Corepressors selectively control the transcriptional activity of PPARγ in adipocytes

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Peroxisome proliferator-activated receptor γ (PPARγ) is the master regulator of adipogenesis as well as the target of thiazolidinedione (TZD) antidiabetic drugs. Many PPARγ target genes are induced during adipogenesis, but others, such as glycerol kinase (GyK), are expressed at low levels in adipocytes and dramatically up-regulated by TZDs. Here, we have explored the mechanism whereby an exogenous PPARγ ligand is selectively required for adipocyte gene expression. The GyK gene contains a functional PPARγ-response element to which endogenous PPARγ is recruited in adipocytes. However, unlike the classic PPARγ-target gene aP2, which is constitutively associated with coactivators, the GyK gene is targeted by nuclear receptor corepressors in adipocytes. TZDs trigger the dismissal of corepressor histone deacetylase (HDAC) complexes and the recruitment of coactivators to the GyK gene. TZDs also induce PPARγ-Coactivator 1α (PGC-1α), whose recruitment to the GyK gene is sufficient to release the corepressors. Thus, selective modulation of adipocyte PPARγ target genes by TZDs involves the dissociation of corepressors by direct and indirect mechanisms.

[*Keywords*: PPARγ; corepressor; TZD; adipocyte]

Received September 20, 2004; revised version accepted December 17, 2004.

Peroxisome proliferator-activated receptor γ (PPARγ) is the master regulator of adipogenesis (Chawla et al. 1994; Tontonoz et al. 1994a,b; Hu et al. 1995). Like other nuclear receptors (NRs), PPARγ contains a central DNA-binding domain (DBD), a C-terminal ligand-binding domain (LBD), and two transcription-activation function motifs (N-terminal ligand-independent AF-1 motif, C-terminal ligand-dependent AF-2 motif) (Rosen and Spiegelman 2001). PPARγ binds DNA as a heterodimer with retinoid X receptor (RXR), and the heterodimer binds preferentially to direct repeats of the consensus NR half-site spaced by one base pair known as DR-1 motifs. Many adipocyte genes, such as the adipose-specific fatty-acid-binding protein (aP2) (Tontonoz et al. 1994a), contain DR-1-type PPARγ response elements (PPRE) in their promoter regions (for review, see Frohnert et al. 1999).

Potential endogenous ligands for PPARγ include FFAs and eicosanoids, which bind and activate PPARγ with relatively low affinity (Rosen and Spiegelman 2001). The physiological ligand for PPARγ has not been identified, and appears to be expressed at lower levels in mature adipocytes than during early adipogenesis (Tzameli et al. 2004). Of great clinical interest, thiazolidinediones (TZD) antidiabetic drugs have far greater affinity for PPARγ than any known endogenous ligand (Lehmann et al. 1995). Activation of gene expression by TZD binding to PPARγ involves the recruitment of coactivators that function similarly with other NRs, including p160/SRC family members, the mediator complex via Med220 (also known as PBP, TRAP220, and DRIP205), and histone acetyltransferases CBP and p300 (McKenna and O'Malley 2002). Another PPARγ coactivator, PGC-1, is notable for the major role that it plays in metabolic regulation (Puigserver and Spiegelman 2003). Interestingly, whereas most coactivators utilize an LXXLL-containing domain ("NR box") to interact with the LBD of NRs (Heery et al. 1997; McInerney et al. 1998), PGC-1 binds to PPARγ in a ligand-independent manner to the hinge region between the DBD and LBD (Puigserver et al. 1998), overlapping the "CoR box" region required for binding of corepressors (Horlein et al. 1995). Another unique aspect of PGC-1 is its target-gene selectivity, for example, potently coactivating the uncoupling protein-1, but not the aP2 gene (Puigserver et al. 1998).

Corepressors N-CoR (Horlein et al. 1995) and SMRT (Chen and Evans 1995) bind to unliganded NRs and repress target-gene expression until ligand triggers their dismissal accompanied by recruitment of coactivators

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Article published online ahead of print. Article and publication date are at http://www.genesdev.org/cgi/doi/10.1101/gad.1263305.

(Glass and Rosenfeld 2000). N-CoR and SMRT utilize NR-box-like motifs (CoRNR boxes) to bind to a NR surface of unliganded NRs overlapping the surface bound by coactivators (Hu and Lazar 1999; Nagy et al. 1999; Perissi et al. 1999; Xu et al. 2002). PPARγ can interact with both N-CoR and SMRT in solution (Hu et al. 2001), but in some contexts is only a weak repressor (Shi et al. 2002), perhaps because the corepressor binding affinity of PPARγ is weaker than other NRs (Reginato et al. 1998) and is modulated by DNA binding (Zamir et al. 1997).

In contrast to adipocyte-specific genes such as aP2, glycerol kinase (GyK) is normally expressed at low levels in white adipose tissue (Wieland and Suyter 1957; Treble and Mayer 1963; Persico et al. 1975; Sargent et al. 1994), but is markedly up-regulated by TZD treatment of adipocytes (Guan et al. 2002; Tordjman et al. 2003; Patsouris et al. 2004). GyK catalyzes the phosphorylation of glycerol to produce glycerol-3-phosphate, the backbone for esterification of FFAs in the production of triglycerides (TGs). Induction of GyK along with PEPCK by TZD augments the futile cycling FFAs in adipocytes, ultimately decreasing FFA release and contributing to the lowering of serum FFA concentration that is one mechanism by which TZD treatment leads to insulin sensitization (Reshef et al. 2003; Tordjman et al. 2003).

Here, we have dissected the mechanism whereby TZDs are required for the induction of GyK, but not aP2, in mature adipocytes. Induction is blocked by a specific PPARγ antagonist, providing pharmacological evidence that induction of GyK is mediated by PPARγ (Guan et al. 2002). Furthermore, the GyK promoter is TZD-responsive in adipocytes, due to a requisite PPARγ/RXRα-binding site. Remarkably, although endogenous PPARγ and RXRα bind to both the GyK and aP2 gene-regulatory regions in adipocytes, they recruit corepressors to GyK and coactivators to aP2. Treatment with TZD ligand dismisses the corepressors and recruits coactivators to the GyK gene, leading to its transcriptional activation. TZDs also induce PGC-1, which is sufficient to dismiss corepressors and activate GyK expression. Thus, selective modulation of adipocyte PPARγ target genes by TZDs involves differential binding of coregulators and dissociation of corepressors by direct and indirect mechanisms.

Results

The GyK gene contains a PPARγ-response element that is functional in adipocytes

aP2 is dramatically induced during adipogenesis (Ntambi et al. 1988; Yang et al. 1989; Tontonoz et al. 1994b), while GyK expression is only slightly increased in 3T3-L1 adipocytes compared with preadipocytes (Guan et al. 2002). The potent PPARγ agonist rosiglitazone markedly induces GyK in adipoctyes, which is blocked by a competitive PPARγ antagonist, while causing only a minimal further increase in aP2 expression (Guan et al. 2002; Tordjman et al. 2003; Patsouris et al. 2004). The mechanism of rosiglitazone induction of GyK was further investigated by fusing a luciferase reporter to the 5′-flanking region and start site of the mouse GyK gene and determining its activity after transfection into 3T3-L1 adipocytes (Fig. 1A). Truncation to 2009 bp upstream of the GyK start site retained robust activation by rosiglitazone, comparable to that of the PEPCK promoter (Tontonoz et al. 1995; Fig. 1A). However, further truncation to –1931 bp abolished the induction by rosiglitazone, localizing the GyK PPRE to between –2009 and –1931 of the GyK gene.

