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PPARγ partial agonist GQ-16 strongly represses a subset of genes in 3T3-L1 adipocytes

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ABSTRACT

Thiazolidinediones (TZDs) are peroxisome proliferator-activated receptor gamma (PPARγ) agonists that improve insulin resistance but trigger side effects such as weight gain, edema, congestive heart failure and bone loss. GQ-16 is a PPARγ partial agonist that improves glucose tolerance and insulin sensitivity in mouse models of obesity and diabetes without inducing weight gain or edema. It is not clear whether GQ-16 acts as a partial agonist at all PPARγ target genes, or whether it displays gene-selective actions. To determine how GQ-16 influences PPARγ activity on a gene by gene basis, we compared effects of rosiglitazone (Rosi) and GQ-16 in mature 3T3-L1 adipocytes using microarray and qRT-PCR. Rosi changed expression of 1156 genes in 3T3-L1, but GQ-16 only changed 89 genes. GQ-16 generally showed weak effects upon Rosi induced genes, consistent with partial agonist actions, but a subset of modestly Rosi induced and strongly repressed genes displayed disproportionately strong GQ-16 responses. PPARγ partial agonists MLR24 and SR1664 also exhibit disproportionately strong effects on transcriptional repression. We conclude that GQ-16 displays a continuum of weak partial agonist effects but efficiently represses some negatively regulated PPARγ responsive genes. Strong repressive effects could contribute to physiologic actions of GQ-16.

1. Introduction

The prevalence of obesity is increasing [1,2]. In 2005, 23% of the world's adult population was overweight and 10% obese [3]. Projections indicate that numbers of overweight and obese individuals will increase from 2005 estimates, totaling 1.35 billion overweight and 573 million obese individuals in 2030 [3]. Obesity-associated insulin resistance (IR) is a component of type 2 diabetes [4]. Current therapies for IR include thiazolidinediones (TZDs) [5], which are strong agonists for peroxisome proliferator-activated receptor gamma (PPARγ) and effectively improve IR and decrease plasma glucose by triggering PPARγ dependent gene expression in adipose tissue and other organs [6]. In addition to glycemic control, TZDs improve lipid profile, lower blood pressure and have anti-inflammatory effects [7,8]. Unfortunately, TZDs also exhibit side effects such as weight gain [9], fluid retention and edema [10–12], congestive heart failure [13,14] and bone loss [15,16]. Rosi is associated with myocardial infarction [17] and concerns have been raised about pioglitazone and bladder cancer [18,19].

In light of TZD side effects, efforts have been made to discover selective PPARγ ligands that retain beneficial effects without undesirable side effects. GQ-16, a synthetic compound derived from 5-benzylidene-3-(4-methyl-benzyl)-thiazolidine-2,4-dione, improves glucose tolerance and IR similarly to Rosi in a murine model of obesity and diabetes without inducing weight gain or edema [20]. As such, GQ-16 is part of an emerging class of PPARγ ligands

Abbreviations: PPARγ, peroxisome proliferator activated receptor γ; TZD, thiazolidinedione; DMEM, Dulbecco's modified eagle medium; DMSO, dimethylsulfoxide; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); ANOVA, analysis of variance.

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with improved spectrums of actions in animal models and partial agonist behavior in cultured cells [21–23]. X-ray structural analysis revealed that GQ-16 and similar partial agonists adopt a binding mode that is distinct from Rosi [20]. GQ-16 binds far from activation helix (H) 12 and parallel to H3 and close to the β-sheet region [20]. This contrasts with TZDs, which adopt an orientation perpendicular to H3 that allows direct stabilization of H12 in the active position [23]. Shared insulin sensitizing activities of TZDs and partial agonists are attributed to the ability of both types of compounds to prevent an inhibitory phosphorylation at PPARγser273, which lies within the PPARγ H2-H2′ region and is indirectly stabilized by interactions of both ligand classes with the β-sheet region [24–26]. This results in selective activation of a beneficial sub-class of adipocyte PPARγ target genes that are inhibited by the phosphorylation at Ser273. By contrast, harmful TZD side effects require full agonist activity. Thus, binding mode of partial PPARγ agonists could explain improved profiles.

