Identification of a domain within PPARγ regulating expression of a group of genes containing FGF21 that are selectively repressed by SIRT1 in adipocytes.

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Running Title: PPARγ-responsive genes and SIRT1.
Abstract

Peroxisome proliferators-activated receptor γ (PPARγ) activity is regulated through association with ligands that include the thiazolidinedione class of anti-diabetic drugs as well as derivatives of polyunsaturated fatty acids. Induction of PPARγ target gene expression involves ligand-dependent reconfiguration of the ligand-binding domain (LBD) followed by recruitment of specific transcriptional coactivators. In this study, we have identified an amino acid (F372) within helix 7 of the LBD that is required for the response of PPARγ to endogenous ligands. Additionally, the data show that this amino acid is also required for expression of a novel subset of adipocyte genes (group 2) including FGF21 and that the FGF21 gene is a direct target of PPARγ. Expression of the group 2 genes is selectively repressed by the NAD-dependent deacetylase SIRT1 in mature 3T3-L1 adipocytes since knockdown of SIRT1 through the constitutive expression of a corresponding RNAi enhances their expression without affecting expression of classic adipogenic genes such as adiponectin and FABP4/aP2. It appears that many of the group 2 genes repressed by SIRT1 in mature adipocytes correspond to the same set of genes that are selectively activated by treatment of fat cells with the PPARγ ligand, troglitazone. These data support a role for helix 7 of the LBD of PPARγ in regulating adipocyte function and suggest that inhibition of SIRT1 in adipocytes induces the same insulin-sensitizing action as PPARγ ligands.
Introduction

Peroxisome proliferators-activated receptor γ (PPARγ) is a nuclear receptor expressed in many tissues but is most abundantly produced in adipose tissue where it acts as the master regulator of adipogenesis as well as a regulator of the multiple functions of mature adipocytes (6-8, 21, 32, 37). The transcriptional activity of PPARγ is regulated in part by association with lipophilic ligands that include derivatives of polyunsaturated fatty acids such as eicosinoids as well as the thiazolidinedione class of synthetic insulin sensitizers (19, 20). PPARγ consists primarily of three regulatory domains comprised of a ligand-independent transactivation domain at the N-terminus, a central DNA-binding domain and a C-terminal ligand-binding domain that facilitates ligand-dependent transactivation and heterodimerization with the retinoic acid X receptor (RXR) (18). Heterodimers of PPARγ and RXR bind to DNA consensus sites within the promoters/enhancers of target genes, which consist of direct repeats of the nuclear receptor half site spaced by a single base pair (DR-1). Activation of transcription at these target genes involves a complex process in which the docked PPARγ/RXR heterodimers, following association with ligands, recruits a series of coactivators including the p160/SRC family members that initiate formation of the RNA polymerase II/transcriptional complex involving components of the Mediator complex (11, 24, 29).

Our understanding of the mechanisms by which PPARγ activates transcription has been derived from studies employing synthetic ligands such as thiazolidinediones (TZDs). It is generally accepted that in the unliganded state PPARγ associates with the corepressors NCoR or SMRT to repress target gene expression. Entry of the thiazolidinedione into the large ligand-binding pocket stabilizes helix 12 of the transactivation domain 2 (AF2), which dislodges the
corepressors and forms a binding site for the p160 family of coactivators that facilitates the pharmacological activation of PPARγ target gene expression (24, 27). Recent studies investigating the role of PPARγ in regulating inflammatory genes in macrophages presented an additional model by which thiazolidinediones might organize the recruitment of various nuclear coregulators. In this model, TZDs induce the SUMOylation of PPARγ on K365 within helix 7 of the ligand-binding domain, which targets PPARγ to NCoR/HDAC3 complexes on inflammatory gene promoters (26). These observations suggest that helix 7, in addition to helix 12, might participate in mechanisms by which ligands regulate association of PPARγ with specific coactivators or corepressors. In support of this, our recent studies have identified helix 7 as a component of the functional interaction between β-catenin, the coactivator of the canonical Wnt signaling pathway, and PPARγ (22). Since the endogenous ligand for PPARγ has not as yet been identified, the physiological mechanisms by which PPARγ regulates target gene expression in various cell types are not known.

Differentiation of preadipocytes into adipocytes depends on stimulation of PPARγ activity that is facilitated by a C/EBPβ-associated induction of PPARγ2 gene expression as well as production of endogenous ligands (8). As mentioned above, the physiological ligand for PPARγ has not been identified, but recent studies suggest that signaling pathways involving cAMP, C/EBPβ and xanthine oxidoreductase activate a transient increase in ligand production during the initial 2-4 days of adipogenesis in 3T3-L1 preadipocytes (4, 14, 23, 35). The level/activity of these ligands subsides dramatically during terminal differentiation to the extent that mature adipocytes express low levels of activity. Despite this apparent decrease in endogenous ligand activity, PPARγ is capable of maintaining expression of most of its target genes in mature adipocytes. In this regard, it is interesting that some target genes are expressed
at low levels in adipocytes, but are responsive to activation of PPARγ by TZDs. Specifically, genes coding for glycerol kinase (GyK) and the oxidized LDL receptor (OLR-1) are PPARγ target genes that are normally expressed at low abundance in white adipose tissue and mature adipocytes in culture. Exposure of 3T3-L1 adipocytes to TZDs induces transcription of mRNAs for GyK and OLR-1 (5, 13). Additional studies by Lazar and coworkers (12) have shown that PPARγ is bound to PPAR response elements (PPREs) in the promoter of the transcriptionally inactive GyK gene in mature adipocytes in addition to being bound to the enhancer of the transcriptionally active aP2 gene. The data suggest that endogenous ligands are unable to dislodge corepressors from PPARγ on the GyK gene, but do facilitate this process along with recruitment of p160 coactivators to PPARγ on the aP2 gene. Moreover, it appears that exposure of mature adipocytes to TZDs can activate GyK expression by regulating the switch in corepressor/coactivator recruitment to the PPARγ bound to the corresponding promoter.

In the present study, we investigated mechanisms by which PPARγ might induce expression of select genes in response to different effectors. The data show that PPARγ regulates expression of at least two programs of gene expression during adipogenesis in 3T3-L1 preadipocytes. The one program (group 1) consists of classic adipogenic genes including FABP4/aP2, adiponectin and perilipin and, following its induction; this program continues to be expressed throughout terminal adipogenesis and in mature adipocytes. The other program (group 2) consists of a diverse array of genes some of which appear to be involved in glucose homeostasis and insulin action including FGF21 and the oxidoreductase Ero1-Lα. Expression of these group 2 genes can be selectively activated in mature adipocytes by synthetic PPARγ ligands or suppression of SIRT1 activity. Our studies also show that helix 7 within the ligand-binding domain plays a critical role in the response of PPARγ to endogenous ligands. Mutation
of select amino acids within helix 7 specifically F372 renders PPARγ completely incapable of activating adipogenic gene expression in response to endogenous ligand activity. Exposure of cells expressing the mutant F-PPARγ to thiazolidinediones induces expression of the adipogenic program containing adiponectin and aP2, but is incapable of inducing the program containing FGF21 and Ero1-Lα.

Materials and Methods

Materials: Dexamethasone (DEX), 3-isobutyl-1-methylxanthine (MIX), insulin were purchased from Sigma (St. Louis, MO). Leupeptin, aprotinin, and puromycin were purchased from American Bioanalytical (Natick, MA), while Dulbecco's modified Eagle's medium (DMEM) were purchased from Mediatech, Inc (Herndon, VA), calf serum and TRIzol were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from Gemini Bio-Products and troglitazone was obtained from Biomol International.

