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Proteasomal degradation of retinoid X receptor α reprograms transcriptional activity of PPAR γ in obese mice and humans

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Obese patients have chronic, low-grade inflammation that predisposes to type 2 diabetes and results, in part, from dysregulated visceral white adipose tissue (WAT) functions. The specific signaling pathways underlying WAT dysregulation, however, remain unclear. Here we report that the PPAR γ signaling pathway operates differently in the visceral WAT of lean and obese mice. PPAR γ in visceral, but not subcutaneous, WAT from obese mice displayed increased sensitivity to activation by its agonist rosiglitazone. This increased sensitivity correlated with increased expression of the gene encoding the ubiquitin hydrolase/ligase ubiquitin carboxyterminal esterase L1 (UCH-L1) and with increased degradation of the PPAR γ heterodimerization partner retinoid X receptor α (RXR α), but not RXR β , in visceral WAT from obese humans and mice. Interestingly, increased UCH-L1 expression and RXR α proteasomal degradation was induced in vitro by conditions mimicking hypoxia, a condition that occurs in obese visceral WAT. Finally, PPAR γ -RXR β heterodimers, but not PPAR γ -RXR α complexes, were able to efficiently dismiss the transcriptional corepressor silencing mediator for retinoid and thyroid hormone receptors (SMRT) upon agonist binding. Increasing the RXR α /RXR β ratio resulted in increased PPAR γ responsiveness following agonist stimulation. Thus, the selective proteasomal degradation of RXR α initiated by UCH-L1 upregulation modulates the relative affinity of PPAR γ heterodimers for SMRT and their responsiveness to PPAR γ agonists, ultimately activating the PPAR γ -controlled gene network in visceral WAT of obese animals and humans.

Introduction

From a clinical perspective, visceral obesity predisposes to an increased incidence of type 2 diabetes mellitus (T2DM) and associated cardiovascular diseases (1, 2). The visceral white adipose tissue (visWAT; i.e., epididymal WAT) depot is believed to contribute to the low-grade, chronic inflammatory state that occurs in obese patients and animals and favors the progression toward T2DM. This feature stems from the specific functional properties of adipocytes from this WAT depot, which are highly sensitive to β -adrenergic stimulation and relatively resistant to the antilipolytic effects of insulin compared with subcutaneous adipocytes (3). Indeed, although subcutaneous WAT (scWAT; i.e., inguinal WAT) is predominantly, but not exclusively, a lipid storage tissue exhibiting a high adipocyte plasticity, visWAT also triggers complex endocrine regulations by releasing FFAs, hormones, and cytokines that reach the liver through the portal vein (reviewed in ref. 4). How visWAT functions are affected upon disease progression is unknown, but metabolic challenges increase the release of proinflammatory cytokines and decrease that of insulin-sensitizing adipokines by visWAT.

Results from recent clinical trials (ADOPT, DREAM, and PRO-ACTIVE; ref. 5) indicate that the insulin-sensitizing thiazolidin-

ediones (TZDs) are highly efficient in maintaining glycemic control, and may exert pancreas-sparing and vascular-protective effects in T2DM patients. TZDs are synthetic agonists for PPAR γ (also known as NR1C3), a member of the nuclear receptor (NR) superfamily. PPAR γ is a key regulator of adipocyte differentiation and lipid storage, thereby exerting major effects on energy homeostasis (6). Gene ablation studies have confirmed the major role of adipocyte PPAR γ (adPPAR γ) in mediating the insulin-sensitizing effect of TZDs in obese mice (7–12). Ligand-activated adPPAR γ has a positive effect on glucose homeostasis by favoring scWAT expansion and FFA redistribution to this fat depot (13). Removing FFA from other tissues, including skeletal muscle and visWAT, prevents the so-called lipotoxic effect, which causes insulin resistance and hence translates into a greater insulin sensitivity. TZDs also act directly on visWAT functions by regulating the expression of adipokines (14) and several key metabolic genes (15). Quite intriguingly, however, TZDs exert neither detectable insulin-sensitizing effects nor significant metabolic effects in lean individuals and mice, which suggests that the PPAR γ pathway operates differently in normal and pathological conditions (16–18).

PPAR γ activates target gene transcription by forming obligate heterodimers with the retinoid X receptor (RXR) isotypes – RXR α , RXR β , and RXR γ – onto PPAR-responsive elements (PPREs). PPREs are found in genes controlling key steps in lipid and glucose metabolism, such as the adipose-specific fatty acid

Authorship note: Bruno Lefebvre and Yacir Benomar contributed equally to this work.

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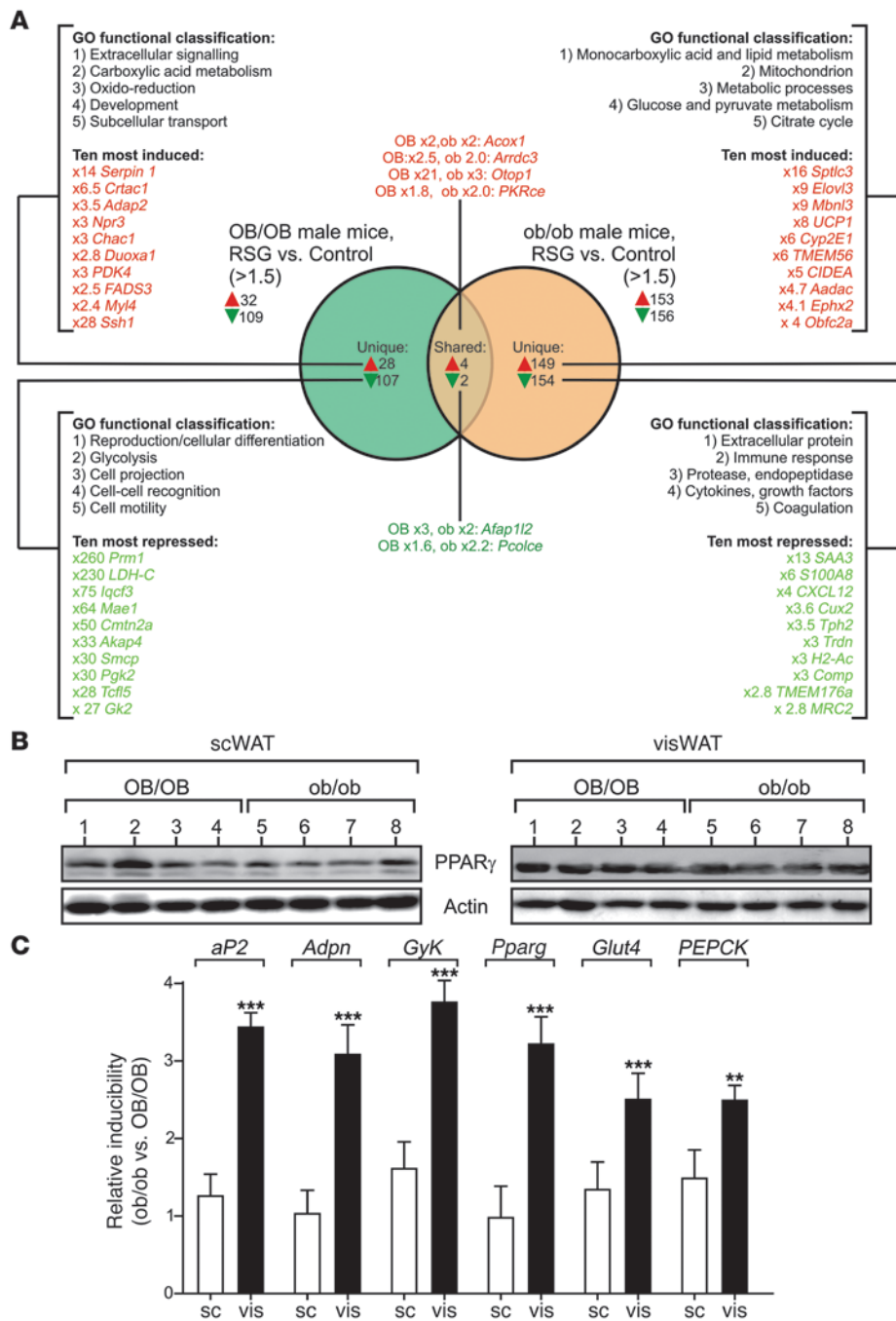


Figure 1

Obese insulin-resistant but not lean mice respond to RSG treatment. Male OB/OB and ob/ob mice were fed a chow diet supplemented or not (control) with RSG corresponding to a 3-mg/kg/d dose. (A) Comparative gene expression profiles in OB/OB and ob/ob visWAT treated or not with RSG. 3 samples from each group were analyzed on Affymetrix microarrays and interpreted using the Agilent GeneSpring GX software. These analysis are summarized here, showing the 10 most upregulated (red) and repressed (green) genes after the 21-day RSG treatment. The 5 most statistically significant Gene Ontology categories are indicated for each subset of genes. (B) PPAR γ is expressed in scWAT and visWAT. Total proteins were extracted from scWAT and visWAT. Proteins (100 g) were analyzed by reducing SDS-PAGE and Western blotting using anti-PPAR γ and anti- β actin antibodies. (C) PPAR γ target genes display enhanced responsiveness to RSG selectively in visWAT from ob/ob mice. scWAT and visWAT depots were removed from OB/OB, ob/ob, treated OB/OB, and treated ob/ob mice. RNAs were extracted and analyzed for their content in mRNA coding for aP2, Adpn, GyK, Pparg, Glut4, and PEPCK by RT-QPCR. Fold inductions by RSG were calculated for each condition and are expressed as the ratio of the induction rate measured in ob/ob WAT depots to that measured in OB/OB WAT depots. Data represent mean \pm SEM. ** $P < 0.01$, *** $P < 0.005$.

binding protein (*aP2*), phosphoenolpyruvate carboxykinase (*PEPCK*), or lipoprotein lipase (*LPL*). Of note, RXR α also plays a critical role in adipogenesis in vivo (19). Agonist-mediated activation of PPAR γ induces structural transitions occurring in the ligand-binding domain (LBD) of this receptor, creating a hydrophobic groove and a charge clamp that binds LXXLL motifs found in most coactivators, such as the p160-related coactivator family (SRC1–SRC3; ref. 20), the integrator complex CBP/p300 (21), components of the mediator complex (22), and the metabolically regulated coactivator PGC-1 α (23, 24). Agonist-dependent coactivator recruitment to PPAR γ is concomitant to corepressor dismissal, and both the NR corepressor (NCoR) and the

silencing mediator for retinoid and thyroid hormone receptors (SMRT) have been shown to affect PPAR γ -controlled cellular processes in distinct cellular backgrounds (25–27).