The model outlined above suggests that GQ-16 should preferentially induce a small subset of Rosi responsive genes in adipocytes. We therefore compared effects of GQ-16 on gene expression to those of Rosi in 3T3-L1 adipocytes by performing microarray analysis. We find that GQ-16 displays a continuum of relatively weak partial agonist activity across a range of positively regulated genes, but also displays disproportionately large effects at small subsets of weakly Rosi induced genes and some negatively regulated genes. Similar effects on down-regulated genes are also seen with other partial PPARγ agonists. We propose that capacity of GQ-16 to efficiently down-regulate genes could be important for insulin sensitizing effects.

2. Materials and methods

2.1. Materials

GQ-16 (5-(5-bromo-2-methoxy-benzylidene)-3-(4-methyl-benzyl)-thiazolidine-2,4-dione) was synthesized as described [27]. Rosi was purchased from Sigma-Aldrich and MRL24 [24,28] and SR1664 [24] were provided by Dr. Patrick Griffin.

2.2. Cell culture

3T3-L1 preadipocytes were cultured in 6 well plates at 37 °C and 5% CO_2 in DMEM 3T3-L1 Preadipocyte Medium (ZenBio Inc, Cat. No PM-1-L1) containing high glucose (4.5 g/L D-glucose), HEPES pH 7.4, bovine calf serum, penicillin, streptomycin and amphotericin B. Two days after confluence, medium was removed and replaced with DMEM/Ham's F-12 medium (1:1, v/v) 3T3-L1 Differentiation Medium (ZenBio Inc, Cat. No DM-2-L1) containing glucose (3.15 g/L D-glucose), HEPES pH 7.4, fetal bovine serum, biotin, pantothenate, penicillin, streptomycin, amphotericin B, and MDI induction cocktail containing dexamethasone, 3-isobutyl-1-methylxanthine, and insulin. After three days, cells were harvested or the 3T3-L1 Differentiation Medium was \ replaced with DMEM/Ham's F-12 medium (1:1, v/v) 3T3-L1 Adipocyte Medium (Cat. No AM-1-L1) containing glucose (3.15 g/L D-glucose), HEPES pH 7.4, fetal bovine serum, biotin, pantothenate, human insulin, dexamethasone, penicillin, streptomycin and amphotericin B until harvesting. On day seven, cells were stained with Oil Red O or treated with vehicle (DMSO), Rosi 100 nM or GQ-16 10 μM.

2.3. cDNA preparation

Total RNA was extracted on day three or eight of 3T3-L1 differentiation using Aurum Total RNA Fatty and Fibrous kit (BioRad), as per manufacturer's instructions. 1 μg of total RNA was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad).

2.4. Microarray hybridization

Mouse whole genome expression arrays were purchased from Illumina (BeadChip Array MouseWG-6v2). cRNA synthesis and labeling were performed using Illumina® TotalPrep™-96 RNA Amplification Kit (Ambion). Biotin labeling *in vitro* transcription reaction was performed at 37 °C for 14 h. Biotinylated cRNA samples were hybridized to arrays at 58 °C for 18 h according to protocol, and scanned with an iScan Reader. Raw data were obtained from GenomeStudio and subsequently background-subtracted and quantile-normalized using the lumi package [29] and analyzed with the limma package [30] within R [31]. All analysis were corrected for multiple hypothesis testing [32], and effects determined to be significant with a ≥1.5-fold change in adjusted p-value ≤ 0.05 versus vehicle (DMSO) control. To facilitate comparisons among treatments, data were uploaded into SQLite3 database (http://www.sqlite.org/).

2.5. Gene expression analysis

Gene transcripts were analyzed by qRT-PCR on the Roche LightCycler 480 II Instrument with SYBR Green Mastermix (Roche). Primers were designed to span exon–exon boundaries to eliminate amplification of genomic DNA (sequences in Supplementary Table 1). Normalized Cp values (Critical point; $Cp_{Target} - Cp_{18S}$) of at least 3 biological replicates were analyzed by ANOVA followed by t-tests of vehicle vs. treatment, with effect determined to be significant with a Bonferroni Corrected p-value ≤ 0.05 (GraphPad Software, version 5.01; GraphPad).

