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SUMOylation of Human Peroxisome Proliferator-activated Receptor α Inhibits Its Trans-activity through the Recruitment of the Nuclear Corepressor NCoR*

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Benoit Pourcet[‡§¶‖], Inés Pineda-Torra[**], Bruno Derudas[‡§¶‖], Bart Staels[‡§¶‖], and Corine Glineur[‡§¶‖1]

From the [‡]Université Lille Nord de France, [§]Inserm, U545, and [¶]UDSL, F-59000 Lille, France, the [‖]Institut Pasteur de Lille, F-59019 Lille, France, and the [**]Division of Medicine, University College of London, London WC1 E6JJ, United Kingdom

The nuclear receptor peroxisome proliferator-activated receptor α (PPARα) is a key regulator of genes implicated in lipid homeostasis and inflammation. PPARα trans-activity is enhanced by recruitment of coactivators such as SRC1 and CBP/p300 and is inhibited by binding of corepressors such as NCoR and SMRT. In addition to ligand binding, PPARα activity is regulated by post-translational modifications such as phosphorylation and ubiquitination. In this report, we demonstrate that hPPARα is SUMOylated by SUMO-1 on lysine 185 in the hinge region. The E2-conjugating enzyme Ubc9 and the SUMO E3-ligase PIASy are implicated in this process. In addition, ligand treatment decreases the SUMOylation rate of hPPARα. Finally, our results demonstrate that SUMO-1 modification of hPPARα down-regulates its trans-activity through the specific recruitment of corepressor NCoR but not SMRT leading to the differential expression of a subset of PPARα target genes. In conclusion, hPPARα SUMOylation on lysine 185 down-regulates its trans-activity through the selective recruitment of NCoR.

The nuclear receptor peroxisome proliferator-activated receptor α (PPARα)[2] is a key regulator of energy homeostasis (1–5) and the anti-inflammatory response (6–8). PPARα is expressed highest in tissues with high fatty acid catabolic activity such as liver, heart, kidney, and skeletal muscle, and also in vascular cells (9). PPARα modulates metabolism, especially lipid homeostasis, through its so-called trans-activation activity (10). The use of synthetic PPARα ligands, such as fibrates, improves lipid profiles in dyslipidemic patients (see review in Ref. 11).

The structure of PPARα consists of an N-terminal A/B domain containing a ligand-independent trans-activation function called activating function-1 (AF-1), a DNA-binding C domain (DBD) containing two highly conserved zinc finger motifs, a hinge D region and, at the C terminus, a ligand-binding E domain (LBD), which contains the ligand-dependent activation function called AF-2 (Fig. 2*A*) (13). The D hinge region not only links the DBD with the LBD but is also implicated in corepressor recruitment (12, 14).

PPARα induces gene transcription after heterodimerization with the retinoic X receptor (RXR) and binding via its DBD to specific DNA sequences called peroxisome proliferator response elements (PPREs) in the promoter of its target genes (15). As other transcription factors, PPARα is likely highly mobile in the nuclear environment, and interacts briefly with target sites moving through many states during activation and repression. The binding of ligands to PPARα modifies the conformation of the PPARα LBD unmasking an interaction area for coactivators such as steroid receptor coactivator 1 (SRC1) and the cAMP response element-binding protein (CREB)-binding protein (CBP)/p300, which possess histone acetyl transferase activity (HAT) resulting in chromatin decondensation and target gene activation (13). In the absence of ligand, the PPARα/RXRα complex actively represses the expression of target genes through the recruitment of transcriptional corepressor complexes such as the nuclear receptor corepressor (NCoR) or the silencing mediator for retinoid and thyroid hormone (SMRT) (12–14). The NCoR and SMRT corepressors have been found to exist *in vivo* in multiple, distinct macromolecular complexes. While these corepressor complexes differ in overall composition, a general theme is that they contain histone deacetylase enzymatic activity (13). It is commonly believed so far that NCoR and SMRT down-regulate the same genes. However, it has recently been demonstrated that liver X receptor (LXR)-regulated genes can be modulated in a NCoR- and/or SMRT-specific manner (16). Thus, a subset of genes appear to be regulated specifically either by NCoR or SMRT. Unfortunately, no regulatory mechanism has been proposed yet to explain this phenomenon.

PPARα activity can be regulated by post-translational modifications such as ubiquitination (17) and phosphorylation (see review in Ref. 18). While this study was in progress, Leuenberger *et al.* (19) have shown that the murine PPARα is SUMOylated on lysine 358, and this SUMOylation triggers the interaction with GA-binding protein α bound to the *cyp7b1* promoter resulting in specific down-regulation of this gene. Although this study identified a role for SUMO modification in the regulation of

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[1] To whom correspondence should be addressed: Inserm UR545, Institut Pasteur de Lille, 01 rue du Professeur Calmette, BP 245, Lille 59019, France. Tel.: 33-3-20-87-77-75; Fax: 33-3-20-87-71-98; E-mail: Corine.Glineur@pasteur-lille.fr.

[2] The abbreviations used are: PPARα, peroxisome proliferator-activated receptor α; NCoR, nuclear receptor corepressor; SMRT, silencing mediator for retinoid and thyroid hormone; GST, glutathione *S*-transferase; WT, wild type; NTA, nitrilotriacetic acid; PPRE, peroxisome proliferator response element; BSA, bovine serum albumin.

mPPARα trans-repressive activity, it is unknown whether human PPARα is SUMOylated.

SUMO modifications play an important role in controlling the function of several proteins including transcription factors (20). SUMO proteins are conjugated to proteins through a series of enzymatic steps including conjugation to the E2-conjugating enzyme Ubc9 (see review in Ref. 21). Targeted lysine residues are part of the consensus site ΨK*X*(D/E), where Ψ is a hydrophobic amino acid, K is the modified lysine, *X* represents any residue and D or E is an acidic residue. However, *in vivo* SUMO conjugation needs a fourth class of proteins, the so-called E3-ligating enzymes, such as the protein inhibitor of activated STAT (PIAS) family, which are implicated in the specificity of the substrate recognition by the SUMO pathway. Finally, SENP desumoylase family catalyzes the de-conjugation of SUMO from their substrate.

In this report, we show that hPPARα is conjugated with SUMO-1 *in vitro*, in COS-7 cells and in the human hepatoma cell line HuH-7. In addition, hPPARα directly interacts *in vitro* with the E2-conjugating enzyme Ubc9. Furthermore, we demonstrate that the E3-ligating enzyme PIASy regulates hPPARα SUMO-1 conjugation. The SUMOylation site of human PPARα was mapped to the lysine residue at position 185, located in the hinge region of the receptor. Arginine substitution of this lysine residue increased the transcriptional activity of hPPARα suggesting that SUMOylation of this lysine reduces hPPARα trans-activity, which is explained by a facilitated recruitment of the corepressor NCoR, but not SMRT, upon hPPARα SUMOylation. We also demonstrate that the SUMO pathway specifically decreases NCoR-specific hPPARα target gene expression. Finally, we demonstrate that the hPPARα ligand GW7647 reduces hPPARα SUMOylation.

EXPERIMENTAL PROCEDURES

Materials—DMEM and fetal calf serum (FCS), glutamine and gentamycin were purchased from Invitrogen (Cergy Pontoise, France). The human hepatoma HuH-7 cell line and COS-7 cell lines were purchased from LGC Promochem (Molsheim, France). GW7647 was kindly provided by Glaxo-Smith-Kline (Les Ulis, France). JetPEI was purchased from Ozyme (Saint-Quentin en Yvelines, France). Redivue L-[^{35}S]methionine was purchased from Amersham Biosciences (Saclay, France). The pSG5-hPPARα and J6-TK-Luc were described previously (8). The pSG5-hRXRα, pCI-SMRT, pKCR2-NCoR full-length expression vectors were kindly provided by Dr. P. Lefebvre (Lille, France). The vector VP16-SMRT and VP16-NCoR were kindly provided by Dr M. Schutz (Justus-Liebig-Universität, Giessen, Germany). The pGEX4T2-Ubc9, pSG5-Ubc9 and pSG5-SUMO-1-His$_6$ vectors were kindly provided by K. Tabech (Institut Cochin, Paris, France). The pcDNA3-FLAG-PIASy was kindly provided by Dr A. Dejean (Institut Pasteur, Paris, France). The efficiency of transfection was monitored using the control plasmid pSV-β-galactosidase.