Inspection of this region revealed an imperfect direct repeat of hexameric NR half-sites separated by one base pair, known as a DR-1 sequence (Fig. 1B). This putative PPRE was conserved in the rat and human GyK promoters and highly related to validated PPREs in the aP2 and PEPCK genes (Tontonoz et al. 1994a, 1995), which are known to be regulated by PPARγ and are also imperfect DR-1 sequences (Fig. 1B). The mouse site was efficiently bound by the PPARγ/RXRα heterodimer (but not by either receptor alone), and this binding was effectively competed by the aP2 PPRE, an idealized DR-1 sequence, the wild-type GyK PPRE, and mutants of the wild-type GyK sequence outside of the core DR-1 (Fig. 1C). In contrast, mutations within the half-sites of the DR-1 core in the GyK, aP2, and PEPCK sequences abolished the competition for binding of PPARγ/RXRα to the wild-type GyK sequence (Fig. 1C). Mutation of the DR-1 half-sites in the context of the –2009 bp GyK promoter abolished transcriptional activation by rosiglitazone in 3T3-L1 adipocytes (Fig. 1D). Together, the DNA binding and transfection studies constitute strong evidence that the GyK DR-1 is a functional PPRE.

Endogenous PPARγ binds to the GyK PPRE in vitro and on the GyK gene in adipocytes

We next investigated whether the GyK PPRE was bound by PPARγ in adipocytes. Incubation of adipocyte nuclear extract with a labeled double-stranded oligonucleotide containing the GyK PPRE resulted in a shifted complex that was effectively competed by wild-type, but not mutant PPRE (Fig. 2A). Antibody to PPARγ supershifted the complex, and antibody that recognizes the DNA-binding domain of RXRα abolished the shift (Fig. 2A), together suggesting that the shifted complex contained the endogenous PPARγ/RXRα heterodimer. The binding of PPARγ in intact adipocytes was assessed by chromatin immunoprecipitation (ChIP). Chromatin preparations from 3T3-L1 preadipocytes and adipocytes were cross-linked, sonicated to shear the DNA to an average size of ~400 bp (Fig. 2B), then precipitated with antibody to PPARγ (or IgG) prior to reversal of cross-linking and PCR of genomic fragments corresponding to the GyK and aP2 promoters. Little PPARγ was detected on either gene in preadipocytes, consistent with the low expression of PPARγ. In adipocytes, ChIP revealed the association of endogenous PPARγ with fragments containing the aP2 and GyK PPREs, but not regions ~2 kb from the PPREs (Fig. 2C). Thus, PPARγ is bound in the region of func-

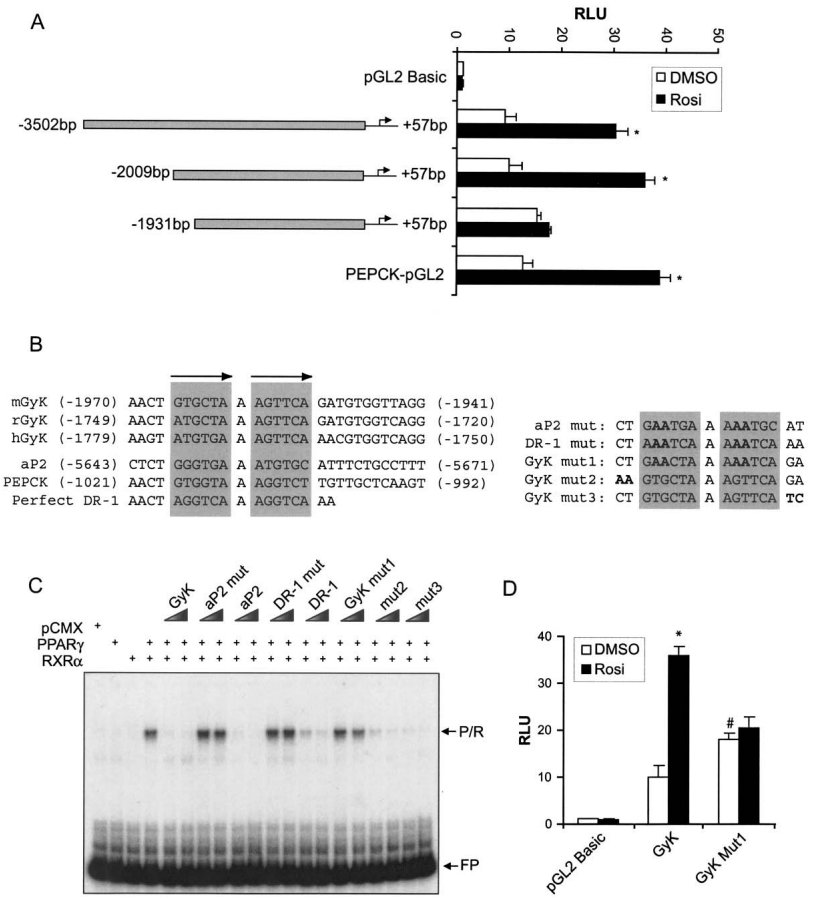

Figure 1. Identification of a functional PPRE in the GyK gene. (*A*) Luciferase reporter assays of transiently transfected 3T3-L1 adipocytes using different truncations of the GyK enhancer/promoter in the pGL2 plasmid vector treated with vehicle (DMSO) or rosiglitazone (rosi). (*) $p < 0.01$ vs. vehicle. (*B*) Alignment of mouse, rat, and human GyK, aP2, PEPCK, and perfect DR-1 PPRE region. (*C*) DNA mobility-shift assay using a ^{32}P-labeled fragment from the GyK enhancer (–2041 to –1886 bp). Competition using cold oligonucleotides containing the GyK, aP2, or ideal DR-1 PPREs, or mutant forms, at 10- and 50-fold molar excess is shown at *right*. Mutation of the PPARγ/RXRα-binding site in the GyK gene abolishes rosiglitazone induction in adipocytes. (P/R) PPARγ/RXRα heterodimer complex; (FP) free probe. (*D*) Luciferase assay of the GyK reporter plasmid (–2009 in Fig. 2A) with (WT) or without (Mut1) the PPRE Mut 1 shown in Figure 2B. (*) $p < 0.001$ vs. vehicle; (#) $p < 0.01$ vs. wild type treated with vehicle. (RLU) Relative luciferase units.

tional PPREs in both the aP2 and GyK promoters in mature adipocytes.

Exogenous PPARγ ligand is required for coactivator recruitment to the GyK gene, but not the aP2 gene

The data thus far demonstrate that the GyK gene contains a functional PPRE to which PPARγ is bound in adipocytes similar to aP2, but does not explain why aP2, but not GyK, is robustly expressed in the mature adipocyte in the absence of a TZD. Consistent with its robust level of expression, the endogenous adipocyte aP2 gene is associated with coactivators including SRC-1, CBP, p300, and Med220, as well as hyperacetylated histones and RNA polymerase II (Fig. 3A). In contrast, although PPARγ and RXRα are associated with the GyK gene in these adipocytes, local histones are hypoacetylated, and there is no major difference in coactivator recruitment to the GyK gene in preadipocytes versus adipocytes. Addition of rosiglitazone leads to coactivator and RNA polymerase II recruitment along with histone acetylation, consistent with the TZD-dependent activation of GyK (Fig. 3B).