3. Results

3.1. GQ-16 effects on adipogenesis

As expected, GQ-16 showed reduced adipogenic potential in 3T3-L1 relative to Rosi, indicated by less Oil Red O staining (Supplementary Fig. 1A) and weaker induction of adipogenesis-related genes (c/EBPα, adipoQ, FABP4, LPL) after several days of treatment with GQ-16 (Supplementary Fig. 1B). While this is consistent with weak GQ-16 partial agonist actions, such effects could complicate analysis of direct versus indirect PPARγ target genes. We therefore focused on fully differentiated adipocytes where PPARγ regulates pathways involved in adipocyte maintenance and function [33]. As expected, expression of adipogenesis-related genes defined in Fig. S1 was largely unchanged after Rosi or GQ-16 treatment in these conditions, except for CEBPα (1.4 fold increase with Rosi) and FABP4 (2 fold increase with Rosi) and 1.5 increase with GQ-16 (Supplementary Fig. 2). By contrast, OLR1, a direct PPARγ target gene involved in lipid metabolism in mature adipocytes [34] displayed Rosi induction after 6 h that gradually increased over time (up to 9 fold after 48 h). GQ-16 responses were smaller and peaked after 12 h with a plateau in response from 24 to 48 h (Supplementary Fig. 2).

3.2. GQ-16 displays weak PPARγ partial agonism on a transcriptome wide level

Based on the OLR1 time course, we performed microarray analysis on mature 3T3-L1 adipocytes treated with ligands for 24 h 1156 transcripts were significantly changed (p-value ≤ 0.05) by 1.5 fold or more with Rosi (544 up and 612 down-regulated), but only 89 gene transcripts met these criteria with GQ-16 (40 up and 49

down-regulated). Of Rosi regulated genes, 46 were flagged as GQ-16 responsive. Thus, only 43 genes were initially flagged as purely GQ-16 specific.

We plotted fold change for Rosi response (x-axis) versus GQ-16 (y-axis) to better understand ligand responses detected on the array (Fig. 1). This revealed a linear relationship (filled red line); albeit with lower slope than expected from 1:1 relationship between ligands (dashed blue line). Rosi responsive genes (red crosses) clustered close to the red line that describes relationships between Rosi and GQ-16, along with some highly induced genes flagged as responsive to both ligands (black squares). Others clustered close to the axis that denotes no GQ-16 response. Thus, many, but not all, Rosi responsive genes also display weak GQ-16 responses. Inspection of data using an alternate analysis procedure (supplemental methods) confirmed this impression and allowed us to group genes into categories with respect to response to Rosi and GQ-16. Some genes responded exclusively to Rosi (Supplementary Table 2), whereas others displayed Rosi responses and weaker responses to GQ-16, which often failed to meet our initial arbitrary cut-offs for fold change, significance or both (Supplementary Table 4). Small gene sets were also flagged as GQ-16 specific or exhibit opposite responses to ligands (Supplementary Tables 3 and 5). Our data are therefore consistent with the idea that GQ-16 elicits a continuum of weak partial agonist responses at PPARγ target genes and that degree of GQ-16 regulation varies on a gene by gene basis relative to Rosi.

3.3. Some genes display disproportionately large responses to GQ-16

The microarray analysis did highlight genes with disproportionately large responses to GQ-16, as seen by proximity to the line describing predicted 1:1 relationship between ligands. This group included genes regulated by both ligands (black squares, Supplementary Tables 4 and 5) and genes that were apparently uniquely responsive to GQ-16 (Fig. 1 blue points, Supplementary Table 3). Of genes flagged as responsive to Rosi and GQ-16, one large subgroup were weakly induced by both ligands and another subgroup were strongly repressed by both ligands. Inspection of original data revealed that some repressed genes flagged as uniquely GQ-16 responsive (Fig. 1; blue points) actually exhibited responses to both ligands, with Rosi responses not flagged because they failed to meet selection cut-offs (Supplementary Tables 3 and 4).