Site-directed Mutagenesis—The pSG5-hPPARα K138R, K185R, K216R, K310R, K358R, K449R expression vectors were generated using pSG5-hPPARα WT as template and the QuikChange XL-II Site-directed Mutagenesis kit (Stratagene, Amsterdam, Netherlands). The point mutations were introduced by using the following synthetic oligonucleotide pairs of primers: K138R: forward: 5′-CGA CTC AAG CTG GTG TAT GAC AGG TGC GAC CGC AGC TGC AAG ATC C-3′ and reverse: 5′-GGA TCT TGC AGC TGC GGT CGC ACC TGT CAT ACA CCA GCT TGA GTC G; K185R: forward: 5′-GAG AAA GCA AAA CTG AGA GCA GAA ATT CTT ACC-3′ and reverse: 5′-GGT AAG AAT TTC TGC TCT CAG TTT TGC TTT CTC-3′; K216R: forward: 5′-GGG TCA TCC ATG GAA AGG CCA GTA ACA ATC C-3′ and reverse: 5′-GGA TTG TT CTG GCC TTT CCA TCG AGG ATG ACC C-3′; K310R: forward: 5′-GAA CGA TCA AGT GAC ATT GCT AAG ATA CGG AGT TTA TGA GGC C-3′ and reverse: 5′-GGC CTC ATA AAC TCC GTA TCT TAG CAA TGT CAC TTG ATC GTT C-3′; K358R: forward: 5′-CTG TGA TAT CAT GGA ACC CAG GTT TGA TTT TGC CAT GAA G-3′ and reverse: 5′-CTT CAT GGC AAA ATC AAA CCT GGG TTC CAT GAT ATC ACA G-3′; K449R: forward: 5′-GCT GGT GCA GAT CAT CAA GAG GAC GGA GTC GGA TGC TGC GC-3′ and reverse: 5′-GCG CAG CAT CCG ACT CCG TCC TCT TGA TGA TCT GCA CCA GC-3′. The mutated cDNAs were entirely sequenced.

Transient Transfection Experiments—HuH-7 cells, cultured in 24-well plates (5×10^4 cells per well), were transfected with 10 ng of J6-TK-Luc, 100 ng of pSV-β-galactosidase, 5 ng of pSG5-hPPARα (WT or K185R) expression vectors and indicated amounts of pKCR2-NCoR, pCI-SMRT, VP16-NCoR, or VP16-SMRT using JetPEI transfection reagent (Ozyme, Saint-Quentin en Yvelines, France) according to the manufacturer's instruction. After 24 h, cells were incubated in medium containing 0.2% fetal calf serum; 0.2% fatty acid-free BSA (Sigma) and Me_2SO or 600 nM of GW7647 (kindly provided by GlaxoSmithKline). After 24 h, cells were lysed with 100 μl of reporter lysis buffer (Promega, Charbonnières, France) according to the manufacturer's protocol, and the luciferase activity was analyzed with Mithras LB940 luminometer (Berthold Technologies, Thoiry, France). As transfection control, β-galactosidase activity was analyzed as previously described.

siRNA Transient Transfection—ON-TARGETplus SMARTpool siRNA human PPARα (J-003434) (5′-CCCGUUAUCUGAAGAGUUC-3′, 5′-GCUUUGGCUUUACGGAAUA-3′, 5′-GACUCAAGCUGGUGUAUGA-3′ and 5′-GGGAAACAUCCAAGAGAUU-3′) and ON-TARGETplus SMARTpool siRNA human Ubc9 (J-004910) (5′-GGGAAGGAGGCUUGUUUAA-3′, 5′-GAAGUUUGCGCCCUCAUAA-3′, 5′-GGCCAGCCAUCACAAUCAA-3′ and 5′-GAACCACCAUUAUUUCACC-3′) were purchased from Dharmacon (Thermo-Fisher Scientific, Saint Herblain, France); siRNAs human NCoR (5′-CCAUGCAUCUAAAGUUGAATT-3′) and human SMRT (5′-CCGAGAGAUCACCAUGGUATT-3′) were purchased from Ambion (Applied Biosystem, Courtabœuf, France). HuH-7 were transfected for 24 h with 50 nM of siRNA using Dharmafect1 transfection reagent (Dharmacon, Thermo-Fisher Scientific) according to the manufacturer's protocol. Knock-down efficiencies of each siRNA were analyzed by RT Q-PCR (Fig. 1, *A* and *B* and supplemental Fig. S1).

GST Pulldown Assay—GST pulldown assays have been performed as previously described (22). Briefly, BL21-Star

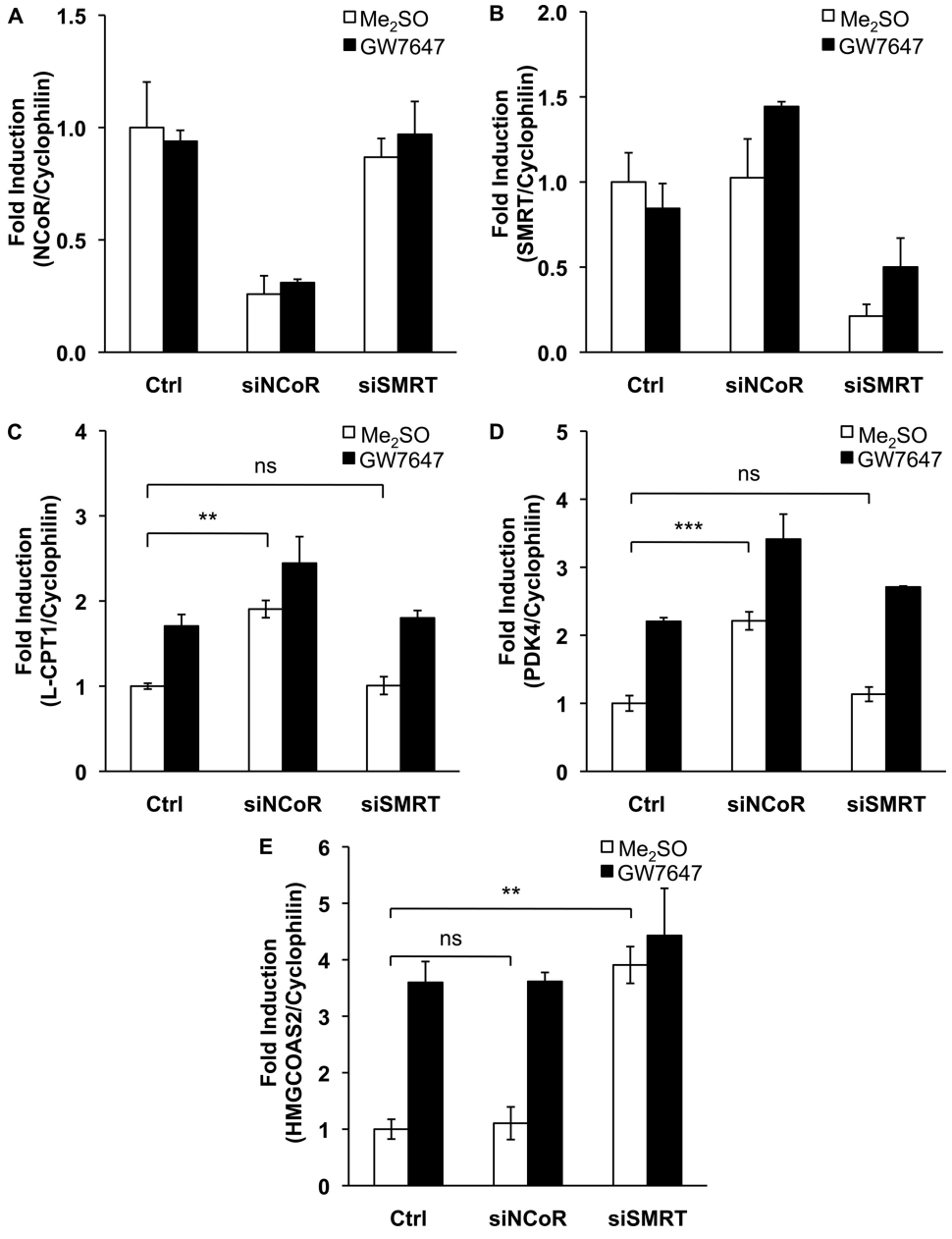

FIGURE 1. **Selective effect of decreased expression of NCoR and SMRT on PPARα target genes in HuH-7 cells.** HuH-7 cells were transfected with control, NCoR, or SMRT siRNA and treated with GW7647 (600 nM) or vehicle ($MeSO_2$). RNA was extracted, and the expression of *NCoR* (*A*), *SMRT* (*B*), *L-CPT1* (*C*), *PDK4* (*D*), and mitochondrial *HMGCOAS2* (*E*) genes was measured by real-time quantitative PCR. Each bar is the mean value ± S.D. of triplicate determinations. Statistical differences are indicated (*t* test; Scramble *versus* siRNA Me_2SO: **, $p < 0.01$; ***, $p < 0.001$; *ns*, nonsignificant).