Antibodies: Monoclonal anti-PPARγ antibody and polyclonal C/EBPα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), polyclonal aP2 serum was kindly provided by Dr. D. Bernlohr, (University of Minnesota) while anti-perilipin antibody was kindly provided by Dr. A. Greenberg (Tufts University, Boston, MA) and polyclonal anti-ACRP30 (adiponectin) was obtained from Affinity BioReagents (Golden, CO). Anti-Ero1 polyclonal antibody was purchased from Abnova Co., (Taiwan, China).
**Plasmids and cell lines:** Replacement of phenylalanine 372 of PPARγ1 with an alanine was achieved by performing site-directed mutagenesis of the pBabe-WT-PPARγ plasmid using QuickChange II XL kit (Stratagene) following the manufacturers instructions. pSUPER-SIRT1 siRNA plasmid was generously provided by Dr Jim Xiao of Boston University School of Medicine and consisted of the vector recently described (28). Generation of appropriate retrovirus particles was as follows: HEK-293T cells were grown to 70% confluence in 100mm-diameter dishes at which stage they were transfected with the DNA-FUGENE cocktail consisting of 36µL Fugene 6, 6µg retrovirus plasmid, 6µg pVPack-VSV-G vector, 6µg pVPack-GAG-POL vector and 164µL DMEM without FBS. Twenty-four hours later, the medium was replaced with 6mL fresh DMEM containing 10% FBS. One day after that, the culture medium containing high-titer retrovirus was harvested and filtered through a 0.45µm pore size filter. The viral filtrate was used to infect both 3T3-L1 preadipocytes (control and SIRT1 siRNA cells) and Swiss 3T3 fibroblasts (WT-, E-, EF-, F-, DD-PPARγ cell lines).

**Cell culture:** The Swiss fibroblast cell lines expressing wild type (WT) and mutant forms of PPARγ (E, EF, F, DD) generated as previously described (22) and murine 3T3-L1 preadipocytes were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (fibroblasts) or 10% calf serum (preadipocytes) until confluent and were then maintained in the same medium for an additional 2 days. Differentiation was induced at 2 days postconfluence (day 0) by adding fresh DMEM containing 10% FBS, 0.5 mM MIX, 1 µM DEX, 1.67 µM insulin with or without 5 µM troglitazone. The immortilized primary brown preadipocytes (gift of Dr CR Kahn, Joslin Diabetes Center, Boston, (9, 10)) were grown to confluence in differentiation medium composed of DMEM containing 10% FBS supplemented with 20 nM insulin and 1 nM 3,3’,5-triiodo-L-
thyronine [T3]). After 2 days post-confluence, cells were induced to differentiate by exposure to DEX, MIX, insulin, 0.125 mM indomethacin and 10% FBS. Cells were refed every 2 days.

**Microarray Gene Chips:** Swiss WT-PPARγ and EF-PPARγ cells were differentiated in the presence or absence of troglitazone for 5 days as described above. Additionally, control and SIRT1 knockdown 3T3-L1 preadipocytes were differentiated as described above for 0, 4 and 10 days (see supplemental data, Tables S1A and S1B). Total RNA was isolated from all cells using Trizol Reagent (Invitrogen) and microarray analysis was performed by the Microarray Resource (Boston University School of Medicine). Briefly, double-stranded cDNA was synthesized from 10 µg of RNA using SuperScript double-stranded cDNA synthesis kit (Invitrogen) and purified using a Phase-Lock Gel (PLG Heavy Brinkmann Instruments, Westbury, NY). Biotin-labeled cRNA was then generated using RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY) and purified using RNeasy affinity columns (Qiagen). After treatment at 94°C for 35 min in 40 mM Tris-acetate, pH 8.1, 100 mM KOAc, 30 mM MgOAc, 15 µg of fragmented cRNA was hybridized to the Affymetrix GeneChip mouse Expression Set MOE430A2.0 array at 45°C for 16 h and 60 rpm using controls supplied by the manufacturer (Affymetrix). Arrays were then washed and stained according to the standard Antibody Amplification for Eukaryotic Targets protocol (Affymetrix). The stained Gene Chip arrays were scanned at 488 nm using an Affymetrix Gene Chip Scanner 3000 (Affymetrix). The scanned images were then quantified and scaled using Microarray Suite 5.0 software (Affymetrix).

**Oil Red O Staining:** The cells were seeded in 35-mm plates, and at the specified stage of differentiation they were rinsed with PBS and fixed with 10% formalin in PBS for 15 min. After two washes in PBS, cells were stained for at least 1 h in freshly diluted Oil Red O solution (6
parts Oil Red O stock solution and 4 parts H$_2$O; Oil Red O stock solution is 0.5% Oil Red O in isopropyl alcohol). The stain was then removed, and cells were washed twice with water and then photographed.

**Western Blot Analysis of Proteins:** Equal amounts of protein extracted from the total cell layer were fractionated on 8% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (PerkinElmer Life Sciences). Following transfer, the membranes were blocked with 10% nonfat dry milk in phosphate-buffered saline, 0.1% Tween 20 and probed with the antibodies corresponding to the various target proteins indicated in each figure. Horseradish peroxidase-conjugated secondary antibodies (Sigma) and an ECL substrate kit (PerkinElmer Life Sciences) were used for detection of specific proteins.

**Analysis of RNA:** Total cellular RNA was prepared using TRIzol reagent (InVitrogen) according to the manufacturer's instructions. cDNAs were made from equivalent amounts of total RNA by using Reverse Transcription System (Promega) as described previously (33). Primer sequences used for amplification were synthesized (Integrated DNA Technologies Inc., Coralville, IA) to be specific for:

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<th>Reverse Primer</th>
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**Plasmid constructs and Luciferase Reporter Gene Assays:** The mouse FGF21 promoter constructs -1537/+54, -1299/+54, and -553/+54 were generated by PCR using C57BL/6NCrl mouse genomic DNA and the following oligonucleotides: -1537 forward, 5’-AAGCCTCACCTTGACACC-3’; -1299 forward, 5’-CAGGAAACAACCCAGCTC-3’; -553 forward, 5’-AGTGCAGACAAAGTCCCCT-3’; +54 reverse, 5’-GGCAGCTGGAATTGTGTT-3’. The PCR-amplified fragments were cloned into Kpn1 and Bgl2 sites of the luciferase reporter plasmid pGL3. For transfection assays, the Swiss fibroblasts (control expressing a pBabe-puro empty vector) or cells expressing a WT-PPARγ were seeded in 24-well plates in triplicate for 24 hr at which time 500 ng of the FGF21 promoter plasmids or pBabe-PPARγ plasmid plus 20 ng of Renilla luciferase plasmid were transfected into each well using FuGENE 6 (DNA: FuGENE 6 = 1:6). Twenty-four hours later, when appropriate, the cells were treated for 48 hours with 1µM GW1929 and were then washed twice with phosphate-buffered saline and lysed with 100 µl of passive lysis buffer. Luciferase/Renilla assays were performed using the Dual-Luciferase Reporter Assay System kit (Promega, Madison, WI) and a Luminoskan Ascent luminometer (Thermo Labsystems, Franklin, MA). The average ratio (from three wells) of luciferase activity (relative light units) to Renilla activity was calculated. The same experiment was repeated at least three times. The final values/standard deviation was calculated based on all repeats.

**Results**

Our previous investigations demonstrated that four amino acids, E367, F372, D378 and D379, within helix 7 of the ligand-binding domain of PPARγ facilitate a functional interaction between PPARγ and β-catenin (22). To gain insight into the potential involvement of helix 7 in regulating the transcriptional activity of PPARγ during adipogenesis, we expressed a series of mutant PPARγ proteins in Swiss 3T3 fibroblasts in which E367, F372, D378 or D379 were
modified to alanine and assessed their ability to induce adipogenic gene expression. First, we observed that ectopic expression of the WT-PPAR\(\gamma\) was capable of inducing the conversion of these fibroblasts into adipocytes simply by exposure to DEX, MIX and insulin without the need for an exogeneous ligand such as troglitazone (Figure 1A). These data suggest that Swiss fibroblasts produce endogenous ligands that can activate the ectopic PPAR\(\gamma\) following exposure to the normal cocktail of adipogenic inducers. In fact, exposure to troglitazone appears to have no additional effect on the morphological features of these Swiss adipocytes (Fig 1A).