PPARγ recruits corepressor complexes to the GyK gene in adipocytes

In addition to abrogating TZD induction, mutation of the GyK PPRE markedly enhanced the basal activity of the GyK gene reporter (Fig. 1D), suggesting that binding of PPARγ actively repressed the gene in the absence of rosiglitazone. ChIP analysis confirmed that endogenous PPARγ was bound to the wild-type GyK promoter, and that this binding was dependent upon the GyK PPRE (Fig. 4A). Moreover, endogenous N-CoR and SMRT are recruited to the wild-type, but not the mutated GyK gene in the absence of exogenous ligand along with histone deacetylase 3 (HDAC3), an integral and functional component of core corepressor complexes (Guenther et al. 2000; Li et al. 2000; Ishizuka and Lazar 2003; Yoon et al. 2003; Fig. 4A). The entire corepressor complex was dismissed from the transfected gene by rosiglitazone (Fig. 4A). Moreover, siRNA knockdown of N-CoR and SMRT increased the basal activity of the GyK promoter in adipocytes (Fig. 4B). Corepressor complexes are also recruited to the endogenous GyK gene in untreated adipocytes, and this was reversed by treatment with rosiglitazone (Fig. 4C). In contrast to the GyK gene, corepressor interaction with the aP2 gene was not detectable above background (Fig. 4C). Furthermore, ChIP reimmunoprecipitation experiments demonstrated that PPARγ was present at the GyK gene in multiprotein complexes containing N-CoR and/or SMRT (Fig. 4D). The effect of rosiglitazone is likely due to dissociation of the corepressor complexes, as the total cellular levels of N-CoR, SMRT, or HDAC3 was similar in both conditions (Fig. 4E). The association of the corepressor HDAC3 complex

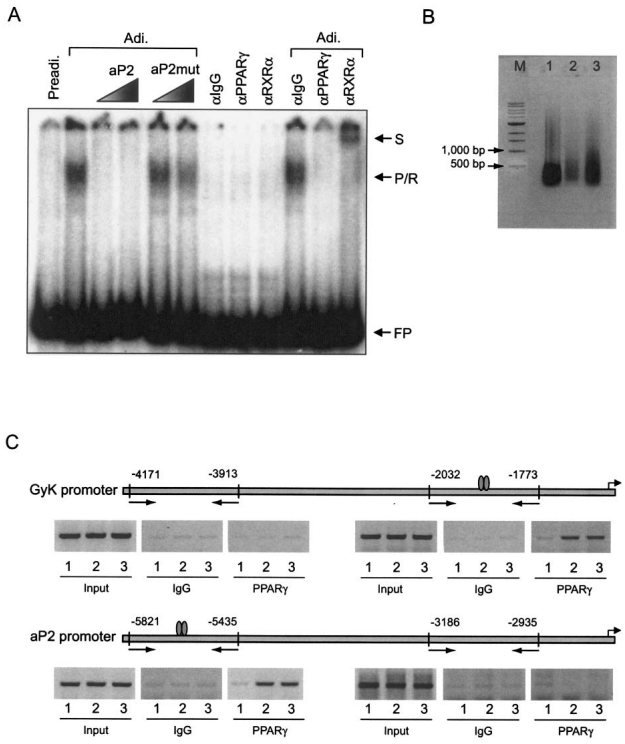

Figure 2. Endogenous adipocyte PPARγ binds to the GyK PPRE in vitro and in living cells. (*A*) DNA mobility-shift assay using nuclear extracts from 3T3-L1 preadipocytes and adipocytes. The same oligonucleotides as in Figure 1B are used. (S) Supershift; (P/R) PPARγ/RXRα; (FP) free probe. (*B*) Sizes of sonicated DNA fragments in preadipocytes, adipocytes treated with DMSO, and rosiglitazone for ChIP analysis. (M) 100-bp marker ladder. (*C*) ChIP analysis of PPARγ association with GyK and aP2 genes. The location of the PPREs and ChIP primers are indicated. (*1*) Preadipocyte. (*2*) Adipocyte treated with DMSO for 48 h. (*3*) Adipocyte treated with rosiglitazone (1 μM for 48 h).

with GyK, but not the aP2 gene, suggested that inhibition of histone deacetylase enzymatic activity might differentially alter expression of these genes. Consistent with this prediction, both trichostatin A (TSA) and sodium butyrate markedly induced gene expression of GyK, with little effect on aP2 (Fig. 4F).

TZDs induce PGC-1α, which activates the GyK gene

The data thus far demonstrate that the adipocyte GyK gene behaves as a classic NR response gene, to which corepressors bind in the absence of ligand and are exchanged for coactivator in the presence of a potent PPARγ ligand. The lack of association of corepressors with the aP2 gene in the normal adipocyte milieu suggests that the conformation of the DNA-bound PPARγ might be different on that gene, with the corepressor-binding site less accessible. Intriguingly, PGC-1 binding overlaps that of corepressors and PGC-1 does not activate the aP2 gene (Puigserver et al. 1998). Furthermore, the expression of PGC-1 is reminiscent of GyK, being very low in white adipose tissue as well as in 3T3-L1 adipocytes, but high in brown adipose tissue (Puigserver et al. 1998). Yet, TZD treatment induces PGC-1α in white adipose tissue in vivo (Wilson-Fritch et al. 2004). Therefore, we considered the potential involvement of PGC-1 in the induction of GyK by PPARγ ligands. PGC-1α is robustly induced by rosiglitazone treatment of 3T3-L1 adipocytes (Fig. 5A). To determine whether PGC-1α expression was sufficient to induce GyK, functionally active, Myc-tagged PGC-1α was ectopically expressed in mature adipocytes by adenoviral delivery (Rhee et al. 2003; Fig. 5B). This was sufficient to induce GyK to nearly the level of rosiglitazone-treated adipocytes (Fig. 5C). Rosiglitazone treatment, in addition to ectopic PGC-1α, led to modest additional stimulation of GyK, likely due to recruitment of a full complement of coactivators, as shown below. Importantly, aP2 expression did not increase significantly due to PGC-1α expression alone or in combination with rosiglitazone (Fig. 5C).