[pGEX4T2-Ubc9] and BL21-star [pGEX4T2] *Escherichia coli* strains were grown in Terrific Broth medium (Invitrogen). GST protein expression was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. Bacteria were mechanically disrupted with FRENCH-Press, and GST and GST fusion proteins were isolated using the pulldown technique. A total of 15 μg of GST, GST-Ubc9 were incubated with 4 μl of [^{35}S]methionine hPPARα WT for 2 h at 4 °C. Finally, bound proteins were boiled at 95 °C, separated by SDS-PAGE, and analyzed by autoradiography.

Western Blotting Analysis—Proteins were resolved by SDS-PAGE electrophoresis and transferred on EtOH-preactivated polyvinylidene fluoride membrane (Millipore, St-Quentin en Yvelines, France). Then, proteins were probed with the corresponding primary antibodies and revealed using horseradish peroxidase-coupled IgG and Immobilon Western detection kit (Millipore). The anti-PPARα and anti-actin antibodies were obtained from Tebu-Bio (Le Perray-en-Yvelines, France). The anti-His_6 antibodies were from ABD Serotec (Oxford, England). The anti-NCoR antibodies were from Affinity Bioreagent (Thermo Fisher Scientific). The secondary antibodies against rabbit and mouse IgG were purchased from Amersham Biosciences (Orsay, France), and the secondary antibodies against goat IgG was obtained from Thermo Fisher Scientific.

Nickel Pulldown Assay—Transfected cells were lysed in denaturating conditions using 6 M-guanidine hydrochloride. His-SUMO-conjugated proteins were recovered with Ni^{2+}-nitrilotriacetic acid (Ni-NTA) beads (Qiagen, Courtaboeuf, France) as previously described (23). Recovered proteins were then separated by SDS-PAGE and analyzed by Western blotting.

In Vitro SUMOylation Assays—*In vitro* SUMO modification was carried out with purified recombinant products provided by SUMOlink kit (Active Motif, Rixensart, Belgium) and [^{35}S]methionine-labeled PPARα proteins generated by *in vitro* transcription/translation in reticulocyte extract (Promega) according to the manufacturer's instructions. Reaction products were fractionated by SDS-PAGE and analyzed by autoradiography.

Coimmunoprecipitation—HuH-7 cells were cross-linked with 1.5 mM ethylene glycol-bis(succinimidylsuccinate) (Thermo Fisher Scientific) for 20 min at room temperature. After ice-cold phosphate-buffered saline washes, cells were lysed with lysis buffer (Tris-HCl, 20 mM, pH 7.5; NaCl, 150 mM; EDTA, 1 mM; EGTA, 1 mM; Triton X-100 1%; protease inhibitors). 300 μg of recovered proteins were incubated with FLAG M2 monoclonal antibodies agarose (Sigma) overnight at 4 °C. Beads were washed four times with ice-cold TBS and eluted with Laemmli buffer. Protein amounts were analyzed by Western blotting.

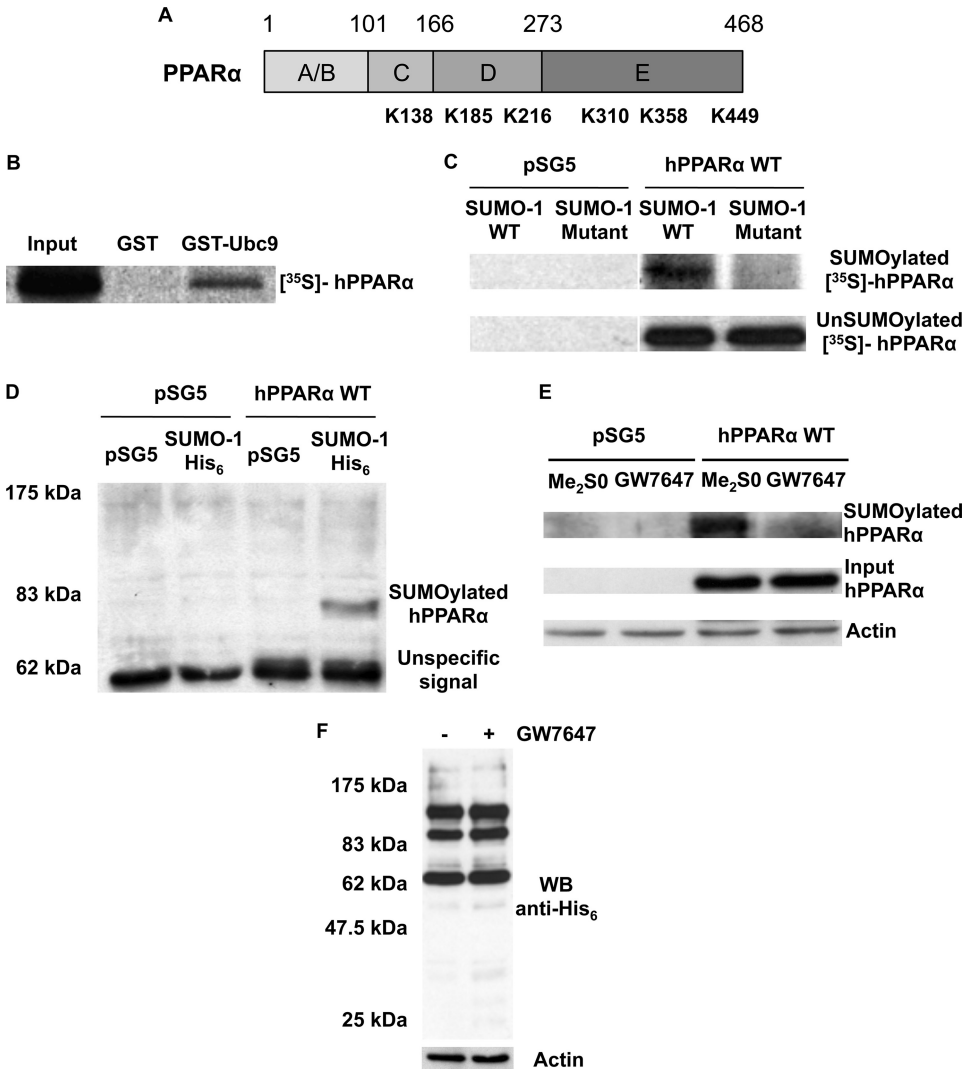

FIGURE 2. **Human PPARα is a substrate for SUMO-1 modification *in vitro* and in HuH-7 cells.** *A*, protein sequence of hPPARα (NM Q07869) was analyzed with the SUMOylation prediction site algorithm SUMOplot™ prediction. *Numbers* correspond to amino acids position. The A/B domain contains AF-1 ligand-independent transcriptional activity; *C*, DNA binding domain; *D*, hinge region; *E*, ligand binding domain containing AF-2 ligand-dependent transcriptional activity. *B*, [^{35}S]methionine hPPARα protein was incubated with GST or GST-Ubc9 proteins. Complexes were precipitated with glutathione-Sepharose, and proteins were analyzed by autoradiography. *C*, [^{35}S]methionine hPPARα WT protein or [^{35}S]methionine reticulocyte lysate (as control) were incubated with SUMO E1-activating enzyme, Ubc9, and SUMO-1 WT protein or unconjugatable SUMO-1 mutant protein provided by the SUMOlink® kit. Proteins were separated by SDS-PAGE and analyzed by autoradiography. *D*, HuH-7 cells were transfected with pSG5-hPPARα WT expression vector and/or pSG5-SUMO-1-His$_6$ expression vectors. After 24 h, cells were lysed in denaturating conditions with HCl-guanidinium. Lysates were incubated with Ni-NTA beads and subsequently eluted with loading buffer. Proteins were separated by SDS-PAGE and analyzed by Western blotting using anti-PPARα antibodies. *E*, HuH-7 cells were transfected with pSG5 control vector or pSG5-hPPARα WT expression vector, and with pSG5-SUMO-1-His$_6$ expression vectors. Cells were treated with Me_2SO as control or GW7647 (600 nM). After 24 h, a SUMOylation test was performed as described above. *F*, HuH-7 cells were transfected with pSG5-SUMO-1-His$_6$ expression vector and treated with GW7647 for 24 h. SUMOylated proteins were analyzed by Western blotting using an anti-His$_6$ antibodies.