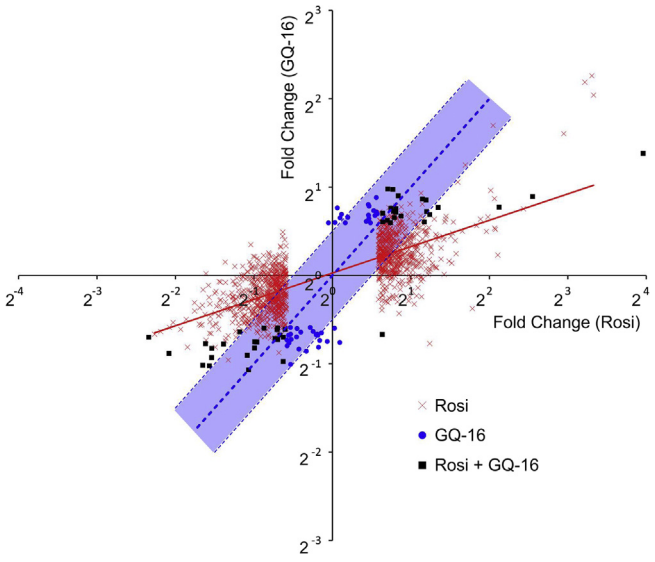

Fig. 1. Rosi and GQ-16 Response in Adipocytes. Fold induction/repression with GQ-16 versus Rosi. Red points; genes that show stronger to exclusive response to Rosi treatment, blue points; genes that show a stronger to exclusive response to GQ-16 treatment, black points; affected by both compounds. Red filled line represents the proportional relationship between overall Rosi response and GQ-16 response. Blue dotted line represents a theoretical one-to-one relationship with shaded area representing a 50% deviation.

We verified selected observations from the array using qRT-PCR (Figs. 2 and 3 and not shown). As expected, GQ-16 displayed a range of weak partial agonist effects at strongly Rosi induced genes (Fig. 2A–C; Acaa1b 13%, Fgf21 13%, Rbp7 11% of Rosi response). We also confirmed that some weakly Rosi induced genes displayed proportionately larger GQ-16 responses (Fig. 2D–F; Elvol3 34%, Gpd1 49%, Irs2 78% of Rosi response). GQ-16 also displayed a continuum of response at negatively regulated genes (Fig. 3). Rosi repressed the orm2, Lgals9 and Vcam1 genes more effectively than GQ-16 (Fig. 3A–C), whereas differences between ligands were less prominent at the Hspb1, Dcn and Insig2 genes (Fig. 3D–F). Attempts to replicate pure GQ-16 responses (Supplementary Table 3) were not successful (not shown). Thus, we were not able to verify existence of target gene that responded to GQ-16 but not Rosi.

3.4. Similar ligand dose responses at induced and repressed PPARγ target genes

To eliminate the possibility that disproportionately large GQ-16 responses at negatively regulated genes were related to differential ligand sensitivity, we determined doses of Rosi and GQ-16 required for induction or repression of selected genes (Supplementary Fig. 4). In all cases, maximal transactivation (Acaa1b, Fgf21) and transrepression (Vcam1, Dcn) required similar doses of Rosi and GQ-16 and only elicited changes in gene expression at the highest doses (10 μM). Notably, ligand dose responses were similar to those seen in transfection assays with full length PPARγ in non-related HeLa cells (Supplementary Fig. 5) and reflected differences between Rosi and GQ-16 in a standard *in vitro* ligand displacement assay (Supplementary Fig. 6). Thus, GQ-16 dependent transactivation and transrepression display similar ligand dose responses.

3.5. PPARγ Partial Agonists Display Effective Transrepression

Finally, we determined whether other partial agonists display similar effects upon gene expression in 3T3-L1 (Fig. 4). We selected MRL24, which adopts similar orientation to GQ-16 in the PPARγ pocket [21–26] and the selective PPARγ modulator SR1664, which lacks conventional H12-dependent PPARγ agonist effects but blocks the inhibitory phosphorylation at Ser273 and displays antidiabetic activity without harmful side effects [24]. While there is no published X-ray structure of PPARγ in complex with the latter compound, hydrogen–deuterium exchange reveals destabilization of H12, explaining absence of agonist effects. In our hands, both ligands exhibited similar weak partial agonist activity to GQ-16 at positively regulated genes (Acaa1b, Fgf21, Elvol3) and repressed selected genes (Vcam1, Orm2, Lgals2, DCn1) as efficiently as GQ-16.