Additionally, the western blot shown in Figure 1B, lanes 1 and 5 shows abundant expression of the adipogenic proteins, C/EBP\(\alpha\), perilipin and aP2, and a low level of \(\beta\)-catenin production in the Swiss-WT-PPAR\(\gamma\) cells induced to differentiate in the presence or absence of troglitazone. The data also show that the relative abundance of transcriptionally active WT-PPAR\(\gamma\) is very low due to its rapid turnover. The mutant PPAR\(\gamma\) corresponding to E367A (E-PPAR\(\gamma\)) retained the ability to induce adipogenesis in the presence or absence of troglitazone (Fig 1A), which included degradation of \(\beta\)-catenin (Fig 1B). It is interesting, however, that this alteration appears to stabilize PPAR\(\gamma\) in the absence of ligand, while exposure to troglitazone results in a significant decrease in its abundance (Fig 1B, compare lanes 2 and 6). The most interesting data came from analyzing expression of the mutant PPAR\(\gamma\) corresponding to E367A and F372A (EF-PPAR\(\gamma\)). Figure 1 demonstrates that mutation of F372 to alanine, in addition to E367A, completely destroys the ability of PPAR\(\gamma\) to respond to an endogenous ligand since Swiss cells expressing EF-PPAR\(\gamma\) remain as fibroblasts (Fig 1A) and do not express adipogenic genes or down regulate \(\beta\)-catenin (Fig 1B). Additionally, this mutant PPAR\(\gamma\) appears to be quite stable. More importantly, exposure of the Swiss-EF-PPAR\(\gamma\) cells to troglitazone induces their conversion into adipocytic cells (Fig 1A) and expression of C/EBP\(\alpha\), perilipin and aP2 (Fig 1B). These data are
consistent with the notion that F372 and E367 within helix 7 participate in the response of PPARγ to endogenous ligands, whereas responses to exogenous synthetic ligands such as troglitazone are less dependent on these amino acids. Mutation of both D378 and D379 to alanine completely destroys the ability of PPARγ to respond to both endogenous and exogenous ligands, since the corresponding mutant PPARγ is incapable of inducing either morphological conversion or adipogenic gene expression (Fig 1A and Fig IB, lanes 4 and 8).

The data presented in Figure 1B suggested to us that analysis of mRNA expression in Swiss WT-PPARγ versus Swiss-EF-PPARγ cells might permit the identification of PPARγ target gene programs responding to endogenous versus exogenous ligands. Consequently, total RNA was harvested from Swiss fibroblasts expressing either WT- or EF-PPARγ proteins 5 days following exposure to the adipogenic inducers in the presence or absence of troglitazone and subjected to oligonucleotide microarray analysis employing affymetrix chips. The data reveal that the abundance of 1767 genes of the ~22,690 represented on the array differed at least 2 fold between the highly differentiated Swiss-WT-PPARγ cells and the undifferentiated Swiss-EF-PPARγ cells (minus troglitazone). A cluster analysis of these genes is shown in Figure 1C in which the genes that are highly expressed in WT-PPARγ cells (minus troglitazone) relative to their expression in EF-PPARγ cells (minus troglitazone) are arranged in descending order of their relative abundance. Genes that are highly expressed in adipocytes (WT-PPARγ) compared to fibroblasts (EF-PPARγ minus troglitazone) cluster together and include those coding for adipogenic, lipogenic and mitochondrial proteins. In contrast, many genes are expressed at much lower abundance in the adipocytes (WT-PPARγ cells) compared to the fibroblasts (EF-PPARγ minus troglitazone) and include components of the Wnt signaling pathway as well as inflammatory proteins, several of which have previously been reported to be down-regulated.
during adipogenesis. Figure S1 in supplement represents the relative abundance of select genes present in each of these clusters and reveals that many of the genes display significantly more than a 5-fold difference in abundance between the two cell types. In fact, some mRNAs such as adiponectin (acdc) and Fsp27 are expressed at least $10^4$ fold more abundantly in the adipocytes (WT-PPARγ) as compared to fibroblasts (EF-PPARγ minus troglitazone). Figure 1C also illustrates that treatment of the WT-PPARγ cells with troglitazone does not significantly alter the overall pattern of gene expression, but appears to enhance the level of adipogenic gene expression whilst suppressing even further the fibroblastic mRNAs (Fig 1C, compare lane 2 with lane 1). More importantly, the EF-PPARγ cells that are completely unresponsive to endogenous ligands (-troglitazone) are extensively induced to express multiple adipogenic, lipogenic and mitochondrial genes following their exposure to troglitazone (Fig 1C, compare lane 3 with lane 4). These EF-PPARγ cells also downregulate expression of the fibroblastic genes in response to troglitazone consistent with them attaining an adipocyte-like morphology (Fig 1A).

**Identification of a subset of PPARγ-responsive genes.**

To gain more insight into the gene programs regulated by PPARγ in response to endogenous vs exogenous ligands, a more detailed analysis of individual genes was performed as shown in Tables S1A and S1B in supplement. We also analyzed the profile of mRNAs expressed during the differentiation of 3T3-L1 preadipocytes for comparison with the mRNAs expressed in Swiss-PPARγ cells by performing additional affymetrix array analysis of mRNAs isolated from the preadipocytes at 0, 4 and 10 days of differentiation. Table S1A lists a selection of classic adipogenic genes that are induced to varying extents during adipogenesis in 3T3-L1 preadipocytes (columns 5, 6 and 7), which include genes coding for proteins involved in lipid storage/metabolism (i.e. FABP4) as well as endocrine functions (i.e. adiponectin). All of these
mRNAs are expressed much more abundantly in Swiss fibroblasts expressing WT-PPARγ compared to cells expressing EF-PPARγ. In fact, the difference in the level of expression of these mRNAs in EF-PPARγ cells versus WT-PPARγ cells is comparable to the difference in their expression in preadipocytes versus mature adipocytes (Table S1A, compare columns 1 and 3 with columns 5 and 7). It is also relevant to point out that expression of at least three genes, Resistin (Retn), Hsd11β1 and Orosomucoid (Orm1) is enhanced by WT-PPARγ in Swiss cells in response to endogenous ligands (minus troglitazone) and during adipogenesis in 3T3-L1 preadipocytes. Interestingly, troglitazone significantly attenuates expression of these genes in WT-PPARγ (Table S1A, Retn, Hsd11b1 and Orm1, compare column 2 with column 1) consistent with reports that thiazolidinediones selectively repress expression of these genes following their dramatic induction during adipogenesis in 3T3-L1 cells (2, 3, 34). Taken together, the data in Table S1A (supplement) are consistent with the notion that PPARγ can induce expression of the majority of the classic adipogenic genes in Swiss fibroblasts in response to an endogenous ligand to the same extent as that occurring during normal adipogenesis in 3T3-L1 preadipocytes. Furthermore, mutation of critical amino acids within helix 7 (EF-PPARγ) prevents PPARγ from responding to endogenous ligand activity (Fig 1C and Table S1A of supplement, column 3). As shown in Figure 1C, however, exposure of EF-PPARγ to troglitazone can induce expression of most of these classic adipogenic genes to levels attained in 3T3-adipocytes (Table S1A of supplement, compare column 4 with column 7).

Following a more extensive analysis of the array data, it was observed that not all genes that are highly expressed in 3T3-L1 or WT-PPARγ adipocytes were induced in the EF-PPARγ cells by troglitazone. In fact, it appears that activation of the mutant EF-PPARγ by the exogenous ligand, while capable of converting these fibroblasts into adipocytic cells that contain
small lipid droplets and express many of the markers of mature adipocytes (Fig 1), is incapable of inducing the entire adipogenic program (Table S1B of supplement). More specifically, the data shown in Table S1B suggest that WT-PPARγ induces expression of a group of responsive genes (columns 1 and 2) that are significantly less responsive to stimulation of EF-PPARγ by troglitazone (column 4). This subset of PPARγ target genes (referred to here as Group 2) includes proteins that have not previously been shown to be associated with PPARγ activity such as the ER oxidoreductase Ero1-Lα, FGF21, and genes coding for components of the glycolytic pathway. Table S1B also shows that some of these genes including Mrap, KLF15, Klb (βKlotho) and Pdxp, are induced several fold during adipogenesis, but are unresponsive to troglitazone activation of EF-PPARγ. Other genes are moderately responsive to adipogenic signals in 3T3-L1 preadipocytes (i.e the glycolytic genes) but almost all of these genes are induced in response to troglitazone activation of WT-PPARγ, but not of EF-PPARγ.