PPAR γ transcriptional activity is therefore dependent on its sequential association with multiprotein complexes. It is thus likely that processes controlling protein stability and degradation also influence the overall activity of the PPAR γ complex and may possibly affect its function under conditions of obesity and T2DM. Indeed, components of the ubiquitin-proteasome system (UPS) have been shown to be involved in PPAR γ -mediated transactivation (28). Although a few studies documented dysregulated expression of several UPS components in cardiovascular diseases

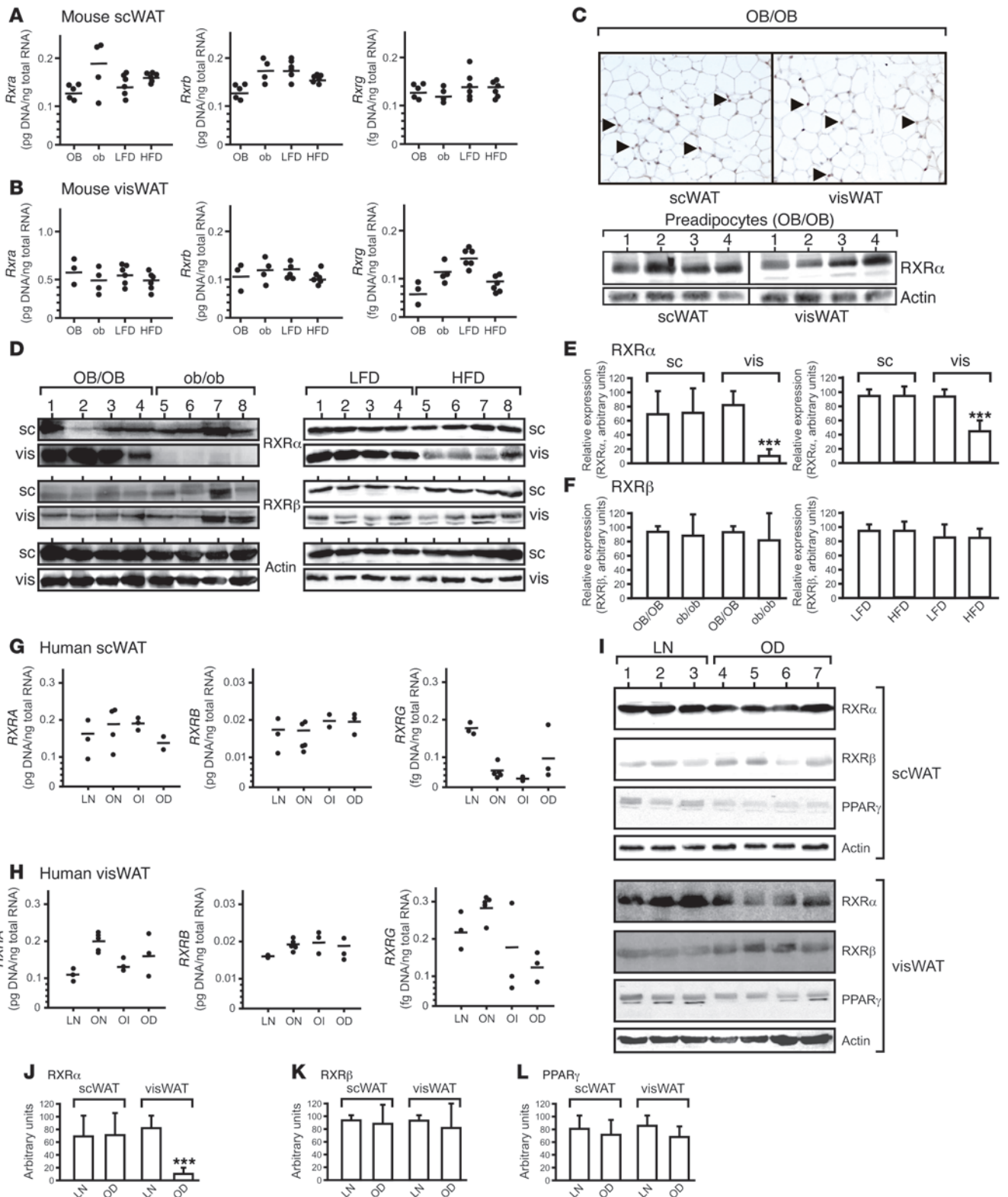




Figure 2

RXR α protein, but not mRNA, expression is downregulated in visWAT from obese mice and from obese diabetic patients. (A and B) RXR mRNA steady-state levels in mouse WAT. mRNAs from scWAT or visWAT were extracted, and *Rxra*, *Rxrb*, and *Rxrg* cDNAs were quantified by QPCR. (C) RXR α protein (arrowheads) in WAT sections. Original magnification, $\times 10$. (D) RXR α protein in mouse WAT. visWAT and scWAT from OB/OB mice, C57BL/6 mice fed LFD or HFD, or ob/ob mice were probed for their content in RXR α and RXR β by Western blot analysis. (E and F) Quantification of data in D. The intensity of each RXR band was normalized to actin. The first sample was arbitrarily set to 100%, and each sample was quantified relative to sample 1. Data represent mean \pm SEM. *** $P < 0.005$. (G and H) RXR mRNA steady-state levels in human WAT. mRNAs from scWAT and visWAT were analyzed as in A. LN, lean normoglycemic; ON, obese normoglycemic; OI, obese glucose intolerant; OD, obese diabetic. (I) RXR α , RXR β , and PPAR γ protein levels in human scWAT and visWAT. visWAT from lean or obese diabetic patients was probed for their RXR α , RXR β , and PPAR γ content by Western blot analysis. (J–L) Quantification of data in I. The intensity of each RXR or PPAR γ band was normalized to β -actin. The first sample was arbitrarily set to 100%, and each sample was quantified relative to sample 1. Data represent mean \pm SEM. *** $P < 0.005$.

(29, 30), a potential contribution of the UPS in the pathogenesis of T2DM and obesity remains unexplored. In the present study, we investigated the relationship among metabolic states, UPS component expression, and the PPAR γ signaling pathway. We report that the expression of the ubiquitin hydrolase/ligase ubiquitin carboxyterminal esterase L1 (*UCH-L1*) was specifically and strongly upregulated in visWAT from obese patients and mice. Mimicking hypoxia in vitro also upregulated *UCH-L1* expression. This enzyme promotes the selective breakdown of RXR α , which correlates with an increased response of PPAR γ to a synthetic agonist in vivo. Moreover, decreasing the RXR α /RXR β ratio in vitro increased PPAR γ transcriptional activity. The molecular basis of this phenomenon was found to be a stable, ligand-insensitive tethering of SMRT to PPAR γ -RXR α heterodimers.

Results

PPAR γ target genes are more sensitive to agonist stimulation in visWAT of obese mice, but not of lean mice. Because previous studies reported an unexpected insensitivity of lean mice or humans to TZD treatment, we investigated whether TZDs have a differential efficiency in WAT of normal lean (OB/OB) versus pathological (ob/ob) mice. Treatment with rosiglitazone (RSG; 3 mg/kg/d) lowered plasma glucose, insulinemia, and, to a lesser extent, triglycerides in 10-week-old male ob/ob mice, but had no effect in OB/OB littermates (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI38606DS1). This suggests that activation of PPAR γ could induce distinct biological responses depending on the energetic status. To assess this at the molecular level, adipose tissue gene expression profiles were examined by oligonucleotide microarray analysis of visWAT mRNAs from OB/OB and ob/ob mice treated or not with RSG (Figure 1A). Although these tissues exhibited similar PPAR γ protein expression levels (Figure 1B), RSG upregulated (> 1.5 -fold, $P < 0.05$) 32 genes in OB/OB visWAT, whereas 153 genes were upregulated in ob/ob visWAT, a substantial fraction of the latter being involved in metabolic control. Of these, only 4 genes were upregulated in both OB/OB and ob/ob visWAT, which suggests that PPAR γ regulates distinct transcriptional networks in normal and pathological tissues. This was con-

firmed by a Gene Ontology functional classification. Importantly, upregulated genes in ob/ob visWAT included known direct target genes for PPAR γ (e.g., *CIDEA*, *UCPI*, pyruvate carboxylase, and malic enzyme), which were not markedly upregulated in visWAT from OB/OB animals. We thus compared the ability of RSG to induce a subset of PPARE-driven adipocyte target genes in scWAT or visWAT from ob/ob mice and OB/OB littermates by real-time PCR. Whereas mRNA levels of the PPAR γ target genes adiponectin (*Adpn*), glycerol kinase (*GyK*), *ap2*, *Glut4*, *Pparg*, and *PEPCK* were upregulated to a similar extent in scWAT from OB/OB and ob/ob mice, they exhibited a much stronger responsiveness to RSG in visWAT from ob/ob mice (Figure 1C), in line with the microarray data. These comparative results thus demonstrate that PPAR γ target genes are more sensitive to agonist-mediated activation in obese than in lean visWAT. In sharp contrast, such an activity shift was not observed in scWAT.