RESULTS

Human PPARα Target Genes Are Regulated in a NCoR- or SMRT-specific Manner—As previously described, LXR target genes can be regulated in a NCoR- and/or SMRT-specific manner (16). However, such mechanism has not been described so far for hPPARα. To investigate this hypothesis, HuH-7 cells were transfected either with siRNA for NCoR or SMRT (Fig. 1). After RNA purification, the expression of hPPARα target genes implicated in different metabolic pathways was analyzed such as fatty acid transport (*L-CPT1*), glycolysis regulation (*PDK4*), and ketogenesis (3-hydroxy-3-methylglutaryl-CoA synthase 2; *HMGCOAS2*). Interestingly, *L-CPT1* and *PDK4* gene expression are up-regulated in the absence of NCoR but not in the absence of SMRT (Fig. 1, *C* and *D*, respectively). Inversely, *HMGCOAS2* expression is increased in the absence of SMRT but not in the absence of NCoR (Fig. 1*E*). These data suggest that *L-CPT1* and *PDK4* are NCoR-sensitive hPPARα target genes, whereas *HMGCOAS2* is a SMRT-regulated hPPARα target gene.

Because the SUMO pathway is known to enhance interaction with NCoR as demonstrated for PPARγ and LXR (24, 25), we investigated whether hPPARα is SUMOylated and whether this SUMOylation could regulate the selective recruitment of corepressors by hPPARα.

Human PPARα Interacts with the SUMO E2-conjugating Enzyme Ubc9—We first assessed direct association of hPPARα with the SUMO E2-conjugating enzyme Ubc9 by GST pulldown. GST-Ubc9 WT was incubated with *in vitro* translated ^{35}S-labeled hPPARα protein. As shown in Fig. 2*B*, PPARα interacts with GST-Ubc9 but not with GST alone, indicating that hPPARα interacts directly with Ubc9 *in vitro*.

Human PPARα Is a Substrate for SUMO-1 Modification in Vitro and in Vivo—While this study was ongoing, it has been shown that murine PPARα is SUMOylated (26). No data are however available concerning human PPARα. SUMOplot™ prediction algorithm analysis identified six putative SUMOylation sites (Lys-138, Lys-185, Lys-216, Lys-310, Lys-358, Lys-449) in human PPARα, which are conserved between species (Fig. 2*A*). To examine whether hPPARα can be SUMOylated *in vitro*, ^{35}S-labeled hPPARα was incubated with the SUMO machinery enzymes provided by the SUMOlink kit. As control, unconjugatable SUMO-1 mutant protein was used instead of SUMO-1 wild-type (WT) protein. As shown in Fig. 2*C*, hPPARα is SUMOylated in the presence of SUMO-1 WT, but not the SUMO-1 mutant.

To examine whether hPPARα is SUMOylated in a cellular context, HuH-7 hepatoma cells were transfected with hPPARα WT and SUMO-1-His_6 expression vectors (Fig. 2*D*). Histidine-tagged SUMOylated proteins were then isolated from whole HuH-7 cell extracts using Ni-NTA beads. When SUMO-1-His_6 was co-expressed with hPPARα, an additional high molecular mass band (72 kDa) corresponding to SUMO-1-conjugated hPPARα was observed (Fig. 2*D*), suggesting that hPPARα is mono-SUMOylated in hepatic cells.

SUMOylation of hPPARα Is Ligand-regulated—The presence of ligand has been shown to regulate the SUMOylation of several nuclear receptors such as PPARγ (26). Hence, we investigated the effect of the PPARα-specific ligand GW7647 on the SUMO-1 modification of hPPARα. After transfection of HuH-7 cells with hPPARα WT and SUMO-1-His_6 expression vectors, the cells were treated with vehicle (Me_2SO) or GW7647 and SUMOylated PPARα was specifically analyzed by Western blotting using anti-PPARα antibodies. As shown in Fig. 2*E*, SUMOylation of hPPARα strongly decreased in the presence of GW7647 compared with vehicle. These data suggest that ligand binding either impairs the SUMOylation of hPPARα or promotes its desumoylation. To investigate whether GW7647 has an effect on the cellular SUMOylation pattern, HuH-7 cells were transfected with a SUMO-1 expression vector in the presence of GW7647 and histidine-tagged SUMOylated proteins were analyzed by Western blotting using anti-His_6 antibodies. As shown in Fig. 2*F*, GW7647 does not modify the amount of other SUMOylated proteins, suggesting that PPARα ligand GW7647 does not modulate the SUMOylation machinery in a general manner.

PIASy Acts as an E3-ligating Enzyme for hPPARα SUMOylation—PIAS protein family members have been shown to be essential for SUMOylation of nuclear receptors (27). For instance, it has been previously described that PIASy increases SUMOylation of RORα (28). Hence, we investigated the potential role of PIASy in the SUMOylation of hPPARα. In contrast to HuH-7, transfection of SUMO-1 or both SUMO-1/Ubc9 in COS-7 cells does not result in SUMOylation of hPPARα protein (Fig. 3*A*). However, when cells were co-transfected with hPPARα WT, SUMO-1-His_6, Ubc9 and FLAG-PIASy expression vectors (Fig. 3*A*), SUMO-1-modified hPPARα was found demonstrating that PIASy can function as an E3-ligating enzyme leading to the SUMOylation of hPPARα.

SUMOylation of hPPARα Decreases Its Trans-activation Activity—To define the role of the SUMO pathway on hPPARα activity, HuH-7 cells were co-transfected with the J6-TK-Luc reporter vector containing six copies of the J site PPRE from the apoA-II gene promoter and with a hPPARα WT expression vector and increasing amounts of PIASy (Fig. 3*B*). The activity of hPPARα decreased when PIASy was co-transfected, demonstrating that the SUMO pathway regulates human PPARα trans-activation.

The SUMO Pathway Inhibits NCoR-specific hPPARα Target Gene Expression—To address the role of hPPARα SUMOylation on the trans-activation activity of its target genes, HuH-7 cells were transfected with siRNA Ubc9 and/or siRNA hPPARα. The expression of different PPARα target genes was then evaluated. In the absence of Ubc9, the expression of