PGC-1 dismisses corepressors and recruits additional coactivators to the GyK gene

We next determined the effect of PGC-1α expression on coregulator association with the GyK gene using ChIP. PGC-1α dramatically reduced corepressor association with the GyK gene in the absence of exogenous ligand, without altering PPARγ binding (Fig. 6A). PGC-1α itself strongly associated with the endogenous GyK gene but, interestingly, not with aP2 (Fig. 6B). Morevoer, PGC-1α was sufficient to recruit coactivators SRC-1 and p300, as

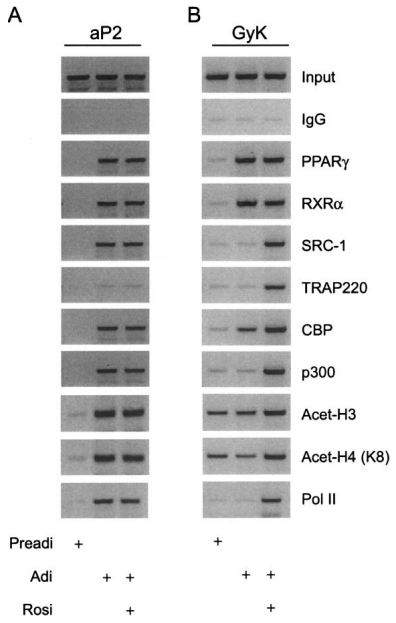

Figure 3. Exogenous ligand is differentially required for coactivator recruitment to PPARγ target genes in adipocytes. ChIP analysis of factor binding and histone acetylation on adipocyte aP2 and GyK genes in preadipocytes and adipocytes ± rosiglitazone treatment. (*A*) aP2. (*B*) GyK.

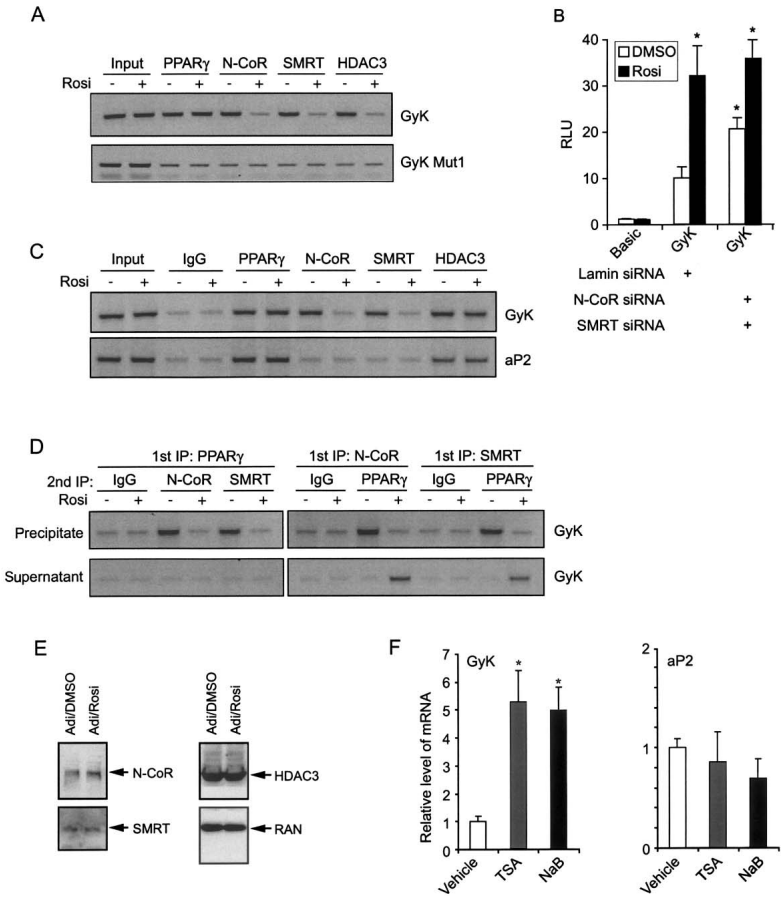

Figure 4. Differential recruitment of corepressors to the aP2 and GyK genes in adipocytes. (*A*) ChIP assay of adipocytes transiently transfected with GyK reporter gene as in Figure 1D. (*B*) Effect of siRNA knockdown of N-CoR and SMRT on basal and rosiglitazone-stimulated expression of GyK-luciferase in 3T3-L1 adipocytes. Lamin siRNA was used as control. (*C*) ChIP assay of endogenous GyK and aP2 genes in adipocytes ± rosiglitazone treatment. (*D*) ChIP re-IP using the supernatants and eluates from ChIP performed for the endogenous GyK gene as in *C*. (*E*) Western blotting showing the protein levels of N-CoR, SMRT, and HDAC3 in adipocytes and rosiglitazone-treated adipocytes. RAN is used as loading control. (*F*) Quantitative real-time PCR measurement of GyK and aP2 mRNA expression in adipocytes treated with vehicle, trichostatin A (TSA, 100 nM) (Shang et al. 2002), and sodium butyrate (NaB, 5 mM) for 48 h. (*) $p < 0.01$ vs. vehicle.

well as RNA polymerase II, to the GyK gene in the absence of rosiglitazone (Fig. 6B). Addition of the potent PPARγ ligand markedly enhanced the SRC-1 binding at the GyK gene (Fig. 6B). Thus, PGC-1α recruitment to the GyK gene is sufficient for activation of the gene, due to corepressor release as well as association of additional coactivators.

Discussion

We have explored the mechanism whereby aP2 and GyK are differentially regulated by adipogenesis per se and a potent exogenous PPARγ ligand, respectively. Both genes contain PPREs bound by PPARγ/RXRα in adipocytes. The key difference is that, in mature adipocytes, PPARγ binding to the aP2 gene is associated with coactivators, histone hyperacetylation, and the presence of RNA polymerase II. In contrast, the GyK gene in the same cellular environment is occupied by corepressors, unless a potent synthetic ligand is available to dismiss corepressors and recruit coactivators. One key coactivator is PGC-1α, which is induced by the ligand, and by itself, is sufficient to displace corepressor binding, recruit additional coactivators, and increase GyK expression.

These findings bring into focus several interesting aspects of gene regulation by PPARγ. First is the apparent importance of corepressor binding in silencing the PPARγ-bound GyK gene in adipocytes. PPARγ binds to both N-CoR and SMRT in solution (Zamir et al. 1997; Lee et al. 2002), and SMRT/N-CoR-derived peptides containing CoRNR motifs bind to PPARγ and are dismissed to varying degrees by different PPARγ ligands (Stanley et al. 2003). Moreover, a synthetic PPARγ antagonist ligand enhances the PPARγ–corepressor interaction (Lee et al. 2002). The recruitment of SMRT/N-CoR to PPARγ at the GyK gene could thus be facilitated by an endogenous molecule with this property. Since the GyK and aP2 enhancers were studied in the same cells, it is likely that the explanation for lack of N-CoR/SMRT binding to the aP2 enhancer is related to the structure of the gene itself. Along these lines, corepressors bind only weakly to the PPARγ/RXRα heterodimer on the acyl CoA oxidase PPRE in the absence of ligand (Zamir et al. 1997), indicating a strong contextual influence on PPARγ–corepressor interaction. However, the difference between GyK and aP2 was not attributable solely to sequence differences in the PPARγ/RXRα-binding site, because replacement of the GyK PPRE with that of aP2 did not significantly alter the basal and rosiglitazone-inducible activities (data not shown). In addition, the GyK promoter was similarly regulated by PPARγ1 and PPARγ2 (data not shown).