RXR α protein steady-state levels are decreased in visWAT of obese, but not lean, humans and mice. PPAR γ transcriptional activity depends on its association with a number of cofactors, including RXRs. We thus reasoned that the increased PPAR γ transcriptional activity in obese visWAT might result from altered expression of a PPAR γ cofactor. Preliminary characterization of the mRNA expression levels of several PPAR γ primary cofactors did not reveal substantial differences between lean and obese visWAT, with the exception of *PGC1*, whose expression decreased by approximately 3-fold in obese visWAT (data not shown). We then monitored *Rxra*, *Rxrb*, and *Rxrg* mRNA levels by quantitative PCR (QPCR) in mouse scWAT and visWAT (Figure 2, A and B). *Rxr* mRNA levels were similar in WAT of OB/OB and ob/ob mice fed a regular diet (LFD) or high-fat diet (HFD). However, RXR α activity has previously been shown to be regulated by protein degradation in several cell types (31–35). We thus investigated whether RXR α polypeptide stability is affected in visWAT and scWAT of OB/OB and ob/ob mice. Immunohistochemical and Western blotting analyses revealed that RXR α was expressed in adipocytes and preadipocytes of both visWAT and scWAT from OB/OB mice (Figure 2C). We thus quantified the expression level of RXR α and RXR β polypeptides, the highest expressed in OB/OB and ob/ob mouse visWAT and scWAT (Figure 2D). Western blot analysis of mouse WAT extracts for RXR α and RXR β revealed that RXR α expression was strongly diminished in ob/ob visWAT, whereas RXR β was expressed, although less abundantly so, at levels similar between OB/OB and ob/ob WAT (Figure 2, D–F). This altered expression was specific for visWAT; scWAT from OB/OB and ob/ob mice displayed similar RXR α and RXR β levels. To rule out the possibility that RXR α downregulation is specific to the ob/ob genetic background, RXR α and RXR β content was also quantified in WAT from mice fed either LFD or HFD. Interestingly, *Rxra*, but not *Rxrb*, protein levels were also specifically decreased in visWAT from HFD-fed mice (Figure 2, D–F). Similarly, we investigated whether RXR polypeptide stability is affected in human scWAT and visWAT biopsies from patients with different levels of obesity and diabetes (Figure 2, G and H). *RXRA*, *RXRB*, and *RXRG* mRNA levels did not differ between scWAT and visWAT biopsies from normal, obese, obese glucose intolerant, and obese diabetic individuals. Western blot analysis of WAT extracts revealed that RXR α , but not RXR β , was much less abundant in visWAT from obese diabetic subjects than in visWAT from lean subjects (Figure 2, I–K). In contrast, *RXRA* and *RXRB* expression were not different in scWAT from lean and obese diabetic individuals (Figure 2, I–K).

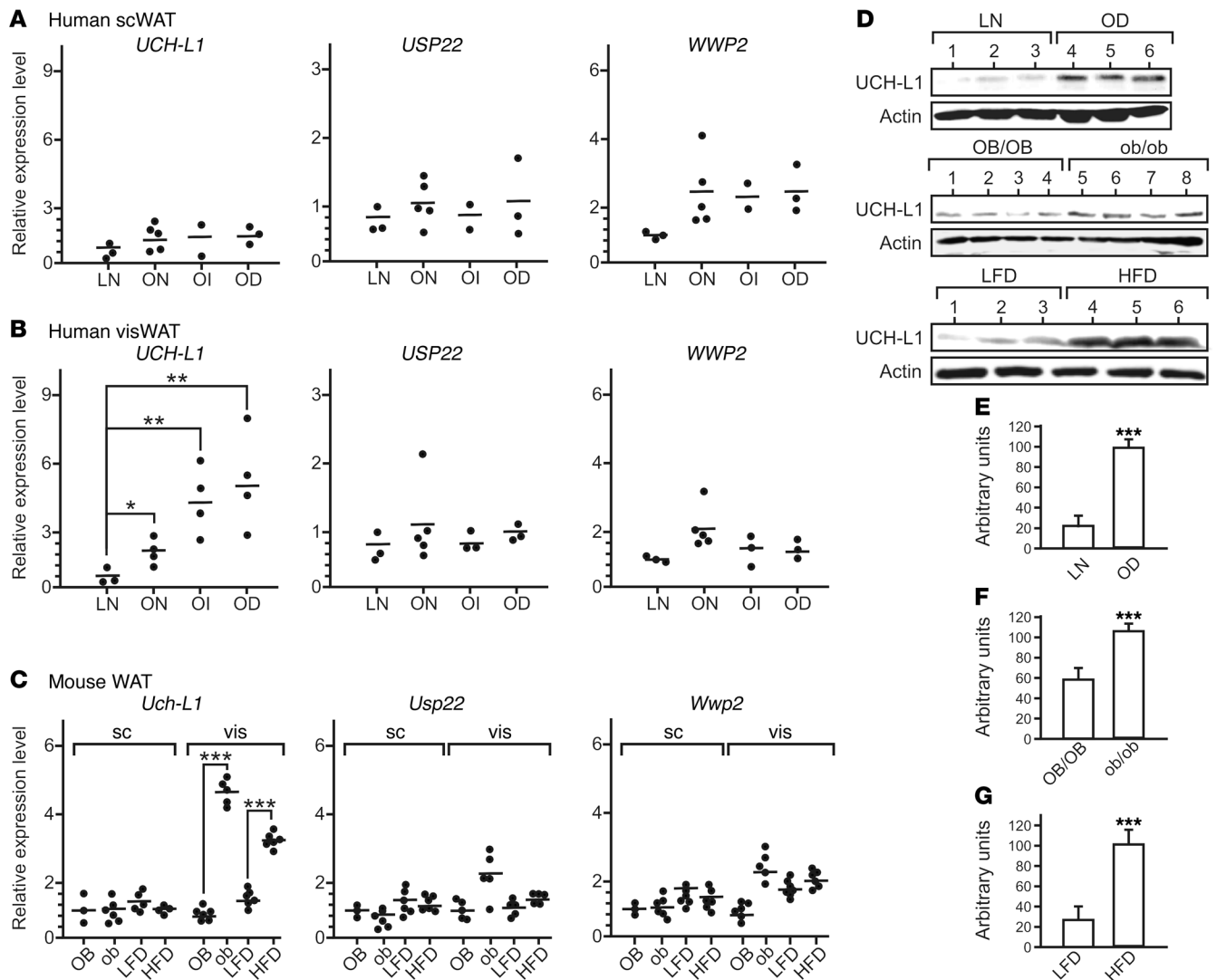


Figure 3 Expression of UCH-L1 is upregulated in visWAT from obese humans and mice. (A–C) Gene expression levels of *UCH-L1*, *USP22*, and *WWP2* were monitored by RT-QPCR using Taqman Assay on Demand sets of primers. Results are expressed relative to a control sample (LN for human WAT, OB/OB for mice) arbitrarily set to 1. (D) UCH-L1 protein expression in human and mouse WAT. Total protein extracts (50 g) were analyzed by Western blot and immunoprobed for UCH-L1. (E–G) Quantification of data in D. The intensity of each UCH-L1 band was normalized to actin. The first sample was arbitrarily set to 100%, and each sample was quantified relative to sample 1. Data represent mean SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

Of note, PPAR γ expression was comparable in all tissues (Figure 2, I and L). Collectively, these findings show that the RXR α protein is specifically degraded in visWAT from obese humans and mice.

Increased UCH-L1 expression in visWAT of obese humans and mice. Because *RXR α* mRNA levels were not altered, we postulated that the severe decrease in RXR α protein might stem from dysregulated expression of components of the UPS during metabolic disease progression. Therefore, UPS component expression was monitored in scWAT and visWAT from lean, obese, obese glucose intolerant, and obese diabetic individuals. Although the expression of components constituting the canonical 26S proteasome was not markedly altered in any of these WAT depots (data not shown), the expression of UCH-L1, an ubiquitin esterase/ligase enzyme, increased in visWAT, but not in scWAT, with progress-

ing stages of the disease (Figure 3, A and B). In contrast, the expression of other UPS components, such as USP22 and WWP2 (known to regulate the stability of transcription factors such as NF κ -B or RNA polymerase 2), was not substantially modified. Because the dysregulated *UCH-L1* expression was depot specific in humans, we investigated whether a similar phenomenon occurs in mouse WAT tissues by comparing the expression of *Uch-L1*, *Usp22*, and *Wwp2* in WAT from OB/OB, ob/ob, LFD-fed, and HFD-fed mice (Figure 3C). Strikingly, a strong upregulation of *Uch-L1* mRNA was observed in visWAT tissues of ob/ob and HFD-fed mice, whereas *Usp22* and *Wwp2* exhibited less pronounced upregulation (5-fold versus 2.5-fold). In contrast, the expression of these genes was similar in scWAT of OB/OB, ob/ob, and HFD-fed mice. This upregulation was also observed at the



protein level (Figure 3D). These data demonstrate that *UCH-L1* is strongly and specifically upregulated in visWAT from metabolically challenged humans or mice.

RXR α polypeptide stability is altered by UCH-L1 upregulation and proteasomal degradation. The correlation between *UCH-L1* upregulation and the strongly reduced *RXR α* polypeptide steady-state levels in obesity led us to speculate that *UCH-L1* could be critical in controlling *RXR α* stability. We therefore transfected 3T3-L1 preadipocytes with expression vectors coding for *RXR α* or *RXR β* , with or without an expression vector coding for *UCH-L1* (Figure 4A). Incubation of *RXR*-transfected cells with the proteasome inhibitor MG132 moderately increased the amount of *RXR α* , but did not influence *RXR β* protein level. A combination of ammonium chloride and leupeptin ($\text{NH}_4\text{Cl}/\text{Leu}$), which inhibits the lysosomal degradation pathway, did not modify *RXR α* and *RXR β* protein levels in *RXR*-transfected cells. Remarkably, *UCH-L1* overexpression in *RXR*-transfected cells induced the breakdown of the *RXR α* polypeptide, whereas *RXR β* stability was unaffected. *UCH-L1*-induced *RXR α* breakdown was prevented by MG132, but not by $\text{NH}_4\text{Cl}/\text{Leu}$, which indicates that *UCH-L1* promotes *RXR α* breakdown through proteasomal degradation. *RXR* ubiquitinylation was then examined in 3T3-L1 preadipocytes transfected with expression vectors coding for *RXR* and HA-tagged ubiquitin. Immunoprecipitation of *RXR* followed by Western blot detection of HA-tagged ubiquitin (Figure 4B), or immunoprecipitation of HA-tagged proteins followed by Western blot detection of *RXR* (Supplemental Figure 2), showed that only *RXR α* was intensively conjugated to HA-ubiquitin and that *UCH-L1* overexpression markedly increased ubiquitinylation of *RXR α* , but not *RXR β* . Furthermore, an in vitro ubiquitinylation assay using purified *RXR*s as substrates showed that *RXR α* was ubiquitinated in an ATP-dependent manner (Figure 4C). Ubiquitin-conjugated *RXR α* was stabilized in the presence of ubiquitin aldehyde, a general inhibitor of ubiquitin hydrolases (36), or of MG132. In sharp contrast, *RXR β* was not detectably conjugated to ubiquitin in similar conditions (Figure 4B and Supplemental Figure 2). Taken together, these data demonstrate that *RXR β* is refractory to proteasome-mediated breakdown. Hypoxia is a feature of obese WAT (reviewed in ref. 37), characterized by induced gene expression and protein stabilization of hypoxia-inducible transcription factors (HIFs). Accordingly, *Hif1a* mRNA was upregulated in visWAT from ob/ob and HFD-fed mice (Figure 4D), whereas *Hif1b* and *Hif2b* expression was selectively increased in visWAT from HFD-fed mice. *Hif2a* expression was unaffected in both types of WAT. Because HIF1 α upregulation in obese WAT correlated with increased *Uch-L1* expression (Figure 3C), and given the occurrence of several HIF-responsive elements in the promoter of the mouse and human *UCH-L1* genes, we tested whether cobalt chloride (CoCl_2), a hypoxia-mimicking compound, also upregulated *UCH-L1* in differentiated 3T3-L1 adipocytes. CoCl_2 caused concomitant upregulation of *Hif1a* and *Uch-L1* mRNAs (Figure 4E) and dose-dependent degradation of the *RXR α* protein (Figure 4F). Furthermore, both MG132 and LDN-54777, a specific inhibitor of *UCH-L1* (38), blocked the CoCl_2 -induced *RXR α* degradation in a dose-dependent manner (Figure 4G). Pulse-chase labeling of 3T3-L1 adipocyte proteins showed that the decay of *RXR α* was linear, with a $t_{1/2}$ of about 8 hours (Figure 4H). CoCl_2 treatment decreased the $t_{1/2}$ of *RXR α* to 4 hours, whereas LDN-54777 clearly prevented the CoCl_2 -induced *RXR α* breakdown ($t_{1/2}$, 7 hours). Taken together, these data argue for a role of *UCH-L1* in the UPS-mediated *RXR α* degradation process, in which hypoxia might play a role.