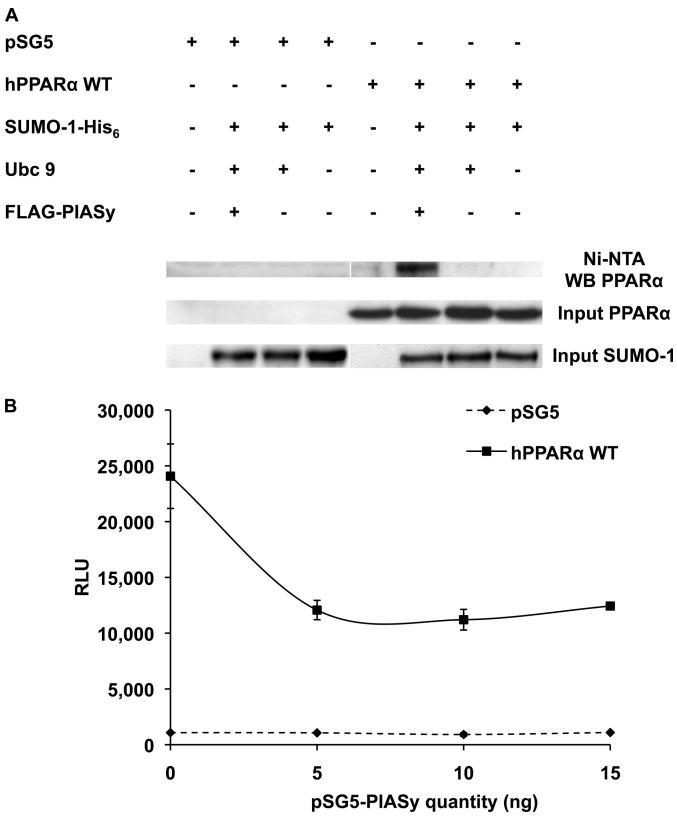

FIGURE 3. **PIASy regulates SUMOylation of human PPARα and inhibits its transcriptional activity.** *A*, COS-7 cells were co-transfected with pSG5 control vector or pSG5-hPPARα WT expression vector and with pSG5-SUMO-1-His_6, pSG5-Ubc9, and pcDNA3-FLAG-PIASy expression vectors as described. Empty vector pSG5 was used as negative control. After 48 h, cell extracts were incubated with Ni-NTA beads to isolate histidine-tagged SUMOylated proteins. The SUMOylated hPPARα proteins and hPPARα input proteins were analyzed by Western blotting using anti-PPARα antibodies. The SUMO-1-His_6 input proteins were analyzed by Western blotting using anti-His_6 antibodies. *B*, HuH-7 cells were co-transfected with pSG5 control vector, pSG5-hPPARα WT expression vectors and with increasing amounts of pcDNA3-FLAG-PIASy expression vectors or pcDNA3-FLAG as controls. After 24 h of transfection, the luciferase and β-galactosidase activities were measured in transfected cell lysates and the ratio luciferase activity/β-galactosidase activity was defined as RLU. Each bar is the mean value ± S.D. of triplicate determinations.

L-CPT1 or *PDK4*, which appears to be more sensitive to NCoR expression (Fig. 1, *C* and *D*), was significantly increased, suggesting that SUMO pathway inhibits their expression (Fig. 4, *A–C*). Interestingly, *HMGCOAS2* gene expression, which was not altered by NCoR silencing (Fig. 1*E*), was not affected by Ubc9 silencing. To evaluate the role of hPPARα on the observed effect of Ubc9 knockdown, we analyzed the expression of these genes in cells cotransfected with both siRNA for PPARα and Ubc9. Our results show that the impact of siRNA Ubc9 on *L-CPT1* and *PDK4* expression is abolished in the presence of siRNA PPARα, suggesting that the regulation of *L-CPT1* and *PDK4* gene expression by SUMOylation is mediated by hPPARα. Conversely, overexpression of PIASy significantly decreased the expression of target genes such as *L-FABP* and *PDK4* induced by hPPARα (Fig. 4, *D–F*), suggesting that activation of the SUMO machinery, and subsequent hPPARα SUMOylation, inhibits hPPARα target gene expression. Again, the SUMO pathway did not modulate the expression of HMGCOAS2. These results in concert with those in Fig. 1 demon-

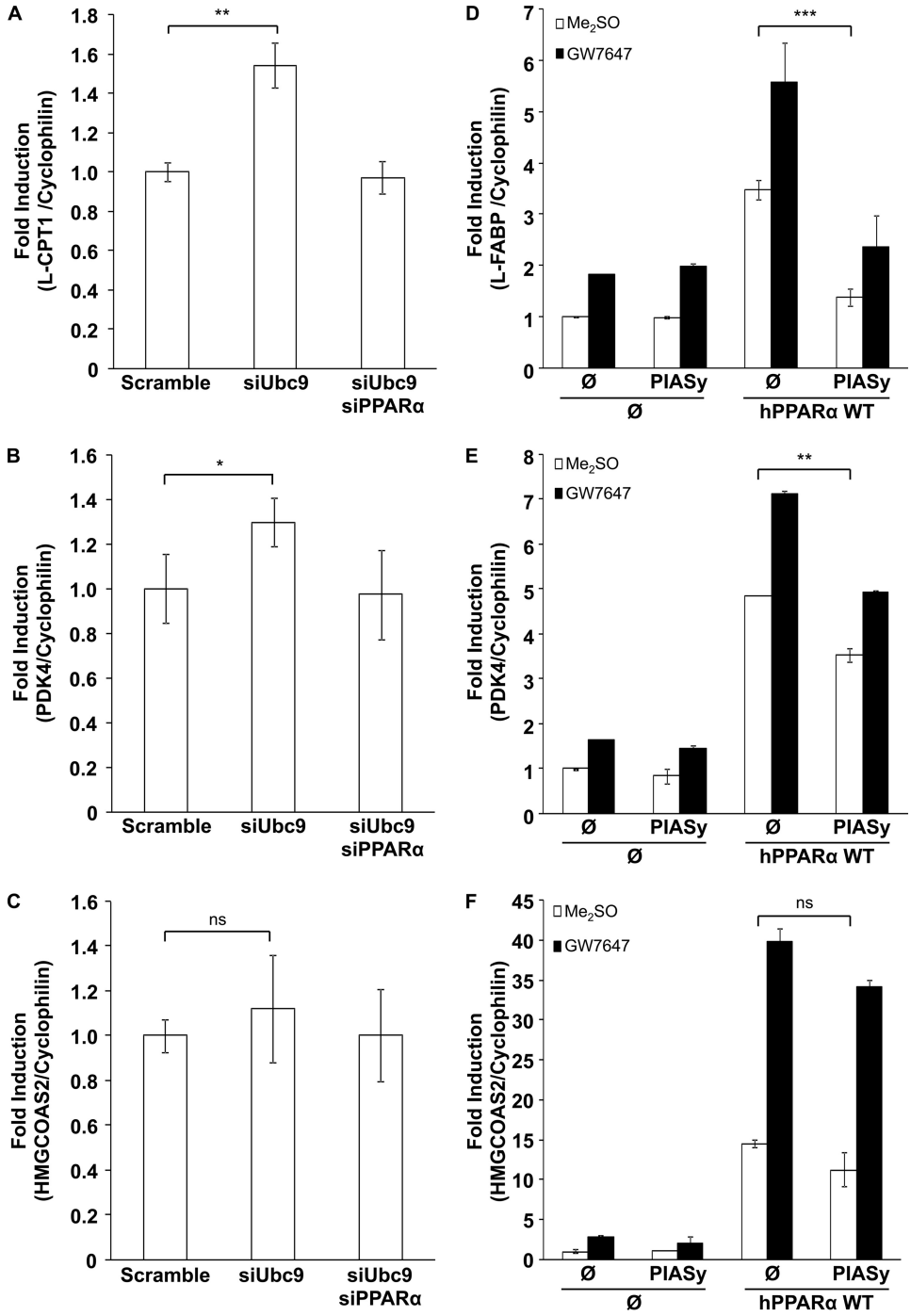

FIGURE 4. **Effect of SUMO pathway modulation on PPARα target genes in HuH-7 cells.** HuH-7 cells were transfected with siRNA Ubc9 and/or siRNA hPPARα. Then RNA was extracted, and the expression of *L-CPT1* (*A*), *PDK4* (*B*), and mitochondrial *HMGCOAS2* (*C*) genes was measured by real-time quantitative PCR. HuH-7 cells were cotransfected with pSG5-hPPARα WT and/or pcDNA3-FLAG-PIASy expression vectors or pSG5 vector and/or pcDNA3-FLAG as controls. After 24 h of transfection, cells were treated with Me_2SO or GW7647 (600 nM) in DMEM medium 0.2% fetal calf serum, 0.2% BSA, for 24 h. Then RNA was extracted and the expression of *L-FABP* (*D*), *PDK4* (*E*), and mitochondrial *HMGCOAS2* (*F*) was measured by real-time quantitative PCR. Each bar is the mean value ± S.D. of triplicate determinations. Statistical differences are indicated (*t* test; Control *versus* PIASy: *, $p < 0.05$; **, $p < 0.01$; *ns*, nonsignificant).

strate that the SUMO pathway selectively inhibits NCoR-specific hPPARα target genes in an hPPARα-dependent manner.

Human PPARα Is SUMOylated on Lysine 185—To identify the SUMOylated site in hPPARα, the six potential acceptor lysines (Fig. 2*A*) were individually replaced by site-directed mutagenesis by an unSUMOylatable arginine, a residue with a similar steric hindrance. Each hPPARα mutant protein was analyzed for their ability to be SUMOylated *in vitro*. As shown in Fig. 5*A*, no band corresponding to SUMOylated protein was visible with the mutant hPPARα K185R, indicating that hPPARα is modified *in vitro* by SUMO-1 in its hinge region on lysine 185.