4. Discussion

GQ-16 is part of a class of PPARγ partial agonists with insulin sensitizing activities and improved side effect profile relative to TZDs [20]. While Amato et al. [20] found that GQ-16 is a weak adipogenic agent and weak partial agonist in transfections and coactivator binding assays, we had not explored effects upon

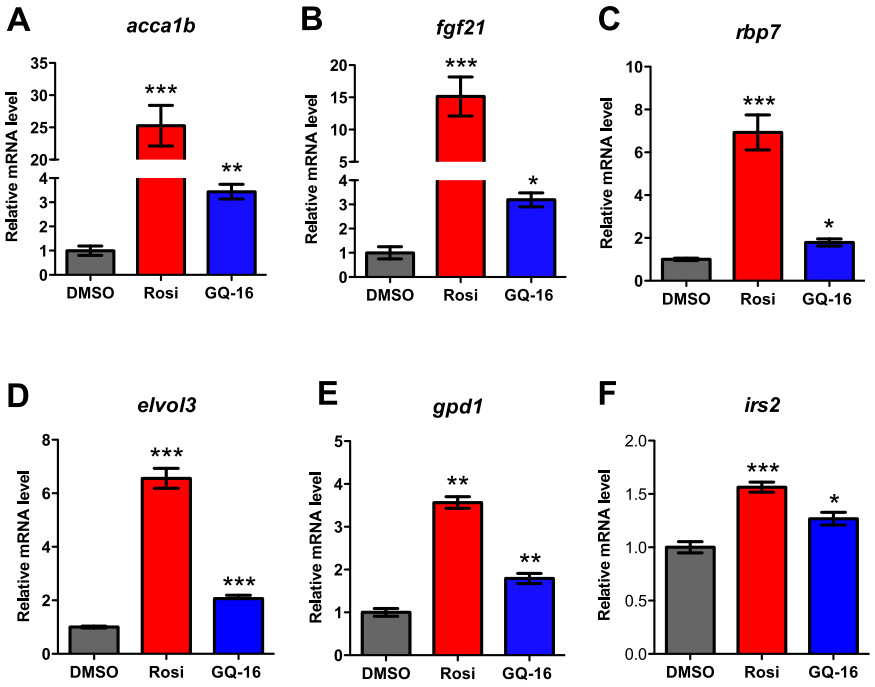

Fig. 2. Validation of microarray analysis. Activated genes. Quantitative RT-PCR (qRT-PCR) analysis of Rosi and/or GQ-16 induction of genes identified in microarray.

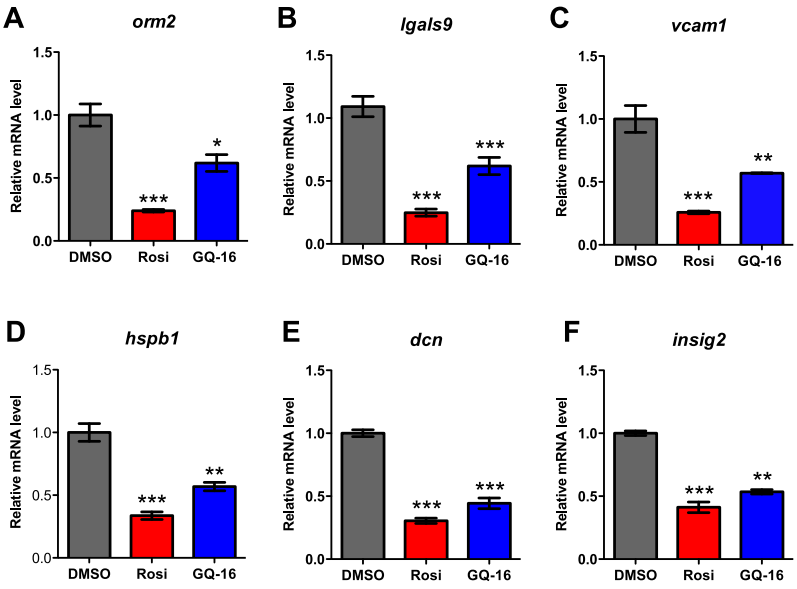

Fig. 3. Validation of microarray analysis. Repressed genes. Representative qPCR analysis showing examples of Rosi or/and GQ-16 repressed genes identified by microarray s.