To confirm the oligonucleotide microarray data, a series of RT-PCR analyses were performed in which the relative abundance of select mRNAs expressed in the Swiss cell lines was measured. Since the data presented in Figure 1 suggest that F372 is the amino acid that appears to be influencing the transcriptional activity of PPARγ, we generated an additional cell line corresponding to Swiss fibroblasts expressing PPARγ in which only F372 was changed to alanine (F-PPARγ cells). We also analyzed Swiss fibroblasts that do not contain an ectopic PPARγ and, therefore, are completely incapable of adipogenesis even in the presence of troglitazone (control cells). Figure 2A shows the constitutive expression of the corresponding PPARγ mRNAs in each of the PPARγ-Swiss cell lines and the absence of any PPARγ mRNA in the control cells. The panel on the left demonstrates expression of select target genes from Table S1A that are induced in the WT-PPARγ cells exposed to endogenous (Figure 2A, lane 3) as well
as exogenous (Figure 2A, lane 4) PPARγ ligands. Expression of most of these genes is unaffected by troglitazone with the exception of EPHX2 that appears to be enhanced even further by the exogenous ligand. This set of classic adipogenic genes, as expected, is not expressed in the EF- or F-PPARγ cells in response to the endogenous ligands (Figure 2A, lanes 5 and 7). Furthermore, activation of these mutant cell lines (EF- and F-PPARγ) by exposure to troglitazone significantly induces expression of all of these genes (Figure 2A, lanes 6 and 8) as shown in Table S1A. In contrast, the subset of genes (Group 2) presented in the panel on the right selected from Table S1B responds quite differently to the action of the mutant PPARγ molecules. These genes are induced to varying extents by endogenous ligands in cells expressing WT-PPARγ and are enhanced many fold by exposure to troglitazone (Figure 2A compare lanes 11 and 12 with 9 and 10). More importantly, this subset of genes is unresponsive to activation of EF- or F-PPARγ by troglitazone as well as the endogenous ligands (Figure 2A, compare lanes 13, 14, 15, 16 with lanes 11 and 12). We also performed western blot analysis of C/EBPα, FABP4/aP2, adiponectin (Group 1, EF-PPARγ-responsive genes) and Ero1-Lα (a Group 2, EF-PPARγ-unresponsive gene) to confirm the RT-PCR data. Figure S2 of the supplement, shows that troglitazone stimulation of all forms of PPARγ including WT-PPARγ, E-PPARγ, EF-PPARγ and F-PPARγ lead to abundant expression of C/EBPα, FABP4/aP2 and adiponectin. In contrast, expression of Ero1-Lα is completely unresponsive to troglitazone stimulation of F-PPARγ or EF-PPARγ, but responds to WT- and E-PPARγ activity. It is interesting that analysis of the proteins in the culture media showed that adiponectin is secreted from cells expressing WT- and E-PPARγ, but is absent from the media of F-PPARγ and EF-PPARγ cells. These data suggest that some Group 2 proteins likely participate in processes
responsible for secretion of adiponectin. In fact, recent studies by others and us have
demonstrated a role for Ero1-Lα in regulating secretion of adiponectin from adipocytes (30, 36).

The Group 2 subset of genes can be selectively activated in response to troglitazone during
the differentiation of Swiss 3T3 fibroblasts into adipocytes.

The data in Figure 2A and Table S1B show that many of the group 2 genes are constitutively
expressed at a low level during normal adipogenesis in response to endogenous ligands, but
appear to be responsive to potent exogenous ligands. To gain a greater insight into mechanisms
regulating these two programs of gene expression, Swiss-WT-PPARγ cells were induced to
differentiate in the absence or presence of troglitazone and expression of select genes was
analyzed each day using RT-PCR technology. Figure 2B demonstrates the constant and
abundant expression of the WT-PPARγ throughout 7 days of differentiation, which resulted in a
robust and sustained induction of the Group 1 genes, such as adiponectin and C/EBPα, in
response to endogenous (- troglitazone) as well as exogenous ligands (+troglitazone).
Interestingly, the Group 2 genes, including Ero1-Lα, Scd3 and FGF21, are transiently expressed
at a very low level during the initial 2-4 days of adipogenesis and are then down-regulated as
differentiation proceeds in the absence of troglitazone. Differentiation of these WT-PPARγ cells
in the presence of troglitazone has a minimal effect on expression of adiponectin and C/EBPα
mRNAs, but enhances as well as maintains expression of Ero1-Lα, Scd3 and FGF21 throughout
the 7-day culture period.

Select group 2 genes are transiently expressed during the differentiation of brown and
white preadipocytes.
The data presented in Figures 2A and 2B were derived from non-adipogenic fibroblasts forced to differentiate into adipocytes by over-expression of PPARγ. We considered it important, therefore, to determine whether this interesting pattern of PPARγ target gene expression occurs in preadipocytes undergoing differentiation into brown as well as white adipocytes in response to activation of endogenous adipogenic transcription factors. To this end, we analyzed expression of genes during the differentiation of 3T3-L1 white preadipocytes and immortalized primary brown preadipocytes. Figure 3 shows the expected induction of PPARγ, C/EBPα, LXRα and adiponectin mRNAs at 2 days following exposure of the preadipocytes to DEX, MIX, insulin and 10% FBS. Furthermore, expression of these adipogenic genes remains at a high level throughout differentiation of both brown and white preadipocytes. To confirm that the immortalized primary brown preadipocytes underwent differentiation into brown adipocytes, we also analyzed expression of PGC-1α and UCP-1, and the data show expression of these mRNAs was initiated at 2 days and was maintained throughout brown adipogenesis (Fig 3B). In contrast, the Group 2 genes that respond poorly to expression of F-PPARγ in the Swiss cells are induced in response to activation of endogenous PPARγ in both the brown as well as the white preadipocytes, however, the level of expression of the corresponding mRNAs drops significantly during terminal adipogenesis as observed in the Swiss-WT-PPARγ cells differentiated in the absence of troglitazone (Fig 2B).