The $RXR\alpha/RXR\beta$ ratio determines PPAR γ responsiveness to agonist in murine adipocytes. In light of the above results, we investigated whether an altered *RXR α* /*RXR β* ratio might directly alter *PPAR γ* responsiveness to agonist challenge in a cell-autonomous manner. *Rxr α* was strongly expressed in 3T3-L1 differentiated adipocytes, whereas *Rxr β* and *Rxr γ* were expressed at much lower levels (Figure 5A). *Ppar γ* , as well as *Ppar α* , were also expressed in differentiated 3T3-L1 cells. RSG treatment readily activated *aP2*, *GyK*, and *Adpn* gene expression (Supplemental Figure 3). ChIP assays using anti-*RXR* or anti-*PPAR* antibodies (Figure 5B) failed to detect *Rxr γ* on the *aP2* and *Adpn* PPREs, in agreement with its low expression level, whereas comparable occupancy by both *Rxr α* and *Rxr β* was detected on these promoters. *Ppar γ* , but not *Ppar α* , also bound to these promoters, and the density of *Ppar γ* and *RXR*s on these promoters was similar in the absence and presence of RSG (Figure 5B). Identical results were obtained for the *GyK* promoter (data not shown). Thus, simultaneous expression of *RXR α* and *RXR β* generates *PPAR γ* -driven promoters on which both *RXR* isotypes can be loaded.

To further assess the role of the *RXR* isotype on *aP2*, *Adpn*, and *GyK* gene responsiveness to RSG, we generated *Rxr α* -depleted 3T3-L1 adipocytes by siRNA-mediated knockdown, which induced a specific *Rxra* mRNA decrease of 70% (Supplemental Figure 4). *RXR α* knockdown resulted in a more pronounced induction of the *PPAR γ* target genes by RSG (*aP2*, 9.0- versus 3.6-fold; *Adpn*, 4.6- versus 2.6-fold; *GyK*, 6.3- versus 4.4-fold; Figure 5C). Conversely, 3T3-L1 CAR cells, which stably overexpress the adenovirus receptor (39), were differentiated into adipocytes and transduced at day 6 with adenoviral particles encoding GFP as a negative control, with *Rxr α* , or with *Rxr β* , allowing for strong overexpression of each *RXR* isotype (Supplemental Figure 5). Interestingly, RSG treatment caused stronger induction of *aP2*, *Adpn*, *GyK*, and *Pparg* in *RXR β* -overexpressing cells compared with GFP-expressing cells (Figure 5D). Inversely, forced expression of *Rxr α* attenuated the induction of *Ppar γ* target genes by RSG (Figure 5D).

The $RXR\alpha/RXR\beta$ ratio determines PPAR γ responsiveness to agonist in different cellular backgrounds. We used QPCR analysis to investigate whether decreased *Rxr α* expression causes a derepression of *PPAR γ* target genes in other cellular backgrounds that exhibit varying *RXR α* /*RXR β* ratios (Figure 5E). 3T3-L1 preadipocytes displayed a *Rxra/Rxrb* mRNA ratio of 10:1, much like the HepG2 hepatocarcinoma and C2C12 myeloblastic cell lines (10:1 and 4:1, respectively). In contrast, the β -insulinoma cell line Min6 expressed comparable levels of *Rxra* and *Rxrb* mRNAs, at a 1:1 ratio. Assessment of the transcriptional activity of *Ppar γ* in these different cell types using the J6 tk-Luc reporter gene showed that a much higher maximal transcriptional activity was reached in Min6 cells than in 3T3-L1, C2C12, and HepG2 cells in response to RSG (Figure 5F). Overexpressing *Rxr α* in Min6 cells significantly blunted the response to RSG, whereas overexpression of *Rxr β* potentiated this response (Figure 5G). A similar pattern was obtained in COS cells and on another PPRE-driven reporter gene, ApoA2 tk-Luc, which demonstrates that the *RXR α* -repressive function was dependent neither on the cell type nor on the response element (Figure 5H). We then used a system in which the P box, located in the DNA-binding domain (DBD) of either *Rxr α* or *Rxr β* , was mutated to confer a high affinity for a glucocorticoid-responsive element (GRE) half site. The *RXR* binding polarity can thus be imposed when using a chimeric direct repeat 1 response element PPRE in which the GRE half site is located in either 3' or 5'. This system was only functional when *RXR* was