It is also possible that the adipocytes contain an endogenous agonist that is sufficient to achieve the activated state of PPARγ on the aP2 gene, but not on GyK,

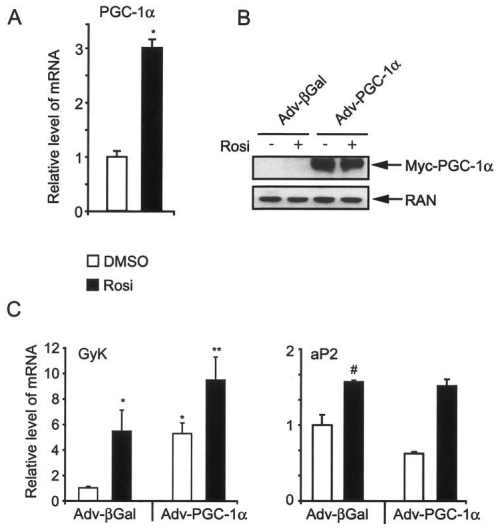

Figure 5. TZD induces PGC-1α and PGC-1α induces GyK in adipocytes. (*A*) Rosiglitazone treatment induces PGC-1α mRNA significantly in adipocytes. (*) $p < 0.001$ vs. vehicle. (*B*) Immunoblot analysis of adenoviral Myc-PGC-1α expression in adipocytes. RAN is used as loading control. (*C*) Adenoviral expression of myc-tagged PGC-1α increases GyK expression, but not aP2, as measured by quantitative real-time PCR. (*) $p < 0.001$ vs. Ad-βGal/vehicle; (**) $p < 0.01$ vs. Adv-PGC-1α/vehicle; (#) $p < 0.05$ vs. Adv/vehicle.

which requires the more potent TZD. Indeed, it has long been assumed that an endogenous ligand is involved in the activation of PPARγ during adipogenesis; a recent study confirms this, but suggests that this ligand is only transiently expressed and is absent from mature adipocytes (Tzameli et al. 2004). Consistent with this, the competitive PPARγ ligand PD068235 does not reduce aP2 gene expression in adipocytes (Camp et al. 2001; Guan et al. 2002). In that scenario, the association of PPARγ/RXRα with the actively transcribed aP2 gene could be constitutively active by virtue of its N-terminal activation domain (Adams et al. 1997; Werman et al. 1997). Indeed, the PPARγ N terminus interacts with p300 (Gelman et al. 1999), which is among the coactivators that we localized to the aP2 gene in the adipocyte. Although not regulated by ligand, this constitutive activity can be regulated by insulin (Werman et al. 1997) as well as phosphorylation by MAP kinase (Adams et al. 1997). The N-terminal activity may also be mediated by a less well-characterized coactivator, PGC-2 (Castillo et al. 1999), whose recruitment could not be assessed by these studies because an antibody is not available.

PGC-1α is induced by TZD and is a strong coactivator for PPARγ bound at the GyK gene. Our findings are consistent with the previous findings that PGC-1 is not a coactivator for PPARγ on the aP2 gene and is normally expressed at low levels in white adipocytes (Puigserver et al. 1998). The sufficiency of PGC-1α in terms of corepressor dismissal and coactivator recruitment in the absence of TZD is consistent with the observation that PGC-1α binds in a ligand-independent manner to a region of PPARγ that is critical for corepressor interaction (Puigserver et al. 1998), and docks with coactivators SRC-1 and p300 (Puigserver et al. 1999). Note that the effects of rosiglitazone and overexpressed PGC-1α were additive, due in part to increased recruitment of coactivators such as SRC-1, indicating that PPARγ ligands induce GyK by direct as well as indirect mechanisms.

Based on these findings, we propose a model for the differential regulation of aP2 and GyK expression during adipogenesis and in response to rosiglitazone treatment of adipocytes (Fig. 7). PPARγ/RXRα heterodimers are recruited to PPREs in both the aP2 and GyK genes in adipocytes, but are associated with different sets of coregulators; corepressors N-CoR/SMRT are recruited along with HDAC3 to GyK, whereas coactivators are recruited to aP2. Addition of a potent exogenous PPARγ ligand not only triggers the conformational change that is classic for the other RXR heterodimer receptors such as TR and RAR, but also induces PGC-1α expression, which specifically binds to PPARγ on the GyK promoter, facilitating corepressor dismissal and coactivator recruitment. This may be critical for PPARγ more than for other NRs, since in some contexts, corepressor binding to PPARγ appears to be less avid and ligand reversible than for other NRs (Reginato et al. 1998). The insulin-responsive glucose transporter GLUT4 is likely to be regulated in a manner similar to GyK, as it is repressed by PPARγ but activated by rosiglitazone treatment of adipocytes (Armoni et al. 2003).

The differential regulation of PPARγ gene targets in adipocytes is reminiscent of the estrogen receptor (ER) in breast cancer cells, wherein ER target genes can be repressed or activated by differential recruitment of corepressors and coactivators, respectively, by selective ER modulators such as tamoxifen (Shang and Brown 2002).

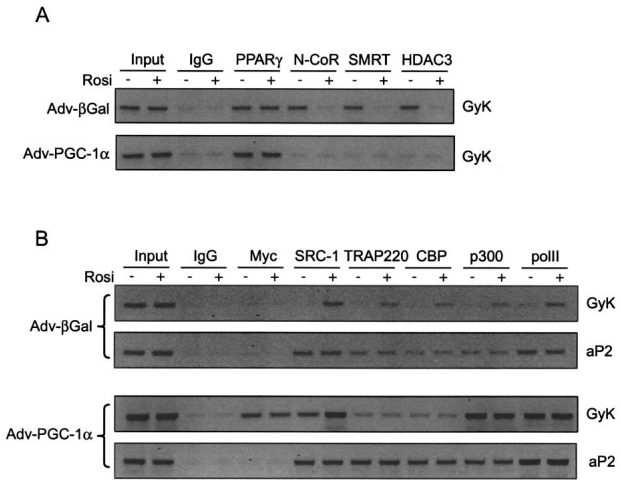

Figure 6. PGC-1α expression facilitates corepressor dismissal on the GyK promoter in adipocytes. (*A*) ChIP analysis for PPARγ and corepressors on endogenous GyK gene in adipocytes infected with adenoviral Myc-PGC-1α or β-galactosidase (βGal) ± rosiglitazone. (*B*) ChIP analysis for coactivators on endogenous GyK and aP2 genes in adipocytes infected with adenoviral Myc-PGC-1α or βGal ± rosiglitazone.

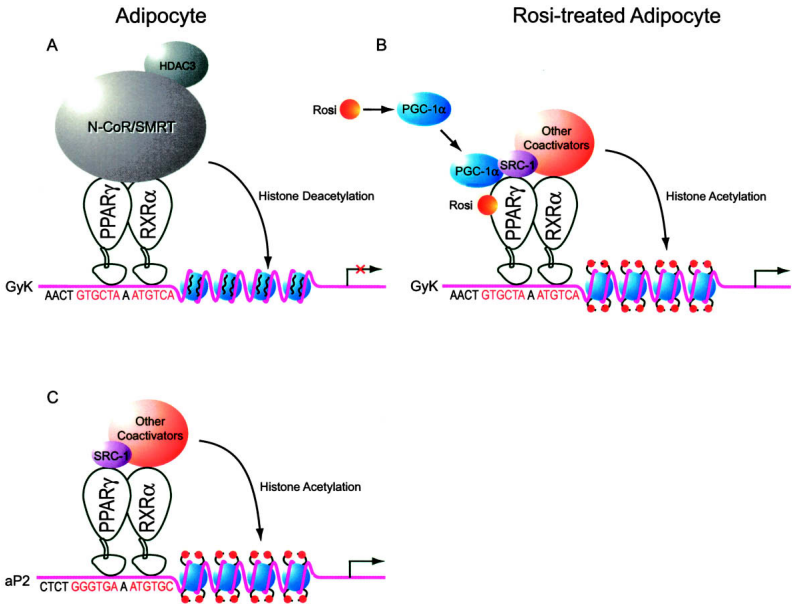

Figure 7. Model of the molecular mechanisms underlying differential ligand requirements for PPARγ activation in adipocytes. In the absence of exogenous PPARγ ligand, PPARγ recruits corepressors N-CoR/SMRT or coactivators to the GyK (*A*) and aP2 (*C*) promoters, respectively. Thus, the expression of GyK is repressed and the basal level of GyK is very low, but aP2 expression is high in mature adipocytes. (*B*) Treatment of adipocytes with rosiglitazone induces GyK in adipocytes by two mechanisms. The direct mechanism involves triggering a conformational change in PPARγ, causing corepressor release and coactivator recruitment. The indirect mechanism involves induction of PGC-1α, which destabilizes the binding of corepressors.