**Differential response of group 1 and group 2 genes to PPARγ agonists and antagonists.**

The fact that mutations within helix 7 rendered PPARγ unresponsive to endogenous ligands and responsive to troglitazone at least for the group 1 genes, encouraged us to determine the effect of other ligands that have previously been shown to possess a range of activities.
Consequently, WT-PPARγ and EF-PPARγ cells were induced to differentiate in the presence or absence of 15δ-PGJ2, FMOC-leu, troglitazone, rosiglitazone or GW1929 for 5 days and expression of select genes was analyzed by RT-PCR. Figure 4A shows that all of the exogenous ligands have little to no additional effect on the expression of select group 1 genes in WT-PPARγ cells since their level of expression is already at a maximum due presumably to the stimulation of the ectopic PPARγ by endogenous ligands (compare lanes 2-6 with lane 1). In contrast, expression of the group 2 genes is enhanced to varying extents by exposure of the WT-PPARγ cells to the exogenous ligands. In the case of the EF-PPARγ cells, exposure to the different ligands resulted in a significantly more varied response than that observed in the WT-PPARγ cells. Specifically, FMOC-leu was incapable of stimulating expression of any of the selected group 1 or group 2 genes, and 15δ-PGJ2 only activated FABP4 expression. The thiazolidinediones, troglitazone and rosiglitazone, induced expression of select group 1 genes including C/EBPα, adiponectin and FABP4, but had a negligible effect on expression of the group 2 genes such as Ero1-Lα and Mrap. Interestingly, GW1929, an extremely potent, synthetic PPARγ ligand in which N-tyrosine moieties have been substituted for the thiazolidinedione head group, is capable of inducing expression of the group 2 genes as well as group 1 genes in the EF-PPARγ cells. These data clearly show a differential response of the two groups of genes to different ligands; we questioned, therefore, whether the genes also show a similar differential response to a PPARγ antagonist. To address this question, WT-PPARγ cells were induced to differentiate in the presence or absence of T0070907 or GW9662 (two PPARγ antagonists) with or without troglitazone and corresponding cellular RNAs were analyzed by RT-PCR. Figure 4B demonstrates that T0070907 and GW9662 moderately attenuate the ability of WT-PPARγ to induce expression of C/EBPα and adiponectin in response to endogenous
ligands. The presence of troglitazone overcomes the inhibitory effect of the antagonists (Fig 4B, compare lanes 5 and 6 with lanes 3 and 4). In contrast, the antagonists almost completely block expression of the group 2 genes including Ero1-Lα, Mrap, Elovl3, Egln1, SCD3, OLR-1 in response to stimulation of WT-PPARγ by endogenous ligands, with T0070907 being the most potent. Again, this effect is overcome somewhat by troglitazone. Taken together, the data in Figures 4A and 4B show that activation of the group 2 genes by PPARγ requires more potent ligands and is significantly more sensitive to antagonists than the group 1 genes. We also considered it important to determine whether mutation of F372 had simply dampened the ligand binding affinity of PPARγ and, consequently, had shifted the dose response to troglitazone significantly to higher concentrations. To investigate this possibility, WT-PPARγ and EF-PPARγ cells were induced to differentiate for 5 days in the presence of increasing concentrations of troglitazone. At this stage, cells were harvested for analysis of select genes by RT-PCR. Figure 4C shows abundant expression of select group 1 genes including C/EBPα, adiponectin and FABP4/aP2 in WT-PPARγ cells due to endogenous ligands (lane 1) with no significant change in expression in response to increasing doses of troglitazone (lanes 2-8). As expected, the group 2 genes, FGF21 and OLR-1, are not expressed in WT-PPARγ without an exogeneous ligand (lane 1), but can be induced in a troglitazone dose-dependent manner (lanes 2-8). Analysis of gene expression in the EF-PPARγ showed that the group 1 genes, C/EBPα, adiponectin and FABP4/aP2, are not expressed in the absence of exogenous ligand (Fig 4C, lane 9) but, as expected, are induced in response to doses of troglitazone (250-500nM) previously shown to be specific for PPARγ (Fig4C, lanes 10-16). Of importance is the observation that expression of FGF21 and OLR1 cannot be activated in the EF-PPARγ by doses of troglitazone (10µM) that far exceed the dose that is specific for PPARγ. These data demonstrate clearly that
mutation of F372 within helix 7 has prevented PPARγ from responding to endogenous ligands, but additionally, has prevented PPARγ from inducing expression of the group 2 in response to the thiazolidinedione, troglitazone.

**PPARγ Directly Regulates Expression of FGF21.**

It is conceivable that the inability of the mutant PPARγ (EF or F) to induce expression FGF21 and OLR1 by troglitazone as well as exogenous ligands is because the corresponding genes might not be direct targets of PPARγ. Other studies, however, have shown a direct induction of the OLR1 gene promoter by PPARγ (5). We considered it important and of significant interest to determine whether the FGF21 gene is also a direct target of PPARγ. To this end, we performed two sets of experiments. First, we determined whether the induction of FGF21 gene expression occurred in the absence of ongoing protein synthesis. To do this, WT-PPARγ cells were induced to differentiate for 5 days without a synthetic ligand at which stage either troglitazone (5µM) or cycloheximide (5µg/ml) was added alone or together for 4, 6 or 8 hrs and at each time RNA was analyzed by RT-PCR analysis. Figure 5A shows significant expression of FABP4/aP2a mRNA at all three times due to its activation by the endogeneous ligand activity during the 5 days of differentiation of the WT-PPARγ cells. In contrast, there is virtually undetectable levels of FGF21 mRNA expression in the absence of an exogenous ligand (Fig 5A, lanes 1, 5 and 9). Interestingly, exposure to troglitazone rapidly induces FGF21 mRNA expression during the 8 hr exposure time (Fig 5A, lanes 2, 6 and 10) and this event occurs in the presence of cycloheximide (Fig 5A, lanes 4, 8 and 12) showing that the FGF21 gene is a direct target of PPARγ. Also of interest is the observation that FGF21 mRNA expression is induced simply due to exposure to cycloheximide (Fig 5A, lanes 3, 7 and 11). This is usually indicative of the existence of a
repressor that is removed due to its rapid turnover in the absence of ongoing protein synthesis. To demonstrate further that PPARγ directly activates FGF21 gene expression, we performed a series of FGF21 gene promoter/luciferase reporter assays. To this end, fragments (-500, -1300 and -1500) of the upstream region of FGF21 gene were cloned into the pGL3 luciferase reporter plasmid as shown in Figure 5B. Analysis of the sequence of the proximal 1500 bp of the gene showed the presence of at least five DR-1 elements that are highly homologous to a consensus PPAR regulatory element (PPRE) and, therefore, have the potential to associate with PPARγ/RXRα heterodimers. Figure 5B shows that transfection of the –500bp fragment plasmid which contains two PPREs into control Swiss fibroblasts in the presence or absence of a potent PPARγ ligand, GW1929, expresses a low basal level of luciferase activity equivalent to a control DR-1/luciferase reporter composed of consensus PPREs. Interestingly, the 1300bp and 1500bp fragments express a higher level of luciferase activity, but the presence of GW1929 has no affect on this activity. Transfection of the reporter plasmids along with a PPARγ expression plasmid, however, resulted in a significant increase in the activity of all three FGF21 gene fragments, which was enhanced even further in the presence of GW1929. We also analyzed FGF21 promoter activity in control (pBabe-puro) and Swiss-WT-PPARγ cells by transfecting each of the luciferase reporter plasmids in the presence or absence of GW1929. The results in Figure 5C are consistent with those in Figure 5B showing that the transcriptional activity of the 500bp fragment of the FGF21 gene is significantly higher in the Swiss cells expressing PPARγ compared to control Swiss cells and, that this activity is enhanced many fold by GW1929. The activity of the 1300 and 1500 bp fragments in WT-PPARγ cells in the presence of GW1929 is only slightly higher than the 500 bp region suggesting that elements within this proximal
promoter are likely responsible for the observed PPARγ-dependent activity of all three fragments.

Expression of many of the Group 2 genes are actively repressed by SIRT1 during terminal adipogenesis.