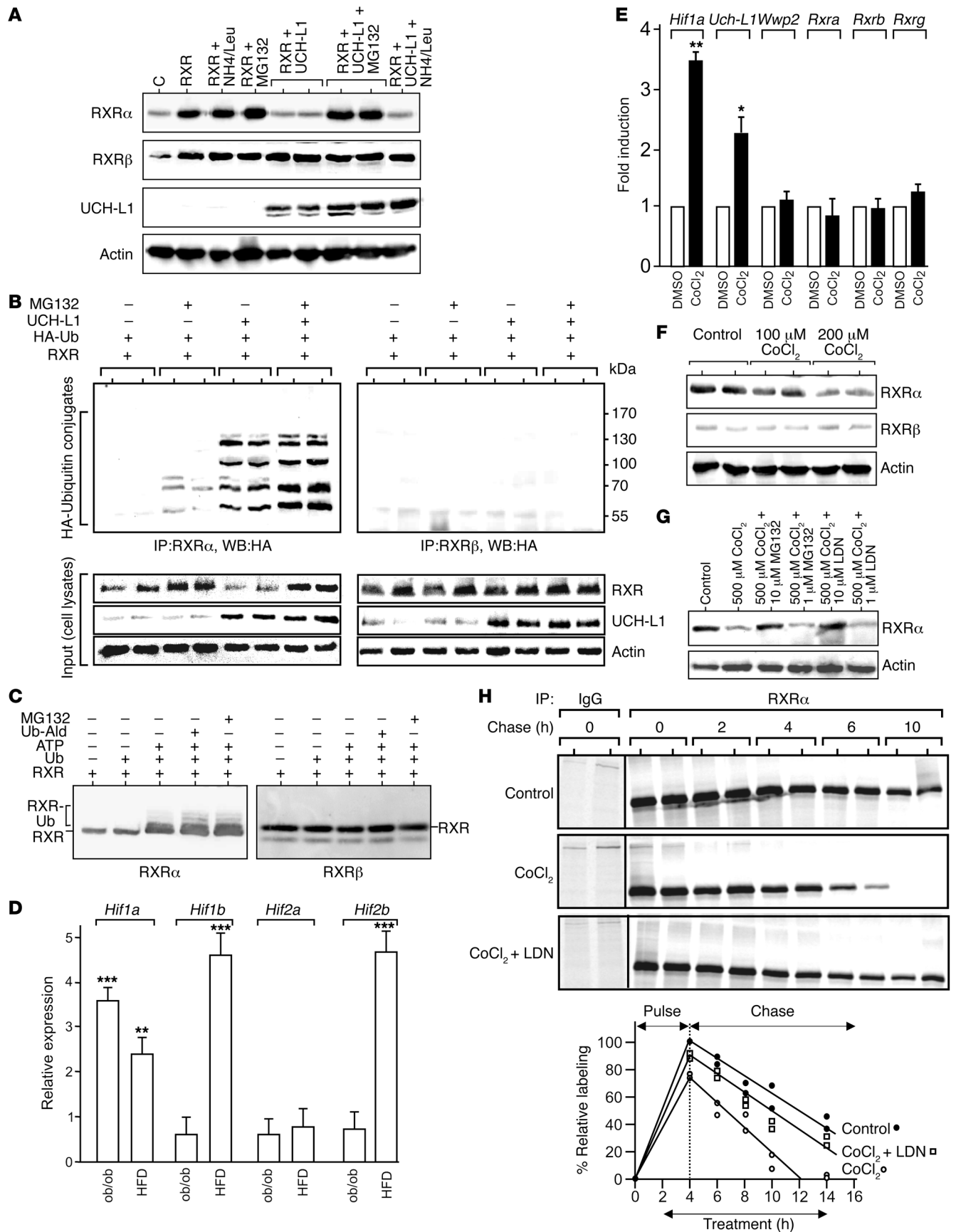




Figure 4

RXR α is selectively degraded through the ubiquitin proteasome system. (A) 3T3-L1 preadipocytes were transfected with expression vectors coding for UCH-L1, RXR α , or RXR β , then treated with 10 μ M MG132 or 10 μ M NH $_4$ Cl/Leu overnight. Whole cell extracts were prepared 48 hours after transfection and analyzed by Western blot. C, control. (B) In vivo ubiquitin conjugation of RXR α or RXR β in 3T3-L1 preadipocytes. Cells were transfected as above with an additional expression vector coding for HA-tagged ubiquitin (Ub) and treated as in A. Whole cell extracts were submitted to immunoprecipitation with an anti-RXR antibody followed by Western blot analysis of HA-conjugated proteins. (C) In vitro ubiquitinylation of RXR α and RXR β . Purified recombinant RXR α or RXR β (50 ng) was incubated for 4 hours with ubiquitin (100 μ g/ml), ATP (0.5 mM), ubiquitin aldehyde (Ub-Ald; 20 μ g/ml), or MG132 (10 μ M) and analyzed by Western blotting. (D) HIF transcription factor mRNA levels in mouse visWAT. *Hif1a*, *Hif1b*, *Hif2a*, and *Hif2b* mRNAs were quantified by RT-QPCR. Expression levels are shown relative to control OB/OB or LFD WAT as appropriate, arbitrarily set to 1. (E) Gene expression levels in 3T3-L1 adipocytes upon CoCl $_2$ treatment for 4 hours. (F) RXR protein levels in 3T3-L1 adipocytes upon CoCl $_2$ treatment for 16 hours. (G) Proteasome and UCH-L1 inhibition protected Rxr α from CoCl $_2$ -induced degradation. 3T3-L1 adipocytes were treated for 16 hours as indicated, and whole cell extracts were analyzed by Western blotting. (H) Pulse-chase labeling of Rxr α in 3T3-L1 adipocytes. Adipocytes were treated as described in Methods. Labeled Rxr α was immunoprecipitated and quantified by autoradiography.

bound on the 3' half site of the chimeric PPRE (ref. 40 and Figure 5I). In this configuration, Ppar γ was more sensitive to a moderate 30-nM concentration of RSG when Rxr β was expressed (Figure 5I).

Taken together, these data indicate that (a) PPAR γ can bind as a dimer with RXR α or RXR β to PPRE-driven promoters in adipocytes; (b) this interaction is not ligand sensitive; and (c) decreased RXR α expression correlates in vitro and in vivo to an increased responsiveness to RSG. RXR α thus acts as a repressor of PPAR γ responsiveness to agonist challenge in murine adipocytes.

Corepressors interact with PPAR γ in vitro and in intact cells. Collectively, these results show that PPAR γ -RXR α heterodimers display lower sensitivity to agonist challenge than do PPAR γ -RXR β dimers. To test whether this might be the result of increased interaction with corepressors, the influence of the RXR isotype on the binding of PPAR γ to SMRT or NCoR was further characterized. A yeast 2-hybrid assay was first performed to assess the interaction of PPAR γ fused to the Gal4 DBD, with the NR interaction domain (NRID) of either SMRT (aa 2,061–2,472) or NCoR (aa 1,906–2,313) fused to the NF- κ B activation domain. SMRT interacted more efficiently with the PPAR γ LBD than did NCoR, whereas interaction of the PPAR γ LBD with the coactivator Med1 (also known as TRAP220 or DRIP205) was negligible (Figure 6A). A similar 2-hybrid assay in mammalian HeLa cells using the PPAR γ LBD fused to the Gal4 DBD and NRIDs fused to the VP16 activation domain (VP16-AD) revealed a similar pattern of interaction (Figure 6B), demonstrating that the preferential interaction of SMRT with PPAR γ is an intrinsic property not affected by the cellular background. SMRT was therefore selected as the representative corepressor in further experiments.

A glutathione S-transferase-pulldown (GST-pulldown) assay was then carried out to assess the interaction between full-length PPAR γ and SMRT. Using an immobilized GST-SMRT fusion protein and radiolabeled PPAR γ , we detected a strong interaction between the proteins in this system (Figure 6C). RSG (Figure 6C) and 2 other PPAR γ agonists, pioglitazone and troglitazone (Supplemental Figure 6A), were unable to promote significant release of SMRT

from monomeric PPAR γ . In similar conditions, RSG promoted the recruitment of Med1, GRIP1 (also known as TIF-2), and PGC-1 α to the PPAR γ LBD (Supplemental Figure 6B), showing that the PPAR γ LBD undergoes appropriate structural transitions. Thus, the PPAR γ -SMRT interaction was not sensitive to PPAR γ agonist binding in this setting. To assess whether a stable interaction with SMRT also occurred on PPAR γ target gene promoters, ChIP assays were performed in 3T3-L1 adipocytes. 3T3-L1 cells express several coactivators, as well as NCoR and SMRT, whose mRNA expression levels did not vary significantly during the differentiation process (Supplemental Figure 7). In differentiated, unstimulated 3T3-L1 adipocytes, SMRT was clearly detected on each promoter, whereas NCoR displayed barely detectable binding (Figure 6D). Upon RSG treatment, SMRT was partially dismissed from both promoters, which indicates that, in the context of an endogenous functional promoter, agonist-activated PPAR γ is able to at least partially release SMRT. To confirm these findings, we monitored the association of 2 SMRT-associated proteins, HDAC3 and SIRT1. Similar to SMRT, both HDAC3 and SIRT1 were partially dismissed from the *aP2* and *Adpn* promoters (Figure 6E). Thus a partial, agonist-induced dismissal of corepressor complex correlated with mixed RXR α /RXR β occupancy at PPAR γ -regulated target genes (Figure 5B).

RXR isotype affects SMRT interaction with the PPAR γ -RXR heterodimer. We hypothesized that the observed increase in PPAR γ -RXR β responsiveness to agonist could result from a specific feature of the RXR α -SMRT interaction. This hypothesis was tested using a 2-hybrid assay in NIH-3T3 cells, which expresses neither PPAR γ nor C/EBP α , but can be fully differentiated into adipocytes upon ectopic expression of these 2 transcription factors (ref. 41 and Figure 7A). A Gal4-SMRT NRID fusion protein was overexpressed together with a PPAR γ LBD-VP16-AD fusion protein in the presence or absence of RXR α or RXR β . The transcriptional activity of the system was monitored with a UAS tk-Luc reporter gene, whose activity was predicted to decline upon dissociation of the PPAR γ -VP16 protein. As a control experiment, we expressed a human RAR-VP16 (hRAR-VP16) fusion protein together with the Gal4-SMRT NRID. This system displayed clear agonist-dependent decreased transcriptional activity (*all trans* retinoic acid; atRA), reflecting the dissociation of the hRAR-VP16 fusion protein from the Gal4-SMRT bait upon agonist binding (Figure 7A). In similar conditions, the PPAR γ -VP16-Gal4-SMRT interaction was not sensitive to increasing concentrations of RSG. Concomitant overexpression of RXR α , RXR β , or RXR γ increased the basal level of interaction of PPAR γ with SMRT. However, RSG decreased system activity only in the presence of RXR β , which indicates decreased SMRT association with the PPAR γ -RXR β heterodimer. Taken together, these data suggest that the PPAR γ -SMRT interaction is disrupted by RSG only when RXR β is integrated in the ternary SMRT-PPAR γ -RXR complex.

RXR isotype affects SMRT-mediated repression of PPAR γ . We concluded from ChIP and 2-hybrid data that the PPAR γ -RXR α interaction with SMRT was ligand insensitive. To further validate this hypothesis in the context of a DNA-bound PPAR γ -RXR heterodimer, we used a modified 2-hybrid assay in NIH 3T3 cells. The J6 tk-Luc reporter gene was cotransfected with PPAR γ and RXR expression vectors, together with an expression plasmid coding either for VP16-AD as a control or for a SMRT-VP16-AD fusion protein (Figure 7B). Preliminary experiments showed that detected responses necessitated a PPRE sequence (data not shown). We first used the RAR α -RXR α heterodimer as a control system. The basal activity of this dimer strongly increased in the presence of VP16-SMRT, indicative of a