Selective PPARγ modulators that block corepressor interaction with PPARγ without recruiting coactivators to all PPARγ gene targets might prove to be useful therapeutic agents that induce genes related to insulin sensitivity while minimizing the side effects, including adipogenesis, that limit the efficacy of TZD drugs. Indeed, the surprisingly insulin-sensitive phenotype of mice lacking one allele of PPARγ (Kubota et al. 1999; Miles et al. 2000) is plausibly explained on the basis of reduced corepressor activity on PPARγ on target genes.

Materials and methods

RNA analysis

Adipocytes were differentiated as described previously (Guan et al. 2002). Eight days post-differentiation, adipocytes were treated with the respective reagents (see figure legends) and total RNA was extracted for real time PCR by TRIzol reagent (Invitrogen) or RNeasy Mini kit (Qiagen). Primers and probes used in the real time PCR were as follows: GyK forward primer, 5′-CGGAGACCAGCCGTGTTAAG-3′; GyK reverse primer, 5′-GTCCACTGCTCCCACCAATG-3′; GyK Probe, 5′-CT GACTGACTTCCATGGCAGCCG-3′; aP2 forward primer, 5′-AAGTGGGAGTGGGCTTTGC-3′; aP2 reverse primer, 5′-CC GGATGGTGACCAAATCC-3′; aP2 probe, 5′-CAGGCATGGC CAAGCCCAACA-3′; 36B4 forward primer, 5′-TCATCCAG CAGGTGTTTGACA-3′; 36B4 reverse primer, 5′-GGCACC GAGGCAACAGTT-3′; 36B4 probe, 5′-AGAGCAGGCCCTG CACTCTCG-3′; PGC-1α (Applied Biosystem).

EMSA

The DNA mobility-shift assays were performed as described previously (Zamir et al. 1997). Two oligonucleotides were used for DNA mobility-shift assay of mouse GyK PPRE. One was the 155-bp product digested by EcoRI and XbaI, which is radiolabeled by Klenow and [α-^{32}P]dATP (Roche Diagnostics). The other was annealed oligonucleotide 5′-AACTGTGCTA AAGTTCAGATGTGGTTAGG-3′ end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (New England Biolabs). For DNA mobility-shift assay of human and rat GyK, 5′-AAGTATGT GAAAGTTCAAACGTGGTCAGG-3′ and 5′-AACTATGCTA AAGTTCAGATGTGGTCAGG-3′ were used, respectively. Recombinant PPARγ/RXRα was produced by in vitro translation using the TNT kit (Promega). Nuclear extracts were prepared using nuclear extract lysis buffer according to the manufacturer's instructions (BD Biosciences Clonetech).

ChIP assays

The chromatin immunoprecipitation was modified from Hartman et al. (2002) as follows: Sonication condition: Set power at 6, sonicate for 14 sec for four times. Total DNA of 400 μg/mL was used for immunoprecipitation. The following antibodies were used for immuniprecipitation: normal rabbit IgG, PPARγ, RXRα, SRC-1, TRAP220, CBP and p300 (Santa Cruz Biotechnology), acetylated Histone H3, acetylated Histone H4 (Upstate Biotechnology), RNA polymerase II (Covance), N-CoR and SMRT (Affinity Bioreagents), HDAC3 (Abcam or Upstate Biotechnology), Myc (Abcam). Total DNA concentrations in chromatin immunoprecipitates were quantitated by PicoGreen dsDNA Quantitation Reagent (Molecular Probes). Primers used for ChIP assays are as follows: aP2 forward primer, 5′-ATGT CACAGGCATCTTATCCACC-3′; aP2 reverse primer, 5′-AA CCCTGCCAAAGAGACAGAGG-3′; GyK forward primer, 5′-CGGAATTCTGATCCCTACTGTGC-3′; GyK reverse primer for endogenous gene and transient transfection ChIP, 5′-GA CACTAGGCCAACTTCTCTGTCAA-3′; Forward primer for transient transfection ChIP (in pGL2 plasmid), 5′-TGTGGTTT GTCCAAACTCATCAATG-3′.

Gene transduction and luciferase assays

3T3-L1 fibroblasts were cultured and differentiated in 12-well plates. Eight days post-differentiation, transient transfections were performed by LipofectAMINE 2000 reagent (Invitrogen) (Frohnert et al. 1999). The reporter plasmids were constructed by inserting PCR products of GyK gene BAC plasmid (Mouse RPCI.22 BAC clones 393 P 18 and 551 O 9 screened by ResGen)

into SmaI site in pGL2-Basic upstream of luciferase initiation site (Promega). Point mutation of GyK PPRE was achieved by Site-directed Mutagenesis kit (Stratagene). Luciferase reporters driven by the PEPCK (~1 kb) and aP2 (~6 kb; data not shown) promoters were used as controls. Two micrograms of reporter plasmids per well were used for transient transfection. Transfection efficiency of 2%~3% was confirmed by eGFP plasmid. β-Galactosidase (β-Gal; 0.5 μg per well) was used for transfection normalization. All of the experiments were done in triplicate. Twenty-four hours after the transfection, the cells were then refed with DMEM containing 10% stripped FBS (Gemini Bioproducts) and treated with either 1 μM rosiglitazone or DMSO for 48 h. Luciferase activities were analyzed by Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. For siRNA experiments, hairpin nucleotides corresponding to N-CoR (AAGAAGGATCCGGCATTTGGA) and SMRT (AAGCTGAAGAAGAAGCAGCAA) were annealed and subcloned into BLOCK-iT U6 RNAi entry vector (Invitrogen). 3T3-L1 adipocytes were transiently transfected with reporter vectors along with the N-CoR and SMRT RNAi vectors. Human lamin siRNA vector was used as control. Efficient knockdown of N-CoR and SMRT was verified in parallel experiments using AML12 mouse hepatoma cells with high-transfection efficiency (data not shown). Results are presented as the mean of triplicate samples ± SD. Statistical significance was determined by Student's *t*-test comparison. Each experiment was repeated at least three times. Adenoviral infections was performed as reported (Michael et al. 2001) at a multiplicity of infection resulting in >95% infection as judged by adeno-GFP.