A recent report demonstrated that activation of SIRT1 in adipocytes triggers lipolysis and loss of fat by mechanisms involving repression of PPARγ activity (28). We questioned, therefore, whether there is a role for SIRT1 in facilitating the differential expression of the Group 1 and Group 2 genes as preadipocytes become mature fat cells. Consequently, we analyzed expression of adipogenic genes during differentiation of 3T3-L1 preadipocytes in which SIRT1 expression is suppressed due to constitutive production of a corresponding SIRT1 RNAi. The western blot in Fig 6A shows the extensive reduction in SIRT1 expression in the RNAi cells compared to the abundant production in a control line of 3T3-L1 cells expressing the vector alone. It is also relevant that expression of SIRT1 increases several-fold during the early phase of adipogenesis in the control cells, but then subsides to preadipocyte levels during the terminal phase. It is also important to point out that there is no significant effect of knockdown of SIRT1 on production of adiponectin. Total RNA was harvested from SIRT1 knockdown as well as control cells at select times throughout differentiation. RNA from preadipocytes (day 0), cells at a mid (4 days) and late phase (day 10) of adipogenesis was subjected to oligonucleotide microarray analysis employing affymetrix chips as discussed above. The relative expression of select mRNAs corresponding to both group 1 and group 2 genes was analyzed as shown in Tables S1A and S1B in supplement, respectively. As discussed above, Table S1A corresponds to a list of classic adipogenic genes (group 1) that are induced during adipogenesis in 3T3-L1 preadipocytes and
are differentially responsive to WT-PPARγ versus EF-PPARγ. Suppression of SIRT1 activity causes a transient increase (~50%) in expression of most of these genes at day 4 of differentiation in 3T3-L1 cells compared to their level of expression at this stage of differentiation in control cells (Table S1A, compare column 9 with 6). This difference in expression correlates with a significant increase in SIRT1 expression during early adipogenesis in control 3T3-L1 preadipocytes (Figure 6A). Interestingly, these genes appear to reach a maximum level of expression by day 4 in the knockdown cells, whereas it requires 10 days for them to reach this maximum in the control cells (Table S1A, compare columns 7 and 10). The data are consistent with the notion that the preadipocytes lacking SIRT1 activity differentiate much faster than control cells reaching terminal adipogenesis within 4 days, compared to 10 days in the controls cells. Table S1B shows the expression profiles of the group 2 mRNAs in control and SIRT1 knockdown 3T3-L1 cells and Swiss-Pγ cells. As observed for the group 1 genes in Table S1A, expression of the group 2 genes also increases at day 4 of differentiation in the knockdown cells even though most of the genes do not normally show an enhanced expression at this stage of differentiation in control cells (Table S1B, compare column 9 with column 6). More important, most of the group 2 genes are expressed at significantly higher levels at day 10 in the SIRT1 knockdown cells compared to control cells (Table S1B, compare column 10 with column 7). In fact, some genes most notably Ero1-Lα (487%), Hig1 (262%) and Trib3 (290%) are enhanced many fold in response to reduction in SIRT1 abundance. Additionally, all the genes coding for glycolytic enzymes as well as the glucose transporter 1 are also induced in the SIRT1 knockdown cells. It is also worth mentioning that the extent of induction of each of these group 2 genes appears to correlate with their level of induction by troglitazone in WT-PPARγ cells (Table S1B, compare columns 1 and 2 with 7 and 10, respectively). To confirm the data
presented in Tables S1A and S1B, Figure 6B shows an RT-PCR analysis of RNA harvested from control and SIRT1 knockdown 3T3-L1 cells at times throughout differentiation. It is quite apparent that the knockdown of SIRT1 has a selective effect on the Group 2 genes compared to the Group 1 genes. Specifically, expression of C/EBPα and adiponectin mRNAs (Group 1) shows a modest increase at the early stage of adipogenesis in the SIRT1 knockdown cells (Fig 6B), as presented in Table S1A, but no significant increase as these cells mature into adipocytes. In contrast, expression of group 2 genes including Ero1-Lα, Scd3, FGF21 and Elovl3 is dramatically enhanced in the SIRT1 knockdown cells (Fig 6B).

**Group 2 Genes are selectively induced in mature adipocytes by exposure to PPARγ ligands**

The data presented in Figure 6B and Table S1B suggested that several of the group 2 genes are actively repressed in mature adipocytes by mechanisms involving SIRT1. We questioned, therefore, whether exposure of such cells to a synthetic PPARγ ligand could overcome the repression and stimulate their expression. To test this notion, normal 3T3-L1 preadipocytes were induced to differentiate following standard procedures and at days 2, 4 and 6 differentiating cells were exposed to troglitazone for 2 days at which time total RNA was harvested for analysis employing RT-PCR. Figure 7A demonstrates an extensive induction of selected group 2 genes at different stages of the differentiation process. Specifically, ELOVL3 is induced by exposure of the 3T3-L1 cells to troglitazone as early as 4 days of adipogenesis and corresponding mRNAs levels remain elevated throughout differentiation. FGF21 and Ero1-Lα gene expression is also enhanced several fold but only occurs in more mature adipocytes. Expression of the selected members of the group 1 genes (C/EBPα, adiponectin and FABP4), however, are essentially unresponsive to the exogenous ligand since the level of expression is already at a maximum due
to their induction by endogenous PPARγ ligands. These data are consistent with the hypothesis that a subset of the group 2 genes including FGF21 and Ero1-Lα are actively repressed by SIRT1 in mature adipocytes and that this repression can be overcome by exposure to troglitazone. The fact that attenuation of SIRT1 (Figure 6B) or exposure to the PPARγ ligand (Figure 7A) does not induce expression of the classic adipogenic genes in group 1 suggest that they are distinct from the group 2 genes since they are presumably in a constant state of optimum transcriptional activity. These data suggest that SIRT1 and PPARγ ligands reciprocally regulate PPARγ activity on group 2 genes; we questioned, therefore, whether SIRT1 might attenuate the response of PPARγ to its ligands. To test this notion, we determined the dose of troglitazone required to induce expression of select group 2 genes in control versus SIRT1 knockdown 3T3-L1 preadipocytes at 4 days of differentiation. Specifically, differentiating cells were exposed to increasing doses of troglitazone for 2 days at which stage RNA was analyzed for expression of select genes by RT-PCR. Figure 7B shows that expression of FGF21 and Egln1 (group 2 genes) is induced in control cells following exposure to doses of troglitazone in range of 1 to 5 µM; in contrast, induction of these genes in SIRT1 knockdown cells requires a significantly lower dose of troglitazone (250nM). These data suggest that SIRT1 attenuates the response of PPARγ to an exogenous ligand. Additionally, Fig 7B also confirms that there is a negligible effect of either knockdown of SIRT1 or ligands on expression of the group 1 genes adiponectin or FABP4.

**Discussion**

Our recent studies have demonstrated that helix 7 within the ligand-binding domain of PPARγ facilitates a functional interaction between β-catenin and PPARγ (22). Additionally, other investigations by Glass and coworkers (26) identified K365 in helix 7 of PPARγ1 as a target for
ligand-dependent SUMOylation, which regulates the repression of inflammatory genes by PPARγ in macrophages. These observations suggested to us that helix 7 might also participate in ligand-dependent control of PPARγ target gene expression during adipogenesis. In the present studies, we show that ectopic expression of a WT-PPARγ in Swiss mouse fibroblasts induces expression of the adipogenic program in response to the normal cocktail of adipogenic inducers, including DEX, MIX, insulin and FBS, without the need for additional stimulation with an exogenous synthetic PPARγ ligand such as troglitazone. In fact, treatment with troglitazone appears to have only a minimal effect on the already robust expression of the adipogenic genes (Fig 1C and Table S1A). In contrast, expression of mutant PPARγ, in which F372 within helix 7 of the ligand-binding domain has been changed to alanine, completely destroys the ability of PPARγ to induce adipogenesis in response to endogenous ligands (minus troglitazone).

Interestingly, F372A-PPARγ can respond to troglitazone, and in doing so, activates expression of many of the genes induced by the WT-PPARγ; although, a subset of these genes are unresponsive to troglitazone-activated F372A-PPARγ (Fig 2A and Table S1B, Group 2 genes). This subset consists of a diverse group of genes encoding a novel set of adipocyte proteins such as Ero1-Lα and FGF21 as well as components of the glycolytic pathway and regulators of glucose uptake. Many of these group 2 genes are constitutively produced at a low level during adipogenesis, but their expression can be activated in mature adipocytes by exposure to potent PPARγ ligands or suppression of SIRT1 activity (Figs 6 and 7). The studies also show that PPARγ directly regulates expression of the FGF21 gene through elements located within the 500bp upstream region of the gene (Fig 5). Taken together, these data are consistent with the notion that PPARγ can differentially regulate multiple programs of gene expression in response to ligands activating different regions of the ligand-binding domain. Moreover, activation of the
group 2 genes by PPARγ requires its association with a potent ligand to overcome the selective, suppressive effects of SIRT1.

