and Oil Red O staining of cells on day 6 was quantified (right). The cells were differentiated into adipocytes for 6 days. B, C3H10T1/2 cells were transfected with control siRNA or two independent siRNAs against Pnpla3. Differentiation levels were assessed by Oil Red O staining (left) and Oil Red O staining of cells on day 6 was quantified (right). The cells were differentiated into adipocytes for 6 days. C, Gene expression in C3H10T1/2 cells expressing two independent siRNAs against Phf16 was measured. Cells were differentiated into adipocytes for 6 days. D, Expression of adipogenic markers in Pnpla3 siRNA expressing cells was quantified by real-time PCR. Data are representative of the two independent experiments. Data shown are means ± SEM and statistical analysis was performed using one-way ANOVA and Student t test (*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$). ANOVA, analysis of variance; Cd36, cluster of differentiation 36; C/ebpα, CCAAT/enhancer-binding protein α; Fabp4, fatty acid binding protein 4; Lpl, lipoprotein lipase; PCR, polymerase chain reaction; Phf16, PHD (plant homeo domain) finger protein 16; Pnpla3, patatin-like phospholipase domain containing 3; Pparγ, peroxisome proliferator-activated receptor γ; siRNA, small interfering RNA

FIGURE 5.

Pnpla3 is a Pparγ downstream gene. A, Real-time PCR analysis of *Phf16* and *Pnpla3* in C3H10T1/2 preadipocytes treated with 30 nM GW1929 and 10 μM troglitazone for 48 hours. B, Expression of *Phf16* and *Pnpla3* in 3T3-L1 cells stably expressing Pparγ-shRNA and control shRNA (ctrl) was determined by real-time PCR. Data are present as means ± SEM and statistical analysis was performed using Student *t* test (*$P < 0.05$; **$P < 0.005$). 3XPPRE, Ppar responsive element; DMSO, dimethyl sulfoxide; Fabp4, fatty acid binding protein 4; PCR, polymerase chain reaction; Phf16, PHD (plant homeo domain) finger protein 16; Pnpla3, patatin-like phospholipase domain containing 3; Pparγ, peroxisome proliferator-activated receptor γ; shRNA, short hairpin RNa

FIGURE 6.

Phf16 is a positive transcriptional cofactor of Pparγ. A, Phf16 promotes Pparγ/RXR activation of luciferase driven by minimal 3XPPRE. Cells were transfected with Pparγ/RXR with various amounts of Phf16. B, Analysis of 3XPPRE-luciferase reporter activity by coexpressing Pparγ/RXR and Phf16 in 293A cells treated with DMSO, 0.5 μM rosiglitazone, or 100 nM GW1929. C, Fabp4 promoter/enhancer (−5.4 kb) driven luciferase reporter activity was stimulated in Phf16 and Pparγ/RXR coexpressing cells treated with 100 nM GW1929. Data shown are means ± SEM and statistical analysis was performed using one-way ANOVA and Student t test (*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$). 3XPPRE, PPAR responsive elements; ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; Fabp4, fatty acid binding protein 4; Phf16, PHD (plant homeo domain) finger protein 16; Pparγ, peroxisome proliferator-activated receptor γ