To demonstrate that lysine 185 is a SUMOylation site *in vivo*, we compared the SUMOylation rate of hPPARα WT and K185R in COS-7 cells (Fig. 5*B*). The cells were transfected with PPARα and SUMO-1, Ubc9, and PIASy expression vectors and SUMOylated PPARα proteins were analyzed by Western blotting after 48 h. The significant reduction of the signal corresponding to the PPARα K185R protein compared with the WT protein confirms that the lysine 185 in hPPARα is a SUMO-1 acceptor site.

The Lysine 185 Is a Relevant Functional Site in the Regulation of hPPARα Transcriptional Activity—To determine the functional effect of the lysine 185 of hPPARα on the nuclear receptor trans-activity, HuH-7 cells were transfected with the J6-TK-Luc reporter vector, pSG5-hPPARα WT, or pSG5-hPPARα K185R expression vectors or pSG5 control (Fig. 6). HuH-7 cells were then treated with the specific hPPARα ligand GW7647. As expected, basal and ligand-induced activities of the mutant hPPARα K185R were significantly higher compared with the WT protein. While both WT and mutant K185R proteins are equally expressed in cells (Fig. 6*B*) in this experiment, we also observed a decrease in hPPARα expression by the ligand that is consistent with our previous studies (17). Thus, SUMO-1 modification of lysine 185 in the hPPARα hinge region contributes directly to the inhibition of hPPARα transcriptional activity.

Human PPARα SUMOylation Regulates Its Interaction with the Corepressor NCoR but Not with the Corepressor SMRT—The SUMO pathway is known to influence protein-protein interactions and, more specifically, to enhance interaction with

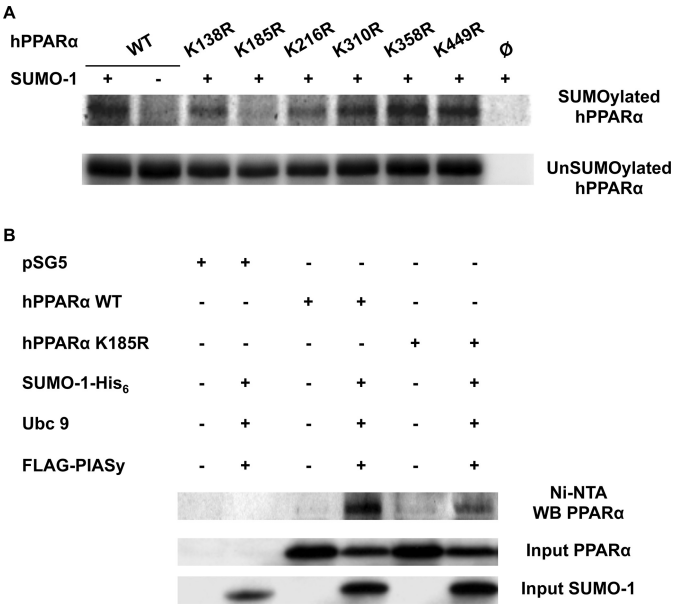

FIGURE 5. **SUMOylation of hPPARα occurs on lysine 185.** *A*, *in vitro* translated [^{35}S]methionine hPPARα WT, hPPARα K138R, hPPARα K185R, hPPARα K216R, hPPARα K310R, hPPARα K358R, hPPARα K449R proteins, and [^{35}S]methionine reticulocyte lysate were incubated with SUMO E1-activating enzyme, Ubc9, and SUMO-1 WT protein or unconjugatable SUMO-1 mutant protein provided with the *in vitro* SUMOlink® kit. Proteins were then separated by SDS-PAGE and analyzed by autoradiography. *B*, COS-7 cells were cotransfected with pSG5 control vector or pSG5-hPPARα WT or pSG5-hPPARα K185R expression vectors and with pSG5-SUMO-1-His$_6$, pSG5-Ubc9, and pcDNA3-FLAG-PIASy expression vectors or pSG5 vector and pcDNA3-FLAG as controls. After 48 h, COS-7 cells were scraped in ice-cold phosphate-buffered saline. One-tenth of cells were lysed in radioimmune precipitation assay buffer and used as input control. The remaining cells were lysed in denaturant binding buffer, and resulting cell extracts were incubated with Ni-NTA beads to isolate histidine-tagged SUMOylated proteins. The SUMOylated hPPARα proteins and hPPARα input proteins were analyzed by Western blotting using anti-PPARα antibodies. The SUMO-1-His$_6$ input proteins were analyzed by Western blotting using anti-His$_6$ antibodies.

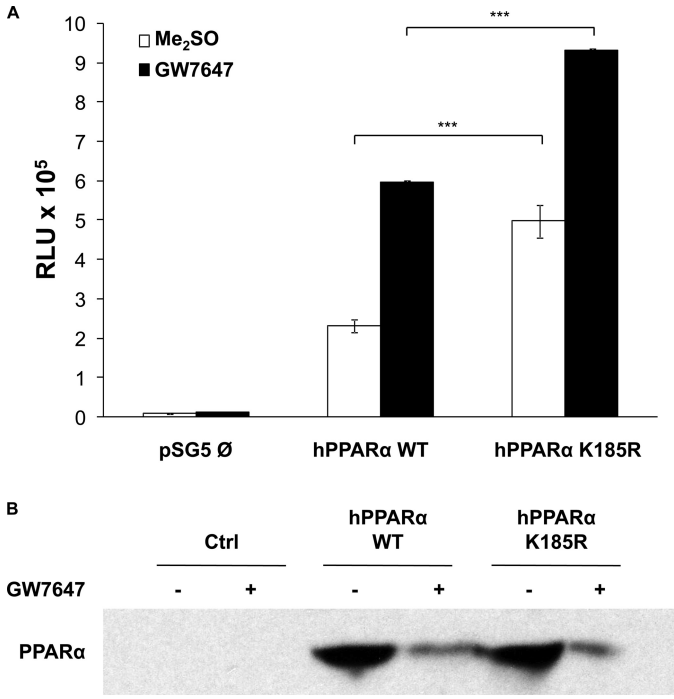

FIGURE 6. **The transcriptional activity of hPPARα K185R is increased compared with hPPARα WT.** *A*, HuH-7 cells were transfected with the pSG5 control vector, or the pSG5-hPPARα WT or pSG5-hPPARα K185R expression vectors, with the pSV-β-galactosidase, with the reporter vector J6-TK-Luc. After 24 h of transfection, cells were treated with Me_2SO or GW7647 (600 nM) in DMEM medium 0.2% SVF, 0.2% BSA for 24 h. The luciferase and β-galactosidase activities were measured in transfected cell lysates, and the ratio luciferase activity/β-galactosidase activity was defined as *RLU*. PPARα protein amounts were evaluated by Western blotting. Each bar is the mean value ± S.D. of triplicate determinations. Statistical differences are indicated (*t* test; PPARα WT *versus* PPARα K185R: ***, $p < 0.001$). *B*, PPARα protein from transfection assay cell lysates was analyzed by Western blotting using anti-PPARα antibodies.

NCoR as demonstrated for PPARγ and LXR (24, 25). In addition, the hinge region of hPPARα has been shown to be implicated in the recruitment of corepressors (14). Therefore, physical interactions of NCoR (Fig. 7) and SMRT (Fig. 8) with the hPPARα WT or hPPARα K185R proteins were investigated.

A mammalian one-hybrid assay was performed by transfecting HuH-7 cells with the J6-TK-Luc reporter vector, the hPPARα WT or hPPARα K185R expression vectors, and increasing amounts of VP16-AD (activating domain) or VP16-NCoR vectors (Fig. 7*A*). The latter encodes the VP16-AD protein fused to the C-terminal domain of NCoR, which includes the nuclear receptor interacting domain. Increasing amounts of VP16-NCoR stimulated hPPARα WT transcription activity more pronouncedly than hPPARα K185R, indicating that the NCoR protein interacts with hPPARα WT with a higher efficiency than hPPARα K185R.