The molecular mechanisms responsible for distinguishing one set of target genes from another likely involves recruitment of different coregulators to PPARγ docked on the promoters/enhancers of the genes. In fact, Lazar and coworkers have recently shown that GγK and OLR1 genes are actively repressed in mature adipocytes by recruitment of NCoR/HDAC3 complexes to PPARγ docked on PPAR response elements (PPRE) in the promoters of the corresponding genes (5, 12). Interestingly, exposure of adipocytes to TZDs dislodges the repressor complexes from these sites by mechanisms involving PPARγ coactivator-1α (PGC-1α) leading to expression of the genes. These authors also demonstrate that other adipogenic genes, such as FABP4/aP2, that are abundantly expressed in mature adipocytes in response to endogenous ligand activity, have PPARγ docked on their enhancers in association with the SRC/p160 family of coactivators. The data presented here are consistent with these observations, but also suggest that a subset of group 2 genes including Ero1-Lα and FGF21 are selectively repressed by mechanisms dependent on SIRT1 activity. A mechanism under consideration involves a regulated SUMOylation of K365 within helix 7 of PPARγ that is docked on the specific set of target genes (Group 2) destined for suppression by SIRT1 in mature adipocytes. SUMOylated PPARγ will then recruit select corepressors such as NCoR/HDAC3 as well as SIRT1 to the target genes that are then subsequently repressed. PPARγ, which is docked on the genes (Group 1) that remain active during this process, likely escapes SUMOylation. Formulation of this model is based on the recent findings of Glass and coworkers, which demonstrated that ligand-dependent SUMOylation of PPARγ on K365 of helix 7 induces the PPARγ-associated repression of inflammatory genes in macrophages (26). K365 could also be a
target of acetylation in which case acetylated K365 would prevent SUMOylation and, consequently, maintain PPARγ in active state. It follows, therefore, that deacetylation of K365 by SIRT1 should facilitate SUMOylation resulting in repression of PPARγ on select target genes. An important question in considering this model is by what means does the SUMOylation process select PPARγ molecules that are docked on the targets that will be repressed during terminal adipogenesis? One possibility is that the environment surrounding the PPREs within these genes facilitates SUMOylation. For instance, the mechanism could involve docking of other nuclear factors that are induced during adipogenesis to sites that are flanking the PPREs. These factors could then participate in recruitment of the SUMOylation machinery to PPARγ and the resulting repression of these genes.

It is also possible that SIRT1 regulates the expression/activity of coregulators whose association with PPARγ is dependent on helix 7 of the ligand-binding domain. In fact, it is reasonable to suggest a role for PGC-1α since Lazar and coworkers have previously shown induction of this coactivator in white adipocytes in response to TZDs (12). Furthermore, these investigators demonstrated the involvement of PGC-1α in the selective activation of the Gyk gene by TZDs. Additionally, other studies have shown that SIRT1 deacetylates PGC-1α and in doing so regulates its ability to modulate the activity of different transcription factors (31). Consequently, it is conceivable that the selective expression of the group 2 genes that includes Gyk and OLR1 in mature adipocytes involves induction of PGC-1α by TZDs and its activation through the suppression of SIRT1 activity.

Another important component of the model explaining how PPARγ activates different programs of gene expression at precise times during adipogenesis is the role played by specific ligands. As stated earlier, the endogenous ligands responsible for stimulating PPARγ activity
during adipogenesis have not as yet been identified. Several studies have, however, identified signaling pathways and transcription factors that appear to regulate ligand production (4, 14, 17, 23, 35). The combined data are consistent with a regulated process induced during the initial days of adipogenesis involving cAMP signaling and enzymes that convert polyunsaturated fatty acids into eicosinoids. It is interesting that ligand activity peaks at 2-4 days of adipogenesis in 3T3-L1 preadipocytes but then rapidly subsides during terminal adipogenesis. Several questions result from these observations; most notably, how does PPARγ continue to maintain expression of most target genes such as FABP4/aP2 in the presence of lower concentrations of these ligands? There are many explanations for this apparent conundrum, including changes in expression and activity of coregulators during adipogenesis that requires lower levels of PPARγ activity to facilitate the associated recruitment/dislodgment process.

The subset of adipogenic genes (group 2) that have been identified in this report contains several members that have not previously been shown to be regulated during adipogenesis or responsive to the activity of PPARγ. In the case of the ER oxidoreductase, Ero1-Lα, studies have recently shown that this protein is involved in regulating the secretion of adiponectin from mature adipocytes (30, 36). Furthermore, expression of Ero1-Lα mediates the nutrient control of adiponectin secretion by responding to the activity of the NAD-dependent deacetylase SIRT1 (30); data that are consistent with the observations presented here showing that the group 2 set of adipogenic genes is regulated by SIRT1. It is also noteworthy that we have identified FGF21 as a direct target of PPARγ since it has recently been shown to be a hormone produced in the liver in response to activation of PPARα and acts as a component of the body’s adaptation to fasting (1, 15). Other studies have also shown that it is a potent regulator of glucose uptake in 3T3-L1 adipocytes and primary human adipocytes (16). Our data show that FGF21 is not only produced
in hepatocytes, but can also be induced in 3T3-L1 adipocytes by exposure to potent PPARγ ligands or suppression of SIRT1 activity, suggesting that this secreted factor might act in both an autocrine as well as a paracrine fashion to regulate insulin-responsive glucose uptake in adipocytes. With regard to FGF21 signaling, the data in Table S1B shows that a gene (Klb-βKlotho) coding for an important component of FGF21 receptor (FGFR1 and 4) complex (25) is also responsive to both PPARγ and SIRT1 activity categorizing it as a member of the group 2 gene family. Additionally, components of the glycolytic pathway and regulators of glucose uptake are also members of this novel group 2 set of adipocytes genes. In fact, studies by others (31), have shown an increase in expression of liver pyruvate kinase and glucokinase in response to knockdown of SIRT1 in hepatocytes. We propose (Fig 7C), therefore, that SIRT1 can control metabolic homeostasis by regulating expression of the group 2 subset of adipocyte genes in response to metabolic effectors resulting in production of multiple proteins including insulin sensitizers such as adiponectin (through Ero1-Lα) as well as intracellular regulators of glucose uptake/metabolism (i.e. FGF21 and βKlotho). It appears that synthetic PPARγ ligands also target this gene program in adipocytes by selectively overcoming the suppressive effects of SIRT1 on these genes.

In conclusion, our studies have identified a novel regulatory region of the ligand-binding domain of PPARγ that facilitates the selective expression of different subgroups of adipocyte genes during the formation of mature fat cells. These findings should provide a greater understanding of the role of PPARγ and its ligands in regulating physiological functions of adipocytes, most notably insulin responsiveness and energy balance. Furthermore, the identification of novel genes that respond to SIRT1 as well as PPARγ activity, such as Ero1-Lα
and FGF21, should provide additional targets for the development of effective therapeutics to combat obesity and its associated disorders.

Acknowledgments

We acknowledge the support of the Genome Center at Boston University School of Medicine and the valuable help of Drs Marc Lenburg and Karen Schlauch in analyzing the microarray data. We are grateful to Dr Kathryn Davis for critical reading of the manuscript and constructive comments. We thank Dr L Guarente for the pSUPER SIRT1 siRNA plasmid. This work was supported by USPHS grants DK51586 and DK58825.

References:


Figure Legends:

Fig1: EF-PPARγ does not respond to endogenous ligands, but is activated by troglitazone. Swiss cells expressing various forms of PPARγ (WT, E, EF, DD) were cultured until confluent, after 2 days, they were exposed to DEX, MIX, insulin, with or without 5 µM troglitazone. A. Day 5 cells were fixed, stained with Oil Red O and photographed. B. Total cellular proteins were collected at day 5 and subjected to western blot analysis with the indicated antibodies. C. Profile of ~1700 mRNAs expressed in Swiss fibroblasts in response to WT- or EF-PPARγ Total RNA of WT-PPARγ and EF-PPARγ cells at day 5 (with or without troglitazone) were isolated using Trizol Reagent (Invitrogen) and microarray analysis was performed as described in materials and methods. Brown corresponds to high- and blue to low-signal intensity.

Fig2A: Identification of two groups of PPARγ-responsive genes: Group 1 (including adiponectin) is responsive whereas Group 2 (including Ero1 and FGF21) is completely unresponsive to troglitazone activation of EF-PPARγ or F-PPARγ. Swiss fibroblasts (C) and Swiss-PPARγ (WT, EF, F) cells were cultured until confluent, after 2 days, they were exposed to DEX, MIX, insulin, with or without 5 µM troglitazone for 5 days. Total RNA of cells was isolated using Trizol Reagent (Invitrogen) and subjected to RT-PCR analysis as described in materials and methods.

Fig2B: Troglitazone selectively enhances expression of the Group 2 PPARγ-responsive genes during the differentiation of Swiss fibroblasts expressing WT-PPARγ. Swiss WT-PPARγ cells were cultured in 10% FBS until they reached confluence. After 2 days post-confluence, cells were induced to differentiate by exposure to DEX, MIX, insulin, and 10% FBS with or without troglitazone. At day 0, 1, 2, 3, 4, 5, 6, 7 of differentiation, cells were harvested for RT-PCR analysis as described in materials and methods.