Acknowledgments

We thank all of the Lazar lab members for helpful discussions, J.-E. Kim for troubleshooting of the ChIP technique, and Dr. Myles Brown for helpful suggestions and comments. This work was funded by NIDDK R01 DK49780, and an unrestricted Bristol Myers Squibb Freedom to Discover Award in Metabolic Research (M.A.L.).

References

Adams, M., Reginato, M.J., Shao, D., Lazar, M.A., and Chatterjee, V.K. 1997. Transcriptional activation by PPARγ is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site. *J. Biol. Chem.* **272:** 5128–5132.

Armoni, M., Kritz, N., Harel, C., Bar-Yoseph, F., Chen, H., Quon, M.J., and Karnieli, E. 2003. Peroxisome proliferator-activated receptor-γ represses GLUT4 promoter activity in primary adipocytes, and rosiglitazone alleviates this effect. *J. Biol. Chem.* **278:** 30614–30623.

Camp, H.S., Chaudhry, A., and Leff, T. 2001. A novel potent antagonist of peroxisome proliferator-activated receptor γ blocks adipocyte differentiation but does not revert the phenotype of terminally differentiated adipocytes. *Endocrinology* **142:** 3207–3213.

Castillo, G., Brun, R.P., Rosenfield, J.K., Hauser, S., Park, C.W., Troy, A.E., Wright, M.E., and Spiegelman, B.M. 1999. An adipogenic cofactor bound by the differentiation domain of PPARγ. *EMBO J.* **18:** 3676–3687.

Chawla, A., Schwarz, E.J., Dimaculangan, D.D., and Lazar, M.A. 1994. Peroxisome proliferator-activated receptor γ (PPARγ): Adipose predominant expression and induction early in adipocyte differentiation. *Endocrinology* **135:** 798–800.

Chen, J.D. and Evans, R.M. 1995. A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature* **377:** 454–457.

Frohnert, B.I., Hui, T.Y., and Bernlohr., D.A. 1999. Identification of a functional peroxisome proliferator-responsive element in the murine fatty acid transport protein gene. *J. Biol. Chem.* **274:** 3970–3977.

Gelman, L., Zhou, G., Fajas, L., Raspe, E., Fruchart, J.C., and Auwerx, J. 1999. p300 interacts with the N- and C-terminal part of PPARγ2 in a ligand-independent and -dependent manner, respectively. *J. Biol. Chem.* **274:** 7681–7688.

Glass, C.K. and Rosenfeld, M.G. 2000. The coregulator exchange in transcriptional functions of nuclear receptors. *Genes & Dev.* **14:** 121–141.

Guan, H.P., Li, Y., Jensen, M.V., Newgard, C.B., Steppan, C.M., and Lazar, M.A. 2002. A futile metabolic cycle activated in adipocytes by antidiabetic agents. *Nat. Med.* **8:** 1122–1128.

Guenther, M.G., Lane, W.S., Fischle, W., Verdin, E., Lazar, M.A., and Shiekhattar, R. 2000. A core SMRT corepressor complex containing HDAC3 and a WD40 repeat protein linked to deafness. *Genes & Dev.* **14:** 1048–1057.

Hartman, H.B., Hu, X., Tyler, K.X., Dalal, C.K., and Lazar, M.A. 2002. Mechanisms regulating adipocyte expression of resistin. *J. Biol. Chem.* **277:** 19754–19761.

Heery, D.M., Kalkhoven, E., Hoare, S., and Parker, M.G. 1997. A signature motif in transcriptional co-activators mediates binding to nuclear receptors. *Nature* **387:** 733–736.

Horlein, A.J., Naar, A.M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C.K., et al. 1995. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor corepressor. *Nature* **377:** 397–404.

Hu, X. and Lazar, M.A. 1999. The CoRNR motif contols the recruitment of corepressors to nuclear hormone receptors. *Nature* **402:** 93–96.

Hu, E., Tontonoz, P., and Spiegelman, B.M. 1995. Transdifferentiation of myoblasts by the adipogenic transcription factors PPARγ and C/EBPα. *Proc. Natl. Acad. Sci.* **92:** 9856–9860.

Hu, X., Li, Y., and Lazar, M.A. 2001. Determinants of CoRNR-dependent repression complex assembly on nuclear hormone receptors. *Mol. Cell. Biol.* **21:** 1747–1758.

Ishizuka, T. and Lazar, M.A. 2003. The N-CoR/HDAC3 deacetylase complex is required for repression by thyroid hormone receptor. *Mol. Cell. Biol.* **23:** 5122–5131.

Kubota, N., Terauchi, Y., Miki, H., Tamemoto, H., Yamauchi, T., Komeda, K., Satoh, S., Nakano, R., Ishii, C., Sugiyama, T., et al. 1999. PPAR γ mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. *Mol. Cell* **4:** 597–609.

Lee, G., Elwood, F., McNally, J., Weiszmann, J., Lindstrom, M., Amaral, K., Nakamura, M., Miao, S., Cao, P., Learned, R.M., et al. 2002. T0070907, a selective ligand for peroxisome proliferator-activated receptor γ, functions as an antagonist of biochemical and cellular activities. *J. Biol. Chem.* **277:** 19649–19657.

Lehmann, J.M., Moore, L.B., Smith-Oliver, T.A., Wilkison, W.O., Willson, T.M., and Kliewer, S.A. 1995. An antidiabetic thiazolidinedione is a high affinity ligand for the nuclear peroxisome proliferator-activated receptor γ (PPARγ). *J. Biol. Chem.* **270:** 12953–12956.

Li, J., Wang, J., Nawaz, Z., Liu, J.M., Qin, J., and Wong, J. 2000. Both corepressor proteins SMRT and N-CoR exist in large protein complexes containing HDAC3. *EMBO J.* **19:** 4342–4350.

McInerney, E.M., Rose, D.W., Flynn, S.E., Westin, S., Mullen, T.M., Krones, A., Inostroza, J., Torchia, J., Nolte, R.T., Assa-Munt, N., et al. 1998. Determinants of coactivator LXXLL

motif specificity in nuclear receptor transcriptional activation. *Genes & Dev.* **12:** 3357–3368.

McKenna, N.J. and O'Malley, B.W. 2002. Minireview: Nuclear receptor coactivators—An update. *Endocrinology* **143:** 2461–2465.

Michael, L.F., Wu, Z., Cheatham, R.B., Puigserver, P., Adelmant, G., Lehman, J.J., Kelly, D.P., and Spiegelman, B.M. 2001. Restoration of insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1. *Proc. Natl. Acad. Sci.* **98:** 3820–3825.

Miles, P.D., Barak, Y., He, W., Evans, R.M., and Olefsky, J.M. 2000. Improved insulin-sensitivity in mice heterozygous for PPAR-γ deficiency. *J. Clin. Invest.* **105:** 287–292.

Nagy, L., Kao, H.Y., Love, J.D., Li, C., Banayo, E., Gooch, J.T., Krishna, V., Chatterjee, K., Evans, R.M., and Schwabe, J.W. 1999. Mechanism of corepressor binding and release from nuclear hormone receptors. *Genes & Dev.* **13:** 3209–3216.

Ntambi, J.M., Buhrow, S.A., Kaestner, K.H., Christy, R.J., Sibley, E., Kelly, T., and Lane, M.D. 1988. Differentiation-induced gene expression in 3T3-L1 adipocytes. *J. Biol. Chem.* **263:** 17297–17300.