We also assessed the role of SUMOylation in the functional interaction between hPPARα and NCoR by co-transfecting HuH-7 cells with the J6-TK-Luc reporter vector, hPPARα WT or hPPARα K185R, and with increasing amounts of NCoR full-length expression vector (Fig. 7*B*). To appreciate both hPPARα WT and hPPARα K185R sensitivity to NCoR independently of the difference between their respective transcriptional activities, the transcriptional activity of both hPPARα WT and hPPARα K185R in the absence of corepressors was set at 100%, and the transcriptional activity in the presence of each amount of corepressors was calculated relatively to this reference value. The transcriptional activity of hPPARα WT was decreased in a dose-dependent manner by NCoR co-transfection whereas the transcriptional activity of hPPARα K185R was unaffected by the co-expression of NCoR (Fig. 7*B*), showing that the hPPARα K185R mutant is less sensitive to a decrease in activity by NCoR co-transfection compared with hPPARα WT protein. To reinforce these results, HuH-7 cells were cotransfected with a flagged-hPPARα WT or K185R expression vectors and NCoR full-length. Flagged proteins were immunoprecipitated, and associated NCoR proteins were analyzed by Western blotting. The results presented in Fig. 7*C* show that, in contrast to PPARα WT, the SUMOylation-defective hPPARα K185R form did not interact with the NCoR protein, confirming our previous results (Fig. 7*A*). As control, we performed a similar experiment with HuH-7 cells transfected with the pEF-FLAG empty vector and NCoR expression vector. As expected, NCoR proteins were not precipitated in this condition (data not shown).

Similarly, the impact of hPPARα SUMOylation was assessed on the interaction between the corepressor SMRT and hPPARα (Fig. 8). For that purpose, HuH-7 cells were transfected with the J6-TK-Luc reporter vector, the hPPARα WT or hPPARα K185R, and increasing amounts of VP16-AD or VP16-SMRT

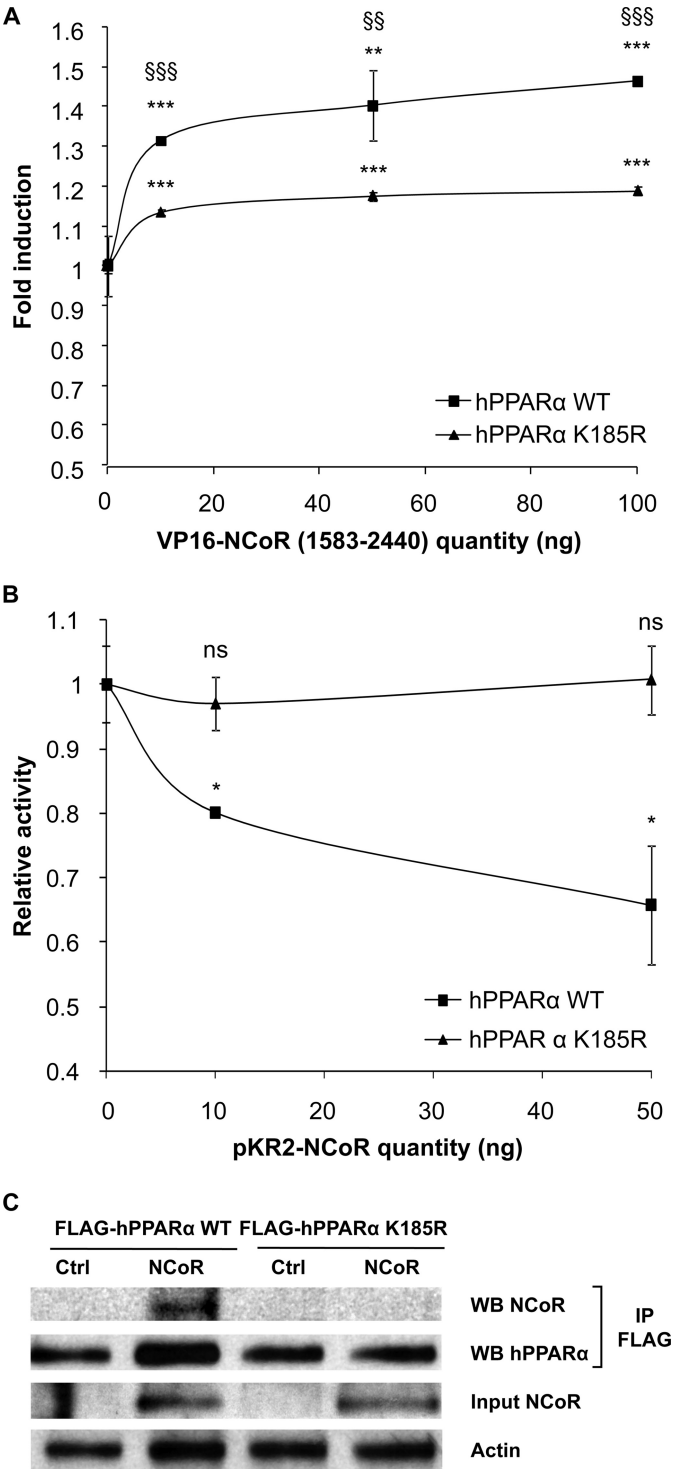

FIGURE 7. **The hPPARα K185R mutant displays a lower physical and functional interaction with NCoR compared with hPPARα WT.** *A*, HuH-7 cells were transfected with the J6-TK-Luc, with pSV-β-galactosidase, with the pSG5 control vector, or the pSG5-hPPARα WT or pSG5-hPPARα K185R expression vectors, and with increasing amounts of VP16-AD or VP16-NCoR vectors. The luciferase and β-galactosidase activities were measured in transfected cell lysates, and the ratio luciferase activity/β-galactosidase activity was determined. Results are expressed in fold induction compared with VP16-AD control curves. Each bar is the mean value ± S.D. of triplicate determinations. Statistical differences are indicated (*t* test; without VP16-NCoR *versus* with VP16-NCoR: **, $p < 0.01$; ***, $p < 0.001$; hPPARα WT *versus* hPPARα K185R: §§, $p < 0.01$; §§§, $p < 0.001$). VP16-AD curves are not represented. *B*, HuH-7 cells were transfected with the J6-TK-Luc, with pSV-β-galactosidase, with the pSG5 control vector, or the pSG5-hPPARα WT or pSG5-hPPARα K185R expression vectors. By contrast to NCoR (Fig. 7), increasing amounts of SMRT similarly activated WT and K185R hPPARα proteins, indicating that the K185R mutation in hPPARα has no effect on the interaction of hPPARα with the corepressor SMRT (Fig. 8*A*).

Additionally, to examine the impact of hPPARα SUMO-conjugation on its functional interaction with SMRT, HuH-7 cells were transfected with reporter vector, hPPARα WT or K185R, with increasing amounts of either pCI-SMRT or pCI as control (Fig. 8*B*). In accordance with the one-hybrid results, the transcriptional activity of both hPPARα WT and hPPARα K185R was decreased to a similar extent by SMRT. Altogether, these data show that the hPPARα K185R protein is still sensitive to repression by SMRT but not to NCoR.

DISCUSSION

In this report, we show that hPPARα target gene expression can be down-regulated by NCoR (*L-CPT1*, *PDK4*) or by SMRT (*HMGCOAS2*) in a gene-specific manner. It has been previously shown that NCoR can be recruited by SUMO-modified nuclear receptors (24, 25). Our study reports that hPPARα binds to the E2-conjugating enzyme Ubc9 providing evidence that PPARα is able to interact directly with SUMO pathway components. Therefore, we investigated whether hPPARα is SUMOylated and whether this SUMOylation could be involved in the regulation of hPPARα target gene expression by NCoR.

Our results show that inhibition of the SUMO pathway, by knocking-down the E2-conjugating enzyme Ubc9, increases the hPPARα target genes *L-FABP* and *PDK4*. Interestingly, expression of the hPPARα target gene *HMGCOAS2* was not changed under similar conditions suggesting that the SUMOylation pathway regulates some, but not all, PPARα target genes. Altogether, these results suggest that the selective recruitment of NCoR by SUMO-modified hPPARα leads to the inhibition of a subset of hPPARα target genes, indicating that nuclear receptor SUMOylation could regulate the NCoR-specific inhibition of nuclear receptor target genes.