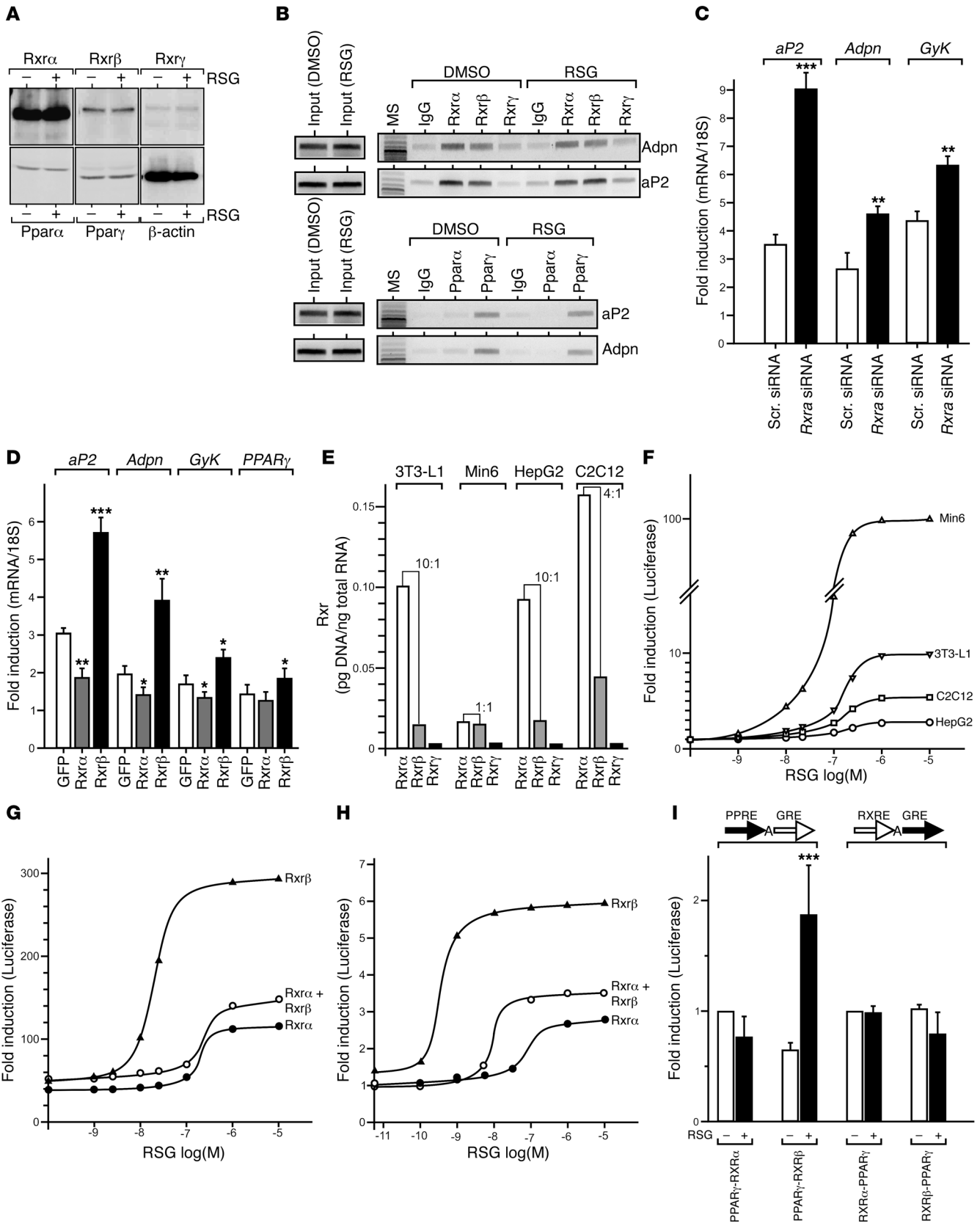




Figure 5

RXR α exerts a repressive effect on PPAR γ -mediated transcription. (A) RXR and PPAR isotype expression in differentiated 3T3-L1 adipocytes. Whole cell extracts (50 g) from control or RSG-treated (2 hours) cells were analyzed by Western blotting. (B) Promoter occupancy by RXR and PPAR isotypes. Differentiated 3T3-L1 cells were treated as in A. RXR and PPAR association to the *aP2* and *Adpn* PPRES was detected by ChIP assay. (C) Rxr α knockdown enhanced Ppar γ responsiveness to RSG in 3T3-L1 adipocytes. 3T3-L1 adipocytes were transfected with control, scrambled, or anti-Rxra siRNAs and treated with 1 μ M RSG 24 hours later. mRNAs were assayed by QPCR for *aP2*, *Adpn*, and *GyK* transcripts. Results are expressed relative to untreated cells. Data represent mean \pm SEM ($n = 3$). ** $P < 0.01$, *** $P < 0.005$. (D) Overexpression of RXR α blunted induction of PPAR γ target genes in 3T3-L1 CAR adipocytes. 3T3-L1 CAR cells were differentiated and transduced at day 5 with adenovirus encoding GFP, Rxr α , or Rxr β . mRNA quantification at day 7 was as in C. Data represent mean \pm SEM ($n = 3$). ** $P < 0.01$, *** $P < 0.005$. (E) Expression levels of RXR isotypes in Min6, 3T3-L1, HepG2, and C2C12 cells. Total RNA from each cell type was analyzed by QPCR for their absolute content in RXR mRNAs. (F) Min6, undifferentiated 3T3-L1, C2C12, and HepG2 cells were transfected with the PPRES-driven J6 tk-Luc reporter gene and stimulated with increasing concentrations of RSG. Luciferase activities are expressed relative to basal expression of the unstimulated reporter system (DMSO), arbitrarily set to 1. (G) Rxr α overexpression blunted Ppar γ responsiveness to RSG in Min6 cells. Min6 cells were transfected with the J6 tk-Luc reporter gene and with 30 ng of either RXR α or RXR β expression vector or 15 ng of each. Luciferase activities were assayed and graphed as in F. (H) Rxr α overexpression blunted Ppar γ responsiveness to RSG in COS cells. Experimental conditions were as in F. (I) RXR β confers RSG responsiveness to a positionally restricted PPAR γ -RXR heterodimer. PPAR γ and mutated RXR α or RXR β (both binding to a glucocorticoid response element half site; ref. 61) were overexpressed in HeLa cells, and the transcriptional activity of the heterodimer was monitored using a reporter gene driven by a composite GRE-PPRES or PPRES-GRE tk-Luc reporter gene. Results are expressed as in F. Data represent mean \pm SEM ($n = 3$). *** $P < 0.005$.

hRAR α -RXR α -SMRT interaction. A saturating concentration of atRA (1 μ M) yielded luciferase activity similar to that observed in control conditions, showing that SMRT was fully dissociated from the RXR-RAR complex upon agonist binding (Figure 7B). When transposed to the PPAR γ -RXR α system, VP16-SMRT expression also strongly increased the basal activity of the reporter gene (5-fold induction) compared with VP16-AD alone. However, in contrast to the RXR α -RAR α dimer, higher transcriptional activity of the PPAR γ -RXR α dimer compared with control conditions was maintained even at a saturating RSG concentration. This additive induction was a result of the cumulative effect of the AF2- and VP16-mediated transcriptional activation domains, strongly suggesting that agonist treatment does not induce the dismissal of VP16-SMRT from the PPAR γ -RXR α heterodimer. RXR γ exhibited behavior similar to that of RXR α (Figure 7C). In sharp contrast, RXR β overexpression generated a system in which saturating RSG concentrations prevented SMRT-VP16 from further activating the reporter gene activity, showing that the liganded PPAR γ -RXR β dimer cannot bind the SMRT moiety. We conclude from the above data that only the PPAR γ -RXR β dimer fully releases SMRT upon agonist binding.

Discussion

The transcriptional control of programs regulating metabolic homeostasis can be substantially altered by manipulating the activity of transcription factors belonging to the NR superfamily.

Among the NRs, PPAR γ plays an important role as a central regulator of lipid and glucose metabolism and is important for maintaining whole-body insulin sensitivity (42). However, how its function may be modulated upon disease progression is unknown. Our results provide what we believe to be a novel paradigm on how a transcriptional regulator may adjust its transcriptional activity in the face of metabolic challenges.

The inverse correlation in visWAT from obese mice and humans between *UCH-L1* expression and RXR α polypeptide stability highlights a regulatory mechanism of the PPAR γ signaling pathway. UCH-L1 exhibits ubiquitin C-terminal hydrolase activity, which, by maintaining a sufficient pool of cellular ubiquitin, sustains protein degradation. Additional roles, such as ubiquitin ligation and stabilization of monoubiquitin, have previously been described for UCH-L1 (reviewed in ref. 43). UCH-L1 substrates are unknown, but its involvement in regulating NF- κ B activity in vascular cells (44) and its varied gene expression in rat pancreatic islets exposed to high glucose concentrations (45) hint at a role in metabolic control. Although we found increased UCH-L1 expression at various stages of disease progression in humans, the mechanism leading hereto is unknown. Hypoxia, which affects obese WAT (37), may be a possible link between pathological states and increased *UCH-L1* expression. In addition, hypoxia has been reported to promote RXR α breakdown in cardiac myocytes (46). In ob/ob and HFD visWAT, we observed increased expression of *Hif1a*, and the UCH-L1 promoter contains several HIF-responsive elements. In agreement with these observations, CoCl₂, an agent mimicking partially hypoxia through HIF1 α induction and stabilization, was found to increase UCH-L1 expression in 3T3-L1 adipocytes and to promote RXR α protein breakdown in a UCH-L1-dependent manner. RXR α is a known target of the UPS in several cell types (31–35), and our present data showed that RXR α was a substrate for in vitro ubiquitin conjugation, whereas RXR β was unable to undergo this posttranslational modification. However, recombinant UCH-L1 neither modified the ubiquitin conjugation rate in the acellular system nor engaged direct protein-protein interaction with RXR α in vitro, as assayed by GST-pull-down assays (data not shown), which suggests that this enzyme promotes RXR α breakdown in an indirect manner. Combined with the fact that visWAT from ob/ob mice is more sensitive to TZD treatment than is visWAT from OB/OB mice, our data raised the possibility that an UCH-L1-dependent RXR expression isotype switch could control PPAR γ transcriptional activity.

As with all other ligand-regulated NRs, PPAR γ exerts its transcriptional activity through the recruitment of coactivators, and PPAR γ has been documented to bind in vitro to a variety of primary coactivators. PPAR γ transcriptional activity is also modulated by corepressor recruitment, and we showed here that PPAR γ -containing heterodimers preferentially recruited SMRT over NCoR. Such a preference has been reported by some in a variety of cell-free and cellular systems (25, 40, 47, 48), but not confirmed by others (26, 49). Although RNA interference studies showed that SMRT repressed PPAR γ transcriptional activity (data not shown), we found that the physical interaction of PPAR γ with SMRT was not affected by agonist binding. Thus, assays involving solely PPAR γ lack a critical component required for the ligand-regulated association of SMRT to PPAR γ , in line with a previous report (50). As an obligate heterodimerization partner conferring specific DNA binding activity to PPAR γ , RXR is a major regulator of PPAR γ activity. Mammalian 2-hybrid assays established that RXR association to PPAR γ allowed for a reversible association of SMRT to the PPAR γ -RXR heterodimer