Fig3: Select group 2 PPARγ-responsive genes are transiently induced during the initial phase of adipogenesis in white 3T3-L1 preadipocytes (A) and immortalized primary brown preadipocytes
(B). A. 3T3-L1 white preadipocytes were cultured in 10% calf serum until they reached confluence. At 2 days post-confluence, cells were induced to differentiate by exposure to DEX, MIX, insulin and 10% FBS. At the indicated days of differentiation, cells were harvested for RT-PCR analysis as described in materials and methods. B. Immortalized brown preadipocytes were grown to confluence in differentiation medium composed of DMEM containing 10% FBS supplemented with 20nM insulin and 1 nM 3’, 5-triiodo-L-thyronine (T3). At 2 days post-confluence, cells were induced to differentiate by exposure to DEX, MIX, insulin, 0.125mM indomethacin and 10%FBS. At the indicated days of differentiation, cells were harvested for RT-PCR analysis as described in materials and methods.

Fig4: The differential response of WT-PPARγ versus EF-PPARγ to select PPARγ ligands and antagonists. A. Swiss-PPARγ (WT and EF) cells were cultured until confluent, after 2 days, they were exposed to DEX, MIX, insulin, with or without the following PPARγ ligands: FMOC-leu (15µM), 15δ-PGD2 (7µM), troglitazone (5µM), rosiglitazone (10µM) and GW1929 (10µM). B. WT-PPARγ cells were differentiated as in A by exposure to DEX, MIX and insulin in the presence or absence of troglitazone with or without either T0070907 (10µM) or GW9662 (10µM) (PPARγ antagonists). C. WT-PPARγ and EF-PPARγ cells were induced to differentiate with DEX, MIX, insulin and the indicated doses of troglitazone. In A B and C, total RNA of cells was isolated at day 5 using Trizol Reagent (Invitrogen) and subjected to RT-PCR analysis of the indicated group 1 and group 2 genes as described in materials and methods.

Fig5: PPARγ directly activates the FGF21 gene. A. Troglitazone activates FGF21 gene expression in absence of ongoing protein synthesis. WT-PPARγ cells were induced to differentiate with DEX, MIX and insulin for 5 days at which time troglitazone (5µM) was added in the presence or absence of cycloheximide (5µg/ml) for the indicated times. Cells were then harvested for extraction of RNA followed by RT-PCR analysis of FGF21 and FABP4/aP2 mRNAs as described in materials and methods. B. Reporter assays were performed in control Swiss fibroblasts following transfection of individual FGF21 luciferase plasmids (-1500, -1300 and –500 bp fragments) along with a PPARγ (pBabe-PPARγ) or control (pBabe-Puro) expression plasmid and a renilla based pGL3 reporter as control in the presence or absence of the potent
PPARγ ligand GW1929. The scheme above shows the presence of putative PPREs in the upstream region of the gene that have the following DR-1 sequences: 1, AGACCAAGGAGCA; 2, AGACCCAAGGCC; 3, TGGCCTGTGGCCA; 4, TGAGCACAAGGC; 5, AGTTCCAGGGCCA. C. Reporter assays were also performed in Swiss fibroblasts stably expressing a WT-PPARγ or a pBabe-puro empty vector (control cells) following transfection of the FGF21 promoter reporter plasmids along with the renilla control vector in the presence or absence of GW1929. In both assays B and C, a set of cells were also transfected with a reporter plasmid consisting of the PPRE from the aP2 gene upstream of luciferase within pGL3 (DR-1). Transcriptional activity of each of the fragments of the FGF21 gene promoter is shown as the ratio of luciferase to renilla activity (Luc/Ren) as described in materials and methods.

Fig6: Suppression of SIRT1 by expression of a corresponding SIRT1 siRNA selectively enhances the expression of Group 2 PPARγ-responsive genes during the differentiation of 3T3-L1 preadipocytes. Control and SIRT1 siRNA cells were cultured in 10% calf serum until they reached confluence. After 2 days post-confluence, cells were induced to differentiate by exposure to DEX, MIX, insulin, and 10% FBS. At the indicated days of differentiation, cells were harvested for western blot analysis (A) and RT-PCR analysis (B) of the indicated gene products as described in materials and methods.

Fig7A: Troglitazone selectively activates expression of group 2 genes in 3T3-L1 adipocytes. 3T3-L1 preadipocytes were differentiated using standard conditions and at day 2, day 4 and day 6 the differentiating cells were exposed to 5µM troglitazone for 2 days. Untreated and treated cells were harvested for analysis of select mRNAs using RT-PCR as described in materials and methods.

Fig7B: Knockdown of SIRT1 in 3T3-L1 preadipocytes enhances the expression of FGF21 in response to exposure to troglitazone. Control and SIRT1 knockdown 3T3-L1 preadipocytes were differentiated for 4 days at which time cells were exposed to the indicated doses of troglitazone for 2 days. Total RNA of cells was isolated at day 6 and subjected to RT-PCR analysis for analysis of the indicated group 1 and group 2 genes as described in materials and methods.
PPARγ functions to regulate adipocyte formation and function. Endogenous ligands activate PPARγ requiring participation of both helix 7 and 12 to orchestrate adipogenesis. In mature adipocytes, SIRT1 mediates hormonal and nutrient control of select PPARγ target genes that are involved in controlling metabolism by suppressing the action of endogenous ligands. The thiazolidinedione (TZD) family of synthetic PPARγ ligands can overcome the suppressive effects of SIRT1 acting through helix 7 as well as helix 12 to induce the metabolic genes.
Fig 1A

WT | E | EF | DD
---|---|----|----
-TROG

+TROG
Fig 1B

- troglitazone

+ troglitazone

WT E EF DD WT E EF DD

1 2 3 4 5 6 7 8

PPARγ

β-catenin

42 kD C/EBPα

30 kD C/EBPα

perilipin

FABP4/aP2
Fig 1C

Troglitazone
- + - +

Adipogenic
Lipogenic
Mitochondrial

ECM
MMPs
Wnt signaling
Cytokines
Inflammatory Proteins

High
Low
Fig 2A

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GROUP 1

1 2 3 4 5 6 7 8

GROUP 2

9 10 11 12 13 14 15 16
Fig 2B

- troglitazone

+ troglitazone

Days

0 1 2 3 4 5 6 7

PPARγ

C/EBPα

Adiponectin

Fabp4

Ero1

Fgf21

Mrap

Scd3

GAPDH

ACCEPTED on January 17, 2021 by guest
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Fig 3.

A. Days of Differentiation

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Fig 4A.
Fig 4B.

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- - - + - + - GW9662
PPARγ
C/EBPα
Adiponectin
Fabp4
Ero1
Fgf21
Mrap
Olr1
Scd3
GAPDH
Fig 4C.
Fig 5A

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Troglitazone
Cycloheximide
Fgf21
Fabp4/aP2
Fig 5B.
Fig 5C.
Fig 6A

Control

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Days

SIRT1

adiponectin

actin
Fig 6B

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Days: 0, 2, 4, 6, 8, 10

Protein expression levels for each sample at different time points:
- PPARγ
- C/EBPα
- Adiponectin
- Fabs4
- Fgf21
- Mraap
- Ndr2
- Egln3
- Scd3
- Elov3
- Ndg2
- Egln1
- GAPDH
Fig 7A

D4  D6  D8
-   -   -
+   +   +
troglitazone

PPARγ

C/EBPα

Adiponectin

Fgf21

Ero1

Scd3

Mrap

Ndg1

Egln3

Elovl3

GAPDH
Fig 7B

Control | SIRT1 RNAi

- nM Trog
- Fgf21
- Egln1
- adiponectin
- Fabp4
- PPARγ
- GAPDH
Fig 7C.

PPAR\(\gamma\)
Helix 7/12

Endogenous Ligands

TZDs

SIRT1

Hormones/Metabolites / Nutrients

Adipogenesis

Metabolic Homeostasis

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