PPARγ-dependent gene expression programs. We chose to investigate ligand effects in fully differentiated 3T3-L1 adipocytes, to avoid confounding indirect effects of ligand treatment upon PPARγ-dependent differentiation processes. Microarray analysis identified 1156 Rosi responsive transcripts that met cut-offs for significant change ($\geq$1.5 fold; adjusted p-value $\leq$ 0.05) and only 89 by GQ-16 treatment. There was an essentially linear relationship between Rosi and GQ-16 activity with a low slope and was consistent with the idea that GQ-16 is a weak partial agonist. There was, however, significant scatter of GQ-16 responses around the straight line relationship, implting a continuum of weak partial agonist responses that vary on a gene by gene basis. Further, we also detected some genes with disproportionally stronger responses to GQ-16. For example, GQ-16 displayed more than 50% of the efficacy of Rosi at weakly induced genes such as Lhfpl2 and Irs2. We also identified genes that were repressed by GQ-16 to nearly the same extent as Rosi (Fig. 3). These preferential effects of GQ-16 were not related to gene-specific differences in dose response and unrelated partial agonists MRL24 and SR1664 also efficiently repress the same genes.

It is easy to rationalize why GQ-16 behaves as a weak agonist; it does not promote efficient coactivator recruitment [20]. It is less clear why GQ-16 displays a continuum of partial agonist activity, with larger effects at some genes. We suspect that GQ-16 may