Patsouris, D., Mandard, S., Voshol, P.J., Escher, P., Tan, N.S., Havekes, L.M., Koenig, W., Marz, W., Tafuri, S., Wahli, W., et al. 2004. PPARα governs glycerol metabolism. *J. Clin. Invest.* **114:** 94–103.

Perissi, V., Staszewski, L.M., McInerney, E.M., Kurokawa, R., Krones, A., Rose, D.W., Lambert, M.H., Milburn, M.V., Glass, C.K., and Rosenfeld, M.G. 1999. Molecular determinants of nuclear receptor–corepressor interaction. *Genes & Dev.* **13:** 3198–3208.

Persico, P.A., Cerchio, M., and Jeffay. H. 1975. Glycerokinase in mammalian adipose tissue: Stimulation by lipogenic substances. *Am. J. Physiol.* **228:** 1868–1874.

Puigserver, P. and Spiegelman, B.M. 2003. Peroxisome proliferator-activated receptor-γ coactivator 1 α (PGC-1 α): Transcriptional coactivator and metabolic regulator. *Endocr. Rev* **24:** 78–90.

Puigserver, P., Wu, Z., Park, C.W., Graves, R., Wright, M., and Spiegelman, B.M. 1998. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* **92:** 829–839.

Puigserver, P., Adelmant, G., Wu, Z., Fan, M., Xu, J., O'Malley, B., and Spiegelman, B.M. 1999. Activation of PPARγ coactivator-1 through transcription factor docking. *Science* **286:** 1368–1371.

Reginato, M.J., Krakow, S.L., Bailey, S.T., and Lazar, M.A. 1998. Prostaglandins promote and block adipogenesis through opposing effects on peroxisome proliferator-activated receptor γ. *J. Biol. Chem.* **273:** 1855–1858.

Reshef, L., Olswang, Y., Cassuto, H., Blum, B., Croniger, C.M., Kalhan, S.C., Tilghman, S.M., and Hanson, R.W. 2003. Glyceroneogenesis and the triglyceride/fatty acid cycle. *J. Biol. Chem.* **278:** 30413–30416.

Rhee, J., Inoue, Y., Yoon, J.C., Puigserver, P., Fan, M., Gonzalez, F.J., and Spiegelman, B.M. 2003. Regulation of hepatic fasting response by PPARγ coactivator-1α (PGC-1): Requirement for hepatocyte nuclear factor 4α in gluconeogenesis. *Proc. Natl. Acad. Sci.* **100:** 4012–4017.

Rosen, E.D. and Spiegelman, B.M. 2001. PPARγ: A nuclear regulator of metabolism, differentiation, and cell growth. *J. Biol. Chem.* **276:** 37731–37734.

Sargent, C.A., Young, C., Marsh, S., Ferguson-Smith, M.A., and Affara, N.A. 1994. The glycerol kinase gene family: Structure of the Xp gene, and related intronless retroposons. *Hum. Mol. Genet.* **3:** 1317–1324.

Shang, Y. and Brown, M. 2002. Molecular determinants for the tissue specificity of SERMs. *Science* **295:** 2465–2468.

Shang, Y., Myers, M., and Brown, M. 2002. Formation of the androgen receptor transcription complex. *Mol. Cell* **9:** 601–610.

Shi, Y., Hon, M., and Evans, R.M. 2002. The peroxisome proliferator-activated receptor δ, an integrator of transcriptional repression and nuclear receptor signaling. *Proc. Natl. Acad. Sci.* **99:** 2613–2618.

Stanley, T.B., Leesnitzer, L.M., Montana, V.G., Galardi, C.M., Lambert, M.H., Holt, J.A., Xu, H.E., Moore, L.B., Blanchard, S.G., and Stimmel, J.B. 2003. Subtype specific effects of peroxisome proliferator-activated receptor ligands on corepressor affinity. *Biochemistry* **42:** 9278–9287.

Tontonoz, P., Hu, E., Graves, R.A., Budavari, A.I., and Spiegelman, B.M. 1994a. mPPARγ2: Tissue-specific regulator of an adipocyte enhancer. *Genes & Dev.* **8:** 1224–1234.

Tontonoz, P., Hu, E., and Spiegelman, B.M. 1994b. Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. *Cell* **79:** 1147–1156.

Tontonoz, P., Hu, E., Devine, J., Beale, E.G., and Spiegelman, B.M. 1995. PPARγ2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene. *Mol. Cell. Biol.* **15:** 351–357.

Tordjman, J., Chauvet, G., Quette, J., Beale, E.G., Forest, C., and Antoine, B. 2003. Thiazolidinediones block fatty acid release by inducing glyceroneogenesis in fat cells. *J. Biol. Chem.* **278:** 18785–18790.

Treble, D.H. and Mayer, J. 1963. Glycerolkinase activity in white adipose tissue of obese-hyperglycaemic mice. *Nature* **200:** 363–364.

Tzameli, I., Fang, H., Ollero, M., Shi, H., Hamm, J.K., Kievit, P., Hollenberg, A.N., and Flier, J.S. 2004. Regulated production of a peroxisome proliferator-activated receptor-γ ligand during an early phase of adipocyte differentiation in 3T3-L1 adipocytes. *J. Biol. Chem.* **279:** 36093–36102.

Werman, A., Hollenberg, A., Solanes, G., Bjorbaek, C., Vidal-Puig, A.J., and Flier, J.S. 1997. Ligand-independent activation domain in the N terminus of PPARγ. Differential activity of PPARγ1and 2 isoforms and influence of insulin. *J. Biol. Chem.* **272:** 20230–20235.

Wieland, O. and Suyter, M. 1957. [Glycerokinase: Isolation and properties of the enzyme.] *Biochem. Z* **329:** 320–331.

Wilson-Fritch, L., Nicoloro, S., Chouinard, M., Chui, P.C., Lazar, M.A., Leszyk, J, Straubhaar, J., Czech, M., and Corvera, S. 2004. Mitochondrial remodeling in adipose tissue associated with obesity and treatment with rosiglitazone. *J. Clin. Invest.* **114:** 1281–1289.

Xu, H.E., Stanley, T.B., Montana, V.G., Lambert, M.H., Shearer, B.G., Cobb, J.E., McKee, D.D., Galardi, C.M., Plunket, K.D., Nolte, R.T., et al. 2002. Structural basis for antagonist-mediated recruitment of nuclear co-repressors by PPARα. *Nature* **415:** 813–817.

Yang, V.W., Christy, R.J., Cook, J.S., Kelly, T.J., and Lane, M.D. 1989. Mechanism of regulation of the 422(aP2) gene by cAMP during preadipocyte differentiation. *Proc. Natl. Acad. Sci.* **86:** 3629–3633.

Yoon, H.G., Chan, D.W., Huang, Z.Q., Li, J., Fondell, J.D., Qin, J., and Wong, J. 2003. Purification and functional characterization of the human N-CoR complex: The roles of HDAC3, TBL1 and TBLR1. *EMBO J.* **22:** 1336–1346.

Zamir, I., Zhang, J., and Lazar, M.A. 1997. Stoichiometric and steric principles govering repression by nuclear hormone receptors. *Genes & Dev.* **11:** 835–846.