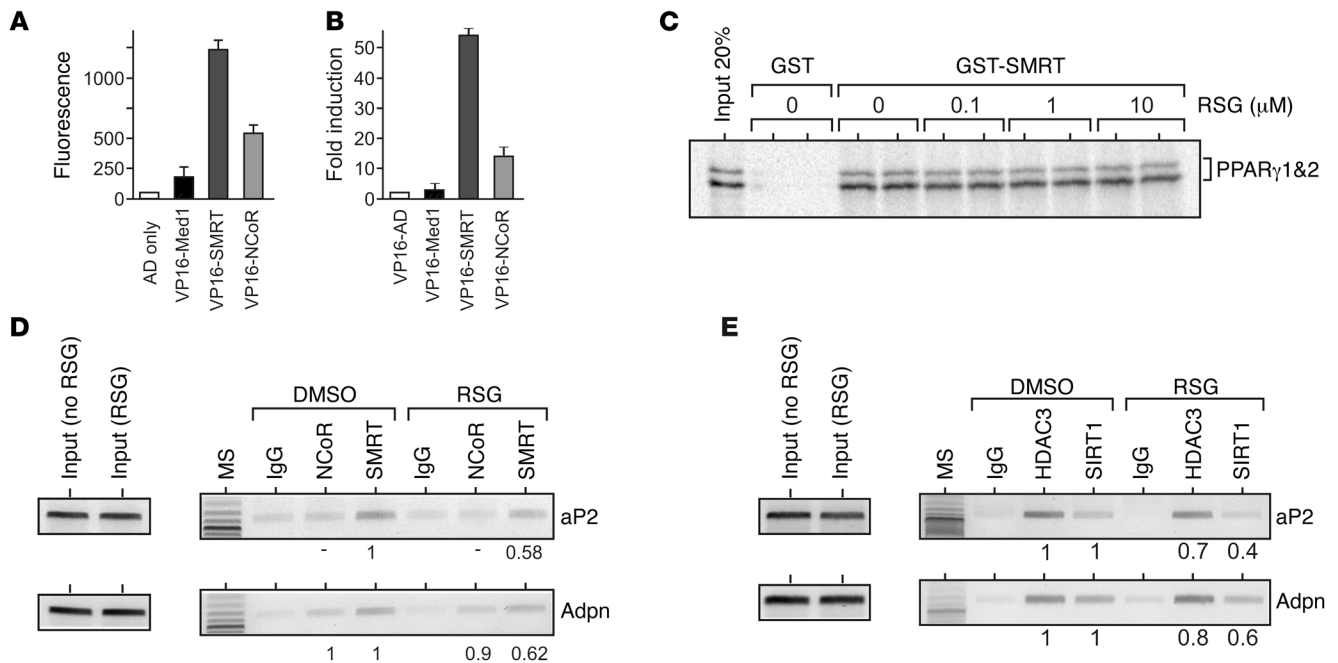
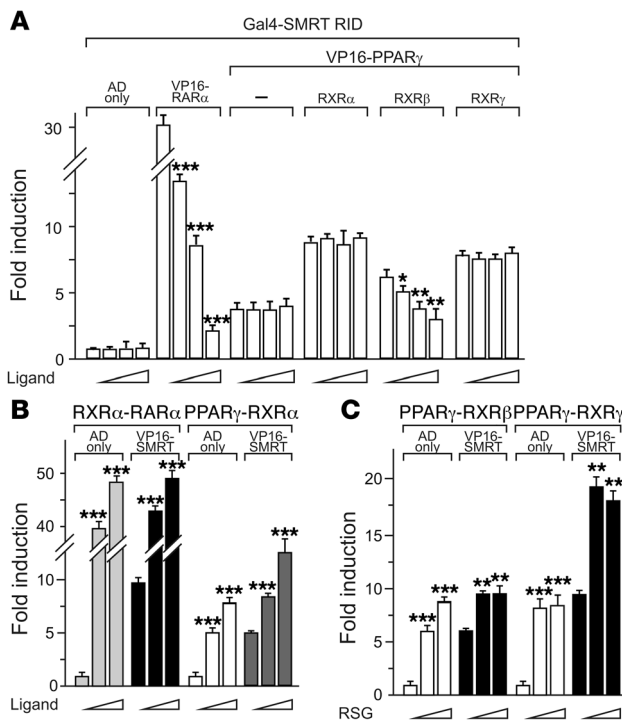


Figure 6 Ligand-dependent dismissal of SMRT from PPAR γ depends on its association with RXR isotypes. **(A)** Cofactor interaction in yeast. Vectors encoding for the VP16-AD alone (AD only), or in frame with the NR1D of Med1 (VP16-Med1), SMRT (VP16-SMRT), or NCoR (VP16-NCOR), were coexpressed with the PPAR γ LBD fused to the Gal4 DBD. Fluorescence is expressed as arbitrary units. **(B)** Cofactor interaction in mammalian cells. An experimental strategy similar to that described in **A** was used to monitor Med1, SMRT, or NCoR interaction with the PPAR γ LBD in HeLa cells. The basal level observed in the presence of VP16-AD and Gal4-PPAR γ LBD was arbitrarily set to 1. **(C)** SMRT interaction with PPAR γ LBD in a cell-free system. A GST-SMRT fusion protein was incubated with radiolabeled PPAR1 and PPAR2, with increasing concentrations of RSG. GST-SMRT-PPAR γ complexes were resolved by SDS-PAGE and autoradiographed. **(D)** Corepressor interaction with aP2 or Adpn promoters in adipocytes. ChIP assays were carried out in differentiated 3T3-L1 cells to detect SMRT or NCoR loading to the aP2 or Adpn promoters. All experiments were carried out at least 3 times. **(E)** Promoter occupancy by deacetylases. ChIP assays were carried out as in **D** to detect either SIRT1 or HDAC3 binding to the aP2 or Adpn PPREs. Numbers below blots indicate level of binding relative to DMSO control, arbitrarily set to 1.

in an isotype-dependent manner. Only RXR β generated a complex able to dismiss SMRT in an agonist-dependent manner, providing a molecular basis for the higher inducibility of PPAR γ in RXR β -enriched cellular backgrounds. This was also true in a mouse adipocyte background, in which RXR α and RXR β normally associate to the PPRE of PPAR γ target genes. The interaction of PPAR γ , as well as that of RXRs, with the PPRE of the endogenous *aP2*, *Adpn*, and *GyK* gene promoters was constitutive and ligand insensitive in differentiated 3T3-L1 adipocytes, in agreement with previous data (26). The mixed composition of PPRE-bound heterodimers, in light of our interaction data, led us to predict that SMRT would be only partially released upon agonist challenge. This was indeed the case, confirmed by the partial release of SMRT-interacting proteins HDAC3 and Sirt1. In agreement with the repressive role of RXR α , siRNA-mediated decrease of RXR α expression led to a higher sensitivity to RSG; conversely, its overexpression in 3T3-L1 adipocytes and other cellular backgrounds invariably blunted responsiveness to RSG. A differential affinity of NRs for distinct corepressors has only been documented for NRs expressed as monomers. For example, restricted SMRT recruitment has been observed for the atRA receptors RAR α , RAR β , and RAR γ (51, 52), and differences are due to the poorly conserved C-terminal F domain (53). However, since neither RXRs nor PPAR γ have an F domain, other structural determinants must regulate the differential affinity of RXR α - versus RXR β -containing heterodimers for SMRT and are yet to be identified.

It has been postulated that the tissue-specific recruitment of nuclear coactivators by specific agonists influences PPAR γ transcriptional activity, a mechanism by which side effects triggered in TZD target tissues, such as kidney, bone, or even adipose tissues, could be circumvented (e.g., refs. 54–56). Our data suggest that PPAR γ operates differently in normal and pathological tissues as a result of heterodimerization with distinct RXR isotypes. Whether this phenomenon also impinges on the ability of PPAR γ to recruit a specific subset of coactivators is unknown, but nevertheless underlines the need for a careful selection of screening procedures aimed at identifying novel PPAR γ synthetic ligands. It also leaves open the possibility to design ligands favoring – or not – heterodimerization with RXR α , thereby predictably moderating the transcriptional response to synthetic PPAR γ ligands. The need for moderated PPAR γ activation has already been underlined by (a) the protective effect of the transcriptionally altered Pro12Ala PPAR γ mutant against the development of insulin resistance and T2DM; (b) the improved insulin sensitivity of heterozygous PPAR γ mice fed a HFD; and (c) the beneficial effects on obesity and insulin resistance upon treatment with PPAR γ antagonists (57).

In summary, our studies elucidated an unexpected mechanism by which the UPS regulates PPAR γ transcriptional activity in pathological states through selective degradation of RXR α . Modifying PPAR γ transcriptional activity in the face of metabolic challenges may be a mechanism by which cells adapt to novel conditions through tran-

**Figure 7**

RXR α overexpression confers ligand sensitivity to the SMRT-PPAR γ interaction. **(A)** NIH 3T3 fibroblasts were transfected with expression vectors for the fusion protein Gal4-SMRT NRID, the full-length PPAR γ fused to the VP16-AD (VP16-PPAR γ), and each RXR isotype. The full-length RAR α fused to VP16-AD (VP16-RAR α) was used as a positive control. The basal level observed in the presence of the VP16-AD and Gal4-SMRT NRID was arbitrarily set to 1. The reporter gene was a pGL3-based vector containing 6 repeats of the UAS yeast sequence. **(B)** Interaction of SMRT with the PPRE-bound PPAR γ -RXR α heterodimer. NIH 3T3 cells were transfected with the expression vectors and reporter gene as in **A**, together with an expression vector coding for the VP16-AD or the VP16-AD fused to the SMRT NRID (VP16-SMRT). At 24 hours after transfection, increasing concentrations of RSG (0, 1, or 5 μ M) were added for 16 hours, and luciferase activities were assayed and quantified as in **A**. The RXR α -RAR α heterodimer was used as a positive control in response to 0.5 or 1 μ M atRA. **(C)** Interaction of SMRT with the PPRE-bound PPAR γ -RXR β or the PPAR γ -RXR γ heterodimer. Transient transfection experiments were carried out as described as in **B** using either a RXR β or a RXR γ expression vector. Data represent mean \pm SEM. $n = 3$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

scriptural reprogramming. Whether RXR α - and RXR β -containing heterodimers control an overlapping or a specific transcriptional program is under investigation. A better understanding of the as yet putative specific roles of RXR isotypes and of their regulation in pathological conditions may help define new therapeutic strategies to treat T2DM and associated comorbidities.

Methods

Chemicals. Isobutylmethylxanthine (IBMX), insulin, dexamethasone, and atRA were obtained from Sigma-Aldrich. LDN-57444 was purchased from Calbiochem. RSG was synthesized at the chemical facilities at Institut de Recherches Servier.

Plasmids. pSG5-hRAR, pSG5-hRXR, pSG5-hRXR, pSG5-hRXR, pSG5-hPPAR γ (DR5)-3-tk Luc, VP16-hRAR (PPRE)6-tk Luc, and Gal4-tk Luc were described elsewhere (58–61). VP16-PPAR γ was provided by B.M. Forman (City of Hope National Medical Center, Duarte, California, USA; ref. 62). Gal4-hSMRT₂₁₁₇₋₂₃₅₇ and VP16-hSMRT₂₁₁₇₋₂₃₅₇ were gifts from A.N. Hollenberg (Beth Israel Deaconess Medical Center, Boston, Massachusetts, USA; ref. 25). pCMX-VP16-NCoR₁₅₈₅₋₂₄₅₃ was provided by R. Renkawitz and M. Schulz (Institute for Genetics, Giessen, Germany; ref. 63). The pCMV-based human UCH-L1, USP22, and WWP2 expression vectors were purchased from OriGene.