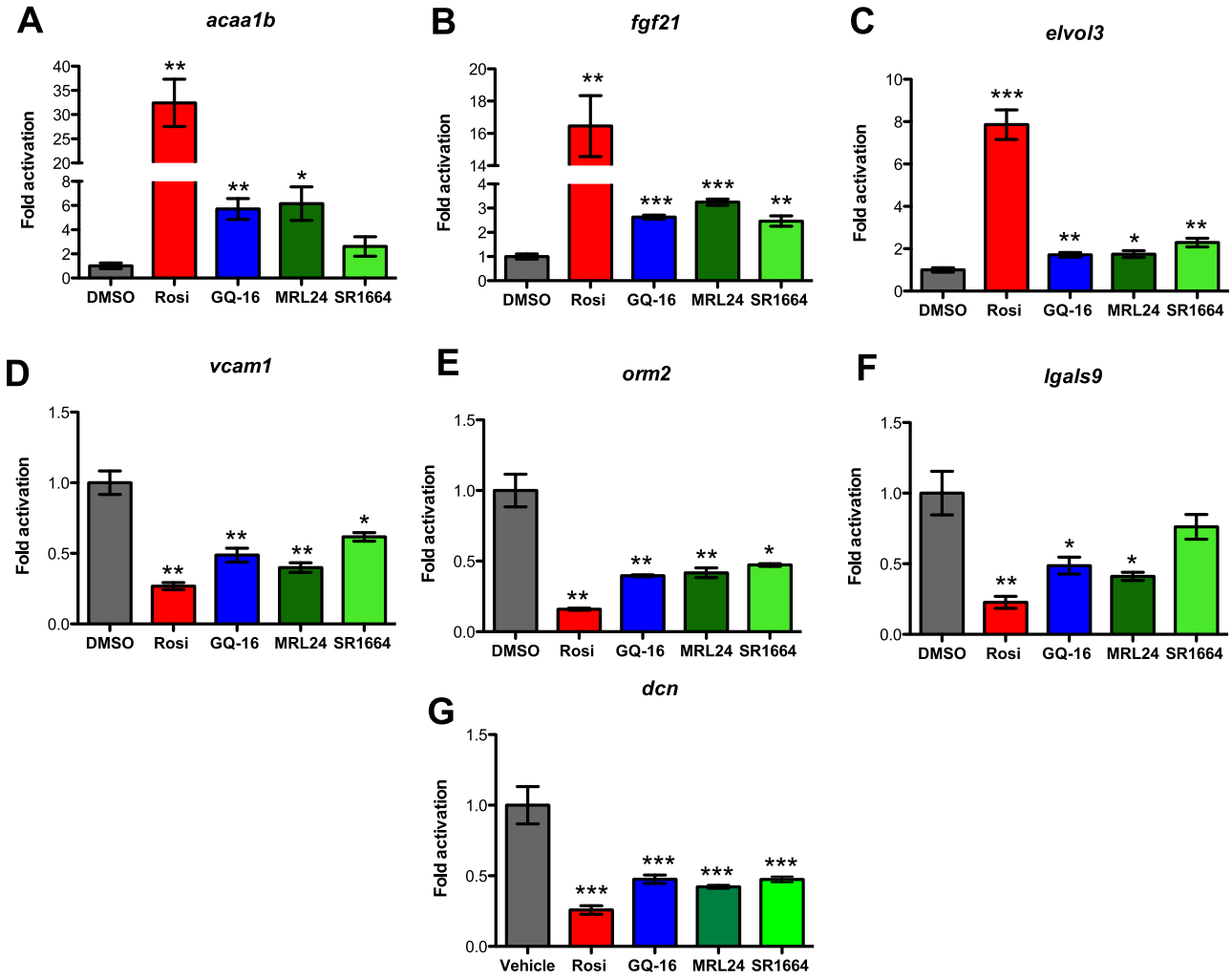

Fig. 4. PPARγ Partial Agonists Display Effective Transrepression. 3T3-L1 cells were differentiated into adipocytes treated with DMSO, Rosi, GQ-16, MRL24 or SR1664 for 24 h. The expression of Acca1b, Fgf21, Elvol3, Orm2 and Legals9 were analyzed by qRT-PCR.

activate genes that are not strongly dependent upon classical coactivator recruitment. Perhaps such genes, including Irs2, display non-classical modes of PPARγ activation as previously suggested [35]. Alternatively, such genes could be sensitive to other mechanisms of ligand-dependent PPARγ activation, proposals include blockade of ser273 phosphorylation [24–26] and variations in corepressor dismissal [36]. Reasons why GQ-16 promotes efficient negative regulation of some PPARγ inhibited genes are less clear. While PPARγ represses pro-inflammatory genes via SUMO-dependent corepressor arrest at target promoters, it is not clear whether GQ-16 efficiently triggers this step at some genes or whether other mechanisms of negative regulation are at play [37].

While this study was designed to detect the likely range of GQ-16 partial agonist activities, it is also important to consider how GQ-16 may influence PPARγ functional outputs [20]. To do this, we cross-referenced datasets with gene sets defined as diagnostic for Rosi agonism or relief of Ser273-dependent repression [24]. We did detect Rosi-specific induction of members of the Rosi agonist set, Idh3A and Abhd12 (Supplementary Tables 2-5), but did not detect GQ-16 effects on the latter gene set, including AdipoQ (Supplementary Figs. 1 and 2). This raises the possibility that GQ-16 partial agonist effects may be independent of relief of inhibitory effects of Ser273 phosphorylation in conditions tested here. We also used GeneCodis software to search for biological processes and pathways that respond selectively to GQ-16 (supplementary data). Gene sets that were GQ-16 specific or differentially regulated by Rosi and GQ-16 (Supplementary Tables 3 and 5) were too small to yield meaningful ontologies. However, analysis of gene sets that respond exclusively to Rosi or to Rosi and GQ-16 (Supplementary Tables 2 and 4) revealed meaningful regulated processes and pathways and possible functional differences between ligands (Supplementary File; GeneCodis). For example, the GO process "metabolic process" was flagged in Rosi and Rosi + GQ-16 datasets, whereas carbohydrate metabolic process was only flagged in the latter. Thus, our data suggest that GQ-16 effects may not exert weak uniform effects on PPARγ responsive gene programs, but would also produce a skewed weak response that preferentially engage subsets of TZD-induced pathways with alterations in adipocyte phenotype.

While it will be important to explore this concept in gene expression studies in animal models, results of our studies of 3T3-L1 adipocytes raise the possibility that the ability of GQ-16 to efficiently repress a subset of negatively regulated PPARγ target genes may be of particular interest [26]. IR is related to an inflammatory adipose tissue phenotype, and PPARγ ligands reverse adipose tissue inflammation via repression of pro-inflammatory target genes in adipocytes, macrophages and other immune cells. Although our studies were not conducted in pro-inflammatory conditions, some

GQ-16 repressed genes are related to inflammatory processes, including orm2 (orosomucoid 2), lgalS9 (Galectin 9), and VCam1 (vascular cell adhesion molecule 1), all of which are involved in cell adhesion and inflammatory processes and hspb1, a heat shock protein involved in stress and tumor necrosis factor α response. It will be interesting to understand how GQ-16 and similar partial agonists influence the onset and establishment of adipose tissue inflammation in animal models.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.07.011.

Transparency document

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