Our *in vitro* and *in vivo* assays demonstrate that PPARα is SUMOylated. In the SUMOylation assays, only one band with a higher molecular mass (72 kDa), corresponding to the size of mono SUMOylated hPPARα, was observed. The *in vitro* SUMOylation assay identified lysine 185 as the major targeted lysine, which is in accordance with the highest prediction score of this site given by the bioinformatic analysis. However, the hPPARα K185R mutant is still slightly SUMOylated in cells (Fig. 5*B*), suggesting that a second minor SUMOylated site could exist. Because the replacement of lysine 185 into an

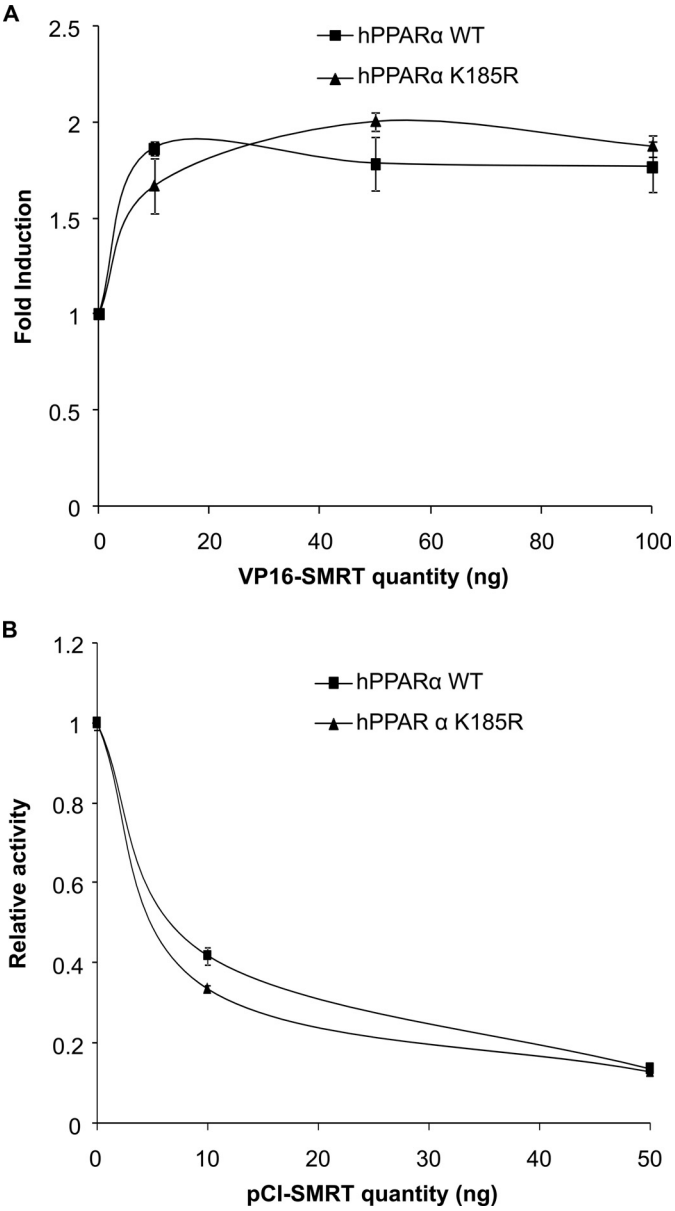

FIGURE 8. **The hPPARα WT and hPPARα K185R proteins display a similar physical and functional interaction profile with SMRT.** *A*, HuH-7 cells were transfected with the J6-TK-Luc, with pSV-β-galactosidase, with the pSG5 control vector, or the pSG5-hPPARα WT or pSG5-hPPARα K185R expression vectors, and with increasing amounts of VP16-AD or VP16-SMRT vectors. The luciferase and β-galactosidase activities were measured in transfected cell lysates, and the ratio luciferase activity/β-galactosidase activity was determined. Results are expressed in fold induction compared with VP16-AD control curves. Each bar is the mean value ± S.D. of triplicate determinations. VP16-AD curve are not represented. *B*, HuH-7 cells were transfected with the J6-TK-Luc, with pSV-β-galactosidase, with the pSG5 control vector, or the pSG5-hPPARα WT or pSG5-hPPARα K185R expression vectors, and with increasing amounts of pCI control vector or pCI-SMRT expression vector. The luciferase and β-galactosidase activities were measured in transfected cell lysates, and the ratio luciferase activity/β-galactosidase activity was determined. Then, pCI-SMRT curves were compared with their respective pCI control curves. Results are expressed as relative inhibition. Each bar is the mean value ± S.D. of triplicate determinations. pCI curves are not represented.

unSUMOylatable arginine residue is sufficient to abolish SUMOylation of hPPARα *in vitro*, lysine 185 must be the major SUMO-1 acceptor site. Similar observations of the presence of hierarchic lysine residues for SUMO-1 conjugation were reported in other proteins such as PPARγ (26), androgen receptor (29), or aryl hydrocarbon receptor (30). Interestingly, we show in Fig. 5*A* that K138R and K216R hPPARα mutants are less SUMOylated *in vitro* compared with the WT protein, suggesting that these two residues could be some SUMOylated lysine targets as well. Unfortunately, these mutants are still strongly SUMOylated in cells (data not shown), suggesting that the lysines 138 and 216 are not SUMOylated *in vivo*. Interestingly, lysine 185 in murine PPARα may not be SUMOylated in NIH3T3 cells (19). This could be due to differences in negative charges downstream the lysine SUMO acceptor shown to be important in the recognition between the substrate and the SUMO machinery (Fig. 9) (31).

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sp|P23204|PPARα MOUSE  170 AIRFGRMPRSEKAKLKAEILTCEHDLKDSET 200
sp|P37230|PPARα RAT    170 AIRFGRMPRSEKAKLKAEILTCEHDLKDSET 200
sp|Q07869|PPARα HUMAN  170 AIRFGRMPRSEKAKLKAEILTCEHDIEDSET 200
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FIGURE 9. **Comparison of the SUMOylation consensus site in mouse, rat, and human PPARα.** PPARα primary protein sequences in mouse, rat, and human were compared by using BLASTP algorithm. (*) represents identical amino acids and (:) represents different amino acids.

In addition, the regulation of SUMO conjugation to a substrate protein upon phosphorylation of the target protein has already been reported for several nuclear receptors such as GR and PPARγ (32, 33). We have previously shown that protein kinase C can phosphorylate hPPARα on serines 179 and 230, which are very close to the lysine 185. The serine 230 is not conserved between mice and human and could be involved in the species-specific SUMOylation of the hPPARα protein.

The mutant hPPARα K185R is transcriptionally more active. Conversely, SUMO E3 ligase PIASy overexpression in HuH-7 cells decreases transcriptional activity of hPPARα and expression of *L-CPT1* and *PDK4* target genes (Fig. 1). In agreement with the results with siRNA NCoR and siRNA Ubc9, PIASy overexpression has no effect on the expression of *HMGCOAS2*, which is a gene specifically regulated by SMRT (Fig. 1). Using the mutant protein hPPARα K185R, we showed that SUMOylation of hPPARα promotes NCoR recruitment without influencing the binding to SMRT. These data suggest that hPPARα SUMOylation helps discriminate among the interactions with different corepressors. Accordingly, overexpression of full-length NCoR inhibits transcriptional activity of PPARα WT without changing the transcriptional activity of hPPARα K185R, whereas overexpression of SMRT inhibits transcriptional activity of both hPPARα WT and hPPARα K185R. Such differential recruitment of corepressors has already been observed with the nuclear receptor LXR α (LXRα), which preferentially recruits either NCoR or SMRT, depending on the target genes (16).

Human PPARα SUMOylation is significantly reduced in HuH-7 cells by ligand treatment. Ligand binding could either promote a conformational change preventing SUMO conjugation, or favor the recruitment of SENP desumoylases. It has previously shown that PPARβ/δ requires SENP1 and various co-regulators to activate gene promoters in response to ligand (34). In contrast to the human protein, mPPARα SUMOylation is not significantly affected by the presence of the PPARα ligand Wy-14,643 in NIH3T3 cells (19). However, mice treated with this ligand show increased SUMOylated PPARα, suggesting

that the ligand could act on PPARα SUMOylation in a cell type-selective manner.

In conclusion, this study provides the first evidence of SUMOylation of hPPARα on lysine 185 resulting in the down-regulation of its transcriptional activity by promoting its interaction with the corepressor NCoR. This is consistent with the ability of the ligand to inhibit hPPARα SUMOylation preventing the binding of NCoR to the nuclear receptor, which leads to its activation. Moreover, this study demonstrates that the SUMO pathway regulates the recruitment of the corepressor NCoR but not SMRT. This differential recruitment leads to a differential inhibition of specific hPPARα target genes. Finally, our work provides further evidence of the relevance of the hPPARα hinge region in the regulation of corepressor recruitment.

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