Cell culture and transfection. NIH 3T3 cells were grown in DMEM (Gluta-max-1, high glucose; Invitrogen) supplemented with 10% FCS (BioWhittaker) and 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen). Transient transfection experiments were performed using Lipofectamine 2000 (Invitrogen). Luciferase assays were performed with the dual luciferase assay system (Promega) according to the manufacturer's guidelines.

Adipocyte differentiation was induced as follows: 3T3-L1 preadipocytes were cultured in DMEM supplemented with 10% FCS until they reached confluence. The medium was then changed, and cells were allowed to grow for 2 additional days. Adipocyte differentiation was then induced by replacing the culture medium by DMEM supplemented with 10% FCS, 0.5 mM

IBMX, 1 μ M dexamethasone, and 10 μ g/ml insulin. 2 days later, the medium was replaced with DMEM supplemented with 10% FCS and 10 μ g/ml insulin for 2 additional days. Cells were then refed at 48-hour intervals with DMEM supplemented with 10% FCS only.

Pulse-chase labeling and immunoprecipitation. Differentiated 3T3-L1 cells were starved in methionine/cysteine-free DMEM supplemented with glutamine and 10% dialyzed FCS for 4 hours. [³⁵S] methionine and cysteine (250Ci, EasyTag EXPRESS³⁵S Protein Labeling Mix; Perkin-Elmer) was added to the culture medium for 4 hours. At 2 hours prior to the end of the pulse, 500 μ M CoCl₂ and/or 10 μ M LDN-57444 was added to the medium. Radioactive media was removed and substituted for regular medium supplemented with 2 mM cysteine and methionine, CoCl₂, and/or LDN-57444. At the indicated times, approximately 10⁷ cells were collected in ice-cold 1 \times PBS, centrifuged, and lysed into 500 μ L RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% NP-40; 0.5% deoxycholate; 0.1% SDS; 10 μ g/ml leupeptin; 10 μ g/ml pepstatin; 5 μ g/ml aprotinin; 5 mM DTT; 5 mM PMSE; and 1 mM benzamide). The homogenate was spun down, and the supernatant was precleared with a protein A/protein G sepharose mix. The supernatant was incubated overnight at 4 $^{\circ}$ C with 5 μ g anti-RXR α antibody (Δ N-197, sc-774; Santa Cruz Biotechnology Inc.). Complexes were precipitated with a protein A/protein G mix, washed, and analyzed by 8% SDS-PAGE.

In vitro ubiquitin conjugation. In vitro ubiquitylation assays were carried out using a S100 HeLa cell extract and reagents according to the manufacturer's instructions (Enzo Life Sciences).

In vivo ubiquitin conjugation. 3T3-L1 preadipocytes were transfected with expression vectors encoding HA-tagged ubiquitin (MT123-Ub HA; refs. 64, 65), RXR α , or RXR β . At 24 hours after transfection, whole cell extracts were prepared in RIPA buffer supplemented with 10 μ M MG132, and HA-tagged proteins were immunoprecipitated using an anti-HA tag antibody (ab9110; Abcam) or anti-RXR antibodies (see below). Ubiquitylated RXR was detected by Western blotting using either anti-RXR or anti-HA tag antibodies as indicated in the figure legends.

Immunofluorescence and Western blot. The anti-RXR α antibody used in immunochemistry experiments was from Perseus (PP-K8508-00). WAT was embedded in paraffin and sectioned (7 μ m). Sections were deparaffinized and rehydrated. After brief heating, the endogenous peroxidase activity was quenched, and sections were then incubated with the anti-RXR antibody (1:200 dilution). Sections were subsequently incubated with biotinylated goat anti-mouse antibody, then streptavidin-HRP



(Vectastain). Antigenic complexes were detected with 3,3'-diaminobenzidine, and sections were mounted in Mowiol (Kuraray). Images were collected with a Leica microscope and a camera coupled to the Leica IM500 image Manager software with $\times 10$ magnification.

Primary antibodies used in Western blotting experiments were directed against RXR α (D-20, sc-553), RXR β (C-20, sc-831), RXR γ (Y-20, sc-555), PPAR γ (H-100, sc-7196; all from Santa Cruz Biotechnology Inc.), and UCH-L1 (3525S; Cell Signaling Technology). Secondary antibodies coupled to HRP were from Sigma-Aldrich. Immune complexes were detected using the ECL+ system from Amersham/GE Healthcare.

Yeast interaction assays. Yeast 2-hybrid experiments, as described by Carmona et al. (54), were carried out at Phenex GmbH.

ChIP assays. ChIP assays were carried out as described previously (58, 66). Primer sequences used in ChIP assays were described elsewhere (26), except for the Adpn promoter region (forward, 5'-CCATGCCTGCAGTC-CATCTA-3'; reverse, 5'-GCTTCTGTCAAGCCATCCTGT-3'). The antibody to SMRT (PA1-844A) was from Affinity Bioreagents, whereas anti-NCoR (sc-1609), anti-PPAR α (sc-9000), anti-PPAR γ (sc-7196), anti-RXR α (sc-553), anti-RXR β (sc-831), and anti-RXR γ (sc-555) were purchased from Santa Cruz Biotechnology Inc.

GST-pulldown assays. GST-pulldown experiments were performed as described previously (67). Data were acquired on a Storm 860 phosphorimager, and band intensities were quantified using ImageQuant TL software.

RNA extraction, Affymetrix analysis, and real-time PCR. Total RNA was prepared using the RNeasy minikit (Qiagen). Total RNA was isolated from mouse WAT or from 3T3-L1 cells using the RNeasy Lipid Tissue kit (Qiagen). Purified RNA was adjusted to 1 g/l, and its integrity was assessed by gel electrophoresis or on an Agilent Bioanalyzer. RNA was purified from visWAT from 3 mice per group (OB/OB, OB/OB plus RSG, ob/ob, and ob/ob plus RSG) and hybridized to Affymetrix 430 2.0 arrays after cDNA labeling. Data were analyzed using the GeneSpring GX software (Agilent). Normalization was performed using the RMA algorithm, followed by a Benjamini-Hochberg false discovery rate statistical analysis. Genes that were significantly upregulated or repressed by more than 1.5-fold were then classified by a Gene Ontology functional classification. For RT-QPCR analysis of transcripts, reverse transcription was performed using random hexamers as recommended by the manufacturer (Promega). cDNAs were analyzed by PCR amplification using the TaqMan PCR master mix (Applied Biosystems) and a mix of actin primers and appropriate FAM probes. Absolute quantification of RXR mRNAs were determined by generating standard curves with known amounts of cloned RXR cDNAs. Actin, aP2, GyK, and Adpn primers and all other probes were purchased from Applied Biosystems (Assay on Demand). PCR (40 cycles) and data analysis were carried out using an ABI Prism 7500 (Perkin-Elmer).

RNA interference. Specific siRNA duplexes targeting RXR α , SMRT, and nonspecific siRNA controls were synthesized by Santa Cruz Biotechnology Inc. siRNAs were transfected using the DeliverX Plus siRNA transfection kit (Panomics) according to the manufacturer's guidelines.

Animal experiments. Mouse RNA and tissue samples were provided by M. Brun and A. Géant (Institut de Recherches Servier, Suresnes, France) and H. Duez (INSERM U545, Lille, France). OB/OB and ob/ob mice (8–10 weeks old) were purchased from Charles River. Mice were housed in a temperature-controlled room (22°C–24°C), with a relative humidity of 36%–80% and 12-hour light/dark cycles. Mice were fed ad libitum with free access to filtered tap water (0.22- μ m filter) and received irradiated pelleted laboratory chow (A03-10; UAR) throughout the study, supplemented or not with RSG to achieve a 3-mg/kg/d intake. Mice were euthanized by cervical dislocation, and visWAT and scWAT were dissected, weighed, and immediately stored in liquid N₂. All procedures were validated by the ethical committee of the Institut Pasteur de Lille and carried out in accordance with European Union (EEC, no. 07430) and French ethical guidelines.

Isolation of stromal vascular fraction from WAT. Cells were prepared from WAT of obese or control mice as previously described (68), with minor modifications. Briefly, tissue was digested at 37°C in 1 \times PBS containing 0.2% BSA and 2 mg/ml collagenase (type II collagenase; Sigma-Aldrich). After filtration of the homogenate through 25- μ m filters, mature adipocytes were separated from pellets (stromal vascular fraction) by centrifugation at 600 g for 10 minutes. After red cell lysis in 140 mM NH₄Cl and centrifugation, stromal vascular fraction cells were pelleted and resuspended in 1 \times PBS. Cells were seeded at 10,000 cells/cm² in DMEM-F12 supplemented with 10% newborn calf serum. Extensive washes were performed 12 hours later, and whole cell lysates were prepared 48 hours later.

Human tissues. Human tissue samples were provided by M.-F. Six and the Centre d'Investigations Cliniques (C.H.R.U. Lille). Human WAT samples were collected from patients undergoing abdominal surgery by laparoscopy or coelioscopy after informed consent was obtained. All procedures were approved by the C.H.R.U. Lille ethical committee and were compliant to the French National Ethics Committee guidelines. Tissue samples from female patients – aged 35–59 years and not receiving any oral antidiabetic treatment – were removed within the first 30 minutes of the surgical procedure and immediately frozen in liquid N₂. Visceral fat was removed from the great omentum, and the subcutaneous fat was taken in the vicinity of the laparotomy incision. Based on biochemical and morphological parameters, patients were classified as lean (BMI, <25; fasting glucose [FG], 6 mM; oral glucose tolerance test [OGTT], <7.8 mM); obese (BMI, >35; FG, <6 mM; OGTT, <7.8 mM); obese glucose intolerant (BMI, >35; FG, between 6 and 7 mM, OGTT, between 7.8 and 11.1 mM) and obese diabetic (BMI, >35; FG, >7 mM; OGTT, >11.1 mM). Glycemia was assayed in the OGTT test 120 minutes after the glucose load.

Statistics. Data are mean \pm SEM. Calculations were carried out using Prism software (GraphPAD Inc.). QPCR, Western blot, and transient transfection experiments were analyzed with the 2-tailed Student's *t* test. Statistical significance of differences between pairs of groups in animal studies was assessed using ANOVA followed by Tukey analysis. A *P* value less than 0.05 was considered